

**A multi-omics study investigating the responses of
Bacillus subtilis to the antibiotic fosfomycin and
the development of cell wall analysis methods**

Dissertation

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Abstract

The rising antimicrobial resistance and a critical shortage of new antibiotics in clinical trials are forcing a re-evaluation of existing drugs for innovative antibiotic therapeutic applications to ensure current standards of infection control. Fosfomycin (FOS) is a long-established antibiotic with broad-spectrum activity against both Gram-positive and Gram-negative bacteria, offering untapped potential for antibacterial therapy, including the containment of multidrug-resistant pathogens, due to its synergistic effects when combined with antibiotics from different classes. A detailed study of the cellular responses to FOS is hypothesised to enhance the understanding of FOS-induced bacterial responses beyond the inhibition of the target MurA, thereby rationalising the development of combination therapies. The probiotic Gram-positive model bacterium *Bacillus subtilis* was used to re-evaluate target inhibition, investigate growth behaviour in the presence of different FOS concentrations and study cellular responses using multi-omics. FOS targets a tightly regulated and generally essential bacterial pathway by blocking the enzyme MurA, which catalyses the first committed step in *de novo* peptidoglycan (PGN) biosynthesis. MurA is a unique enzyme that catalyses the enolpyruvyl transfer of phosphoenolpyruvate (PEP) to UDP-*N*-acetylglucosamine (UDP-GlcNAc) to form UDP-GlcNAc-enolpyruvate. FOS covalently inhibits MurA by binding to a cysteine residue in the active site of the enzyme. Interestingly, in the presence of PEP, the native MurA enzyme is covalently modified at this cysteine residue, protecting it from the attack of FOS. Although MurA crystal structures suggested this post-translational PEP modification, it had not been directly demonstrated. Native protein MS was used to reveal covalent PEP binding to both MurA isoforms present in *B. subtilis* (MurAA and MurAB) and to confirm the resulting protection against FOS. The importance of the glycerol-3-phosphate transporter GlpT for FOS uptake and the bacillithiol S-transferase FosB for FOS detoxification was demonstrated using chromosomal mutants that were at least five times more sensitive to the antibiotic. To understand the metabolic adaptations induced by lower FOS levels that confer tolerance to the antibiotic and the causes of cell lysis in the presence of higher FOS levels, the FOS-induced responses of *B. subtilis* were investigated using a comprehensive multi-omics approach, including transcriptomics, proteomics and metabolomics. The results revealed specific stress-induced responses as well as stress-independent metabolic adaptations in FOS-treated cells. Cell envelope stress, controlled by the alternative sigma factors SigM, V, W, X, Y and the transcriptional regulator Spx, dominates the response to high concentrations of FOS. Interestingly, lipoteichoic acid, teichoic acid and fatty acid synthesis were upregulated under these conditions, along with iron homeostasis. PGN associated hits were affected in different ways: Synthesis was upregulated and degradation enzymes were downregulated. Overall, FOS downregulates catabolic processes. Furthermore, an LC-MS-based cell wall metabolomics workflow was developed to monitor FOS-induced metabolite changes by semi-automated comparison with a standard library containing all soluble precursors of PGN synthesis. Analyses of bacterial metabolites were performed in the context of PGN and beyond. In addition, LC-MS and HPLC analyses were developed and applied to address a wide range of questions related to FOS action, cell wall and sugar metabolism. The study provides a nuanced understanding of the complex cellular responses to FOS and provides valuable new insights into the interplay between growth inhibition, cell lysis and metabolic adaptation under FOS treatment. The results contribute to a more comprehensive understanding of bacterial metabolic adaptation under antibiotic stress, advancing knowledge in the fight against antimicrobial resistance and antibiotic use.

Zusammenfassung

Die derzeitige Zunahme antimikrobieller Resistenzen und der alarmierende Mangel an neuen Antibiotika in klinischen Studien erfordern eine Neubewertung bestehender Antibiotika für therapeutische Anwendungen, um den aktuellen medizinischen Standard aufrechtzuerhalten. Fosfomycin (FOS) ist ein seit langem etabliertes Antibiotikum mit einem breiten Wirkungsspektrum gegen grampositive und gramnegative Bakterien. Durch synergistische Effekte mit Antibiotika aus verschiedenen Klassen bietet es ungenutztes Potenzial für Kombinationstherapien, auch gegen multiresistente Erreger. Die Untersuchung der zellulären Antworten von Bakterien ermöglicht ein tieferes Verständnis der FOS-induzierten bakteriellen Reaktionen, die über die Hemmung des Zielmoleküls MurA hinausgehen. Dies bildet die Grundlage für medizinische Kombinationstherapien. Dazu wurde das Wachstumsverhalten des probiotischen grampositiven Modellbakteriums *Bacillus subtilis* in Gegenwart verschiedener FOS-Konzentrationen mittels Multi-Omics Analysen untersucht. Durch die Hemmung des Enzyms MurA, das den ersten Schritt der *de novo* Peptidoglykan (PGN) Biosynthese darstellt, greift FOS in einen streng regulierten und essenziellen bakteriellen Stoffwechselweg ein. MurA katalysiert die Enolpyruvyl Übertragung von Phosphoenolpyruvat (PEP) auf UDP-*N*-acetylglucosamine (UDP-GlcNAc), wodurch UDP-GlcNAc-Enolpyruvat entsteht. FOS hemmt MurA kovalent durch Bindung an einen Cysteinrest im aktiven Zentrum des Enzyms. Interessanterweise wird das native MurA-Enzym in Gegenwart von PEP an diesem Cysteinrest kovalent modifiziert, wodurch es vor einem Angriff durch FOS geschützt ist. Die posttranslationale Modifikation durch PEP konnte bisher nicht direkt nachgewiesen werden, obwohl bereits existierende Kristallstrukturen von MurA darauf hindeuten. Mittels nativer MS konnte die kovalente Bindung von PEP an beide MurA-Isoformen in *B. subtilis* (MurAA und MurAB) direkt nachgewiesen und der daraus resultierende Schutz vor FOS bestätigt werden. Die Bedeutung des Glycerin-3-Phosphat-Transporters GlpT für die Aufnahme von FOS und der Bacillithiol-S-Transferase FosB für die Entgiftung von FOS wurde durch chromosomale Mutanten nachgewiesen. In einem Multi-Omics Ansatz, bestehend aus Transkriptom-, Proteom- und Metabolom-Untersuchungen, wurden die metabolischen Anpassungen durch die Verwendung niedrigerer FOS-Konzentrationen und die Ursachen der Zelllyse in Gegenwart höherer FOS-Konzentrationen umfassend analysiert. Die Ergebnisse zeigten sowohl spezifische stressabhängige Reaktionen als auch stressunabhängige metabolische Anpassungen in FOS-behandelten Zellen. Zellhüllenstress, der durch die alternativen Sigma-Faktoren SigM, V, W, X, Y und den Transkriptionsregulator Spx kontrolliert wird, dominierte die Antwort. Auffallend ist, dass auch die Lipoteichonsäure-, Teichosäure- und Fettsäuresynthese sowie die Eisenhomöostase hochreguliert waren. Die PGN-Synthese war hochreguliert, während PGN abbauenden Enzyme herunterreguliert waren. Insgesamt regulierte FOS die katabolen Prozesse der Zelle herunter. Um FOS-induzierte Metabolitveränderungen zu untersuchen, wurde ein LC-MS Metabolomics Arbeitsablauf mit Schwerpunkt auf der Zellwandanalyse unter Verwendung einer Standardbibliothek entwickelt, die alle löslichen PGN-Vorstufen enthält. Darüber hinaus wurden LC-MS- und HPLC-Analysen entwickelt und eingesetzt, um Fragestellungen im Zusammenhang mit der Wirkung von FOS, der bakteriellen Zellwand und dem Zuckerstoffwechsel in verschiedenen Projekten zu beantworten. Die vorgestellte Arbeit ermöglicht ein differenziertes Verständnis der komplexen zellulären Reaktionen auf FOS und liefert wertvolle neue Erkenntnisse über das Zusammenspiel von Wachstumshemmung, Zelllyse und metabolischer Anpassung unter FOS-Behandlung. Die Ergebnisse dieser Arbeit tragen zu einem umfassenderen Verstehen bei, wie flexibel Bakterien ihren Stoffwechsel unter antibiotischen Stressbedingungen anpassen und erweitern das Wissen im Umgang mit antimikrobiellen Resistenzen.

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Abbreviations and Symbols

Å	Angstrom (10^{-10} metre)
AUC	area under the curve
BPC	base peak chromatogram
Da	Dalton (atomic unit of mass, also u)
DAP	2,6-Diaminopimelinsäure
EIC	extracted ion chromatogram
FLU	fluorescence
FOS	Fosfomycin
g	gram
GlcNAc	<i>N</i> -acetylglucosamine
h	hour
HPLC	high performance liquid chromatography
IPTG	isopropyl-D-1-thiogalactopyranoside
k	kilo, SI prefix 10^3
L	litre
LC	liquid chromatography
M	molar
$m \cdot z^{-1}$	mass to charge ratio
m	milli, SI prefix 10^{-3}
min	minutes
MS	mass spectrometry
MurNAc	<i>N</i> -acetylmuramic acid
MIC	minimum inhibitory concentration
n	nano, SI prefix 10^{-9}
NAC	<i>N</i> -acetyl-L-cysteine
OD	optical density

OPA	<i>ortho</i> -phthalaldehyde
p	nano SI prefix 10^{-12}
PEP	phosphoenolpyruvate
PGN	peptidoglycan
s	seconds
S	setup
UDP	uridine diphosphate
v/v	volume per volume
α	alpha
β	beta
Δ	genetic deletion
%	percent
°C	degree Celsius
μ	micro, SI prefix 10^{-6}

List of publications

Accepted publications

1. Walter A, **Friz S**, Mayer C. Chitin, Chitin Oligosaccharide, and Chitin Disaccharide Metabolism of *Escherichia coli* Revisited: Reassignment of the Roles of ChiA, ChbR, ChbF, and ChbG. *Microb Physiol.* 2021;31(2):178-94.
2. Neumann N, **Friz S**, Forchhammer K. Glucose-1,6-Bisphosphate, a Key Metabolic Regulator, Is Synthesized by a Distinct Family of alpha-Phosphohexomutases Widely Distributed in Prokaryotes. *mBio.* 2022;13(4):e0146922.
3. Hantke K, **Friz S**. The TonB-dependent uptake of pyrroloquinoline-quinone (PQQ) and secretion of gluconate by *Escherichia coli* K-12. *Mol Microbiol.* 2022;118(4):417-25.

Submitted manuscripts

4. Mund M*, **Friz S***, Esser A, Matzner D, Babczyk A, Menche D, Brötz-Oesterhelt H, Mayer C, Mayer G. Metabolization of α -D-carba-glucosamine in vivo generates antimetabolites of cell wall precursors. *BioRxiv*, 10.1101/2023.02.08.527593.

Manuscripts in preparation - ready for submission

5. **Friz S**, Stahl Y, Stincione P, Franz-Wachtel M, Macek B, Petras D, Mayer C. Native mass spectrometry reveals that MurA carboxyvinyltransferases are primarily phosphoenolpyruvate-bound and therefore fosfomycin insensitive
6. **Friz S**, Stahl Y, Mayer C. Investigating the inhibition of *Bacillus subtilis* growth by fosfomycin: The role of the targets MurAA and MurAB, drug transport, intracellular accumulation and tolerance development
7. **Friz S**, Walter A, Franz-Wachtel M, Harbig T, Macek B, Nieselt K, Mayer C. Comprehending the responses of *Bacillus subtilis* to fosfomycin using multi-omics.
8. **Friz S**, Walter A, Mayer C. A cell wall metabolomics workflow to determine cytosolic peptidoglycan precursor levels in bacteria

*contributed equally

Declaration of personal contribution

Publication 1:

The plasmid pQE32-ChbBCA was cloned and provided by me, and the corresponding HPLC-MS experiments were carried out together with Axel Walter.

Publication 2:

The HPLC-MS measurements were carried out by me, and I assisted with data analysis and visualisation.

Publication 3:

The HPLC-MS measurements were performed by me, and I assisted in the data analysis.

Publication 4:

The metabolomics experiments were designed by me, the HPLC-MS measurements, the analysis and interpretation, and the presentation of the results, were carried out by me. I wrote the manuscript together with Milena Mund.

Publication 5:

The experiments were designed, and the proteins cloned by me. The experiments were performed in collaboration with Yannis Stahl. The results were visualised, and the manuscript was written by me.

Publication 6:

The experiments were planned, carried out and interpreted by me, the results were visualised by me, and the manuscript was written by me.

Publication 7:

The experiments were planned by me, all biological experiments and isolations were performed by me, as well as analysing the omics results and writing the manuscript.

Publication 8:

All biological experiments were designed and performed by me, the standard library was created by me, and the manuscript was written by me.

1 Introduction

Alongside global pandemics, crises and wars, the alarming rise of antimicrobial resistance (AMR) is escalating into a global health crisis. It poses an imminent threat to the effectiveness of existing therapeutic interventions, forcing us to intensively explore the intricate mechanisms underlying bacterial resistance and the urgent need for innovative strategies and methods to combat this pervasive challenge. The rapid global spread of multi- and pan-resistant bacteria - *superbugs* that cause infections that cannot be treated with existing antimicrobial drugs such as antibiotics is forcing us to act. Antimicrobial drugs are the backbone of modern medicine. AMR threatens our ability to treat common infections and perform life-saving interventions such as cancer chemotherapy, caesarean sections, organ transplants and other surgeries. Furthermore, AMR also affects food security by threatening animal and plant health and agricultural productivity. [1, 2]

Bacteria are adapting to commonly used antibiotics, making them less effective or ineffective altogether. As a result, more than 10 million people are expected to die from antimicrobial resistance by 2050 [3, 4]. The importance of acting is indisputable, but the road to success is not trivial. There are only two ways to ensure effectiveness and an adequate supply of antimicrobial drugs: either find and authorise new antibiotics, ideally with new modes of action, or use the existing antibiotics more effectively and in a more targeted manner. As resounding and revolutionary as the success of antibiotics was in retrospect for the areas described, after the time of Alexander Fleming and his Nobel Prize in 1945 for the discovery of penicillin, the so-called golden age of antibiotic natural product discovery, the new discovery of novel antibiotics declined. Although new methods from the fields of synthetic biology, genome mining, simplified and faster diagnostics, all combined with artificial intelligence, are currently creating many possibilities for the future, there are currently only 30 to 40 possible new antibacterial candidates in the clinical trials for approval. By contrast, nearly 4,000 immuno-oncology candidates are in the approval process. In addition, less than a quarter of the antibiotics currently in the approval process have a new mode of action and therefore a high likelihood of low levels of widespread antimicrobial resistance. [5, 6, 7, 8, 9]

There are many targets for antibiotics, but most are found in the metabolic pathways of macromolecules. These can be enzymes, ribosomal RNA or metabolic intermediates of pathways. [10]. One of the most important targets for antibiotics is the bacterial cell wall, where so-called cell wall-active antibiotics such as: fosfomycin (FOS) (which attacks MurA, the first committed biosynthetic enzyme of cell wall biosynthesis), β -lactams (e.g. penicillins which attack penicillin-binding proteins (PBPs)) or glycopeptides (e.g. vancomycin, which attacks lipid II) inhibit the bacterial cell wall synthesis [10, 11].

1.1 Bacterial cell wall

The importance of the bacterial cell wall was known long before the discovery of antibiotics. Almost 140 years ago, the biologist Hans Christian Gram developed the method of Gram staining. Gram staining is one of the best-known and most important differential staining methods in microbiology. It is used to classify bacteria into the groups of Gram-positive and Gram-negative. Gram-positive bacteria appear purple after staining with crystal violet, whereas Gram-negative bacteria appear colourless or slightly red after counterstaining with fuchsin or safranin. [12, 13]

The basis of Gram staining resides in the structure of the bacterial cell wall, which differs considerably between Gram-positive (monoderm) and Gram-negative (diderm) bacteria. The cell wall of Gram-positive bacteria is comparatively simple, consisting of a relatively thick, > 20 nm layer of peptidoglycan (PGN). In addition, polyol phosphates, firstly covalently linked wall teichoic acids (WTA) and secondly non-covalently linked lipoteichoic acids (LTA), protrude from the PGN and the phospholipid bilayer cell membrane. The cell wall of Gram-negative bacteria has a more complex structure. It contains an additional outer membrane composed of double-tailed phospholipids and lipoproteins in the inner leaflet, lipopolysaccharides in the outer leaflet, and integral membrane proteins (e.g. porins). The cell membrane and outer membrane enclose the periplasmic space, which contains a thin 2-6 nm layer of PGN. The periplasmic space is only present in Gram-negative bacteria and is another major difference. [14, 15, 16, 17, 18]

The bacterial cell wall fulfils a number of essential functions for the bacterial cell. It is essential for the stability and shape of the cell and resists the turgor pressure. However, it also has other functions such as controlling cell growth and division, nutrient uptake and protein secretion, including possible movement towards nutrients (i.e. swarming, swimming, twitching and chemotaxis). The bacterial cell wall must protect the cell interior and keep it in balance during all stages of cell growth and differentiation as well as under different environmental influences and conditions (e.g. changes in osmotic pressure). [17, 20, 21, 22]

The central, cell-stabilising component of the bacterial cell wall of Gram-positive and Gram-negative bacteria is PGN and is only missing in a few obligate intercellular bacteria or so-called cell-wall-less (L-) forms. So far, PGN has only been found in bacteria and is even absent in archaea [19]. The importance and the central role of PGN makes it a predestined target for antibiotics, but the cell itself also controls its metabolism via and with the enzymes of PGN *de novo* synthesis.

1.2 Structure of peptidoglycan

In all bacteria, PGN is essentially composed of long chains of alternating β -1,4-linked amino sugars, in particular *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc). This sugar backbone is often linked to another similar amino sugar heteropolymer by short peptide chains linked via the D-lactyl group of MurNAc. This results in a reticulated or net-like structure. The peptide cross-linking varies between bacterial groups.

In *Escherichia coli* and *Bacillus subtilis*, the peptide chain consists of L-alanine (L-Ala), iso-D-glutamic acid (D-Glu), *meso*-diaminopimelic acid (*m*-DAP), two molecules of D-alanine (D-Ala-D-Ala). Cross-linking takes place between an *m*-DAP of one peptide chain (acceptor) and the penultimate D-Ala of the second peptide chain (donor). This reaction, called D, D-transpeptidation, is catalysed by penicillin-binding proteins (PBPs, also known as D,D-transpeptidases), which form a cross-bridge and cleave the terminal D-Ala to form the pentapeptide. There is considerable variation in the peptide composition of bacteria. In *Staphylococcus aureus*, for example, the peptide bound to MurNAc consists of L-Ala, *iso*-D-glutamine (D-Gln), L-lysine and one or two D-Ala residues. The cross-linking also differs in that L-Lys and D-Ala are linked by a pentaglycine peptide (pentaglycine bridge). The structure of the sugar backbone also varies between species. In Gram-negative bacteria, and in *Bacillus* sp. the glycan chains are generally terminated with 1,6-anhydroMurNAc (anhMurNAc), resulting from intramolecular ring formation by a glycosidic linkage between C1 and C6. So far, no anhydroMurNAc has been found in *S. aureus*, but extensive O-acetylation of MurNAc (at C6) is common in this organism. In *B. subtilis*, free carboxylic acid groups in the PGN are amidated under appropriate conditions. In addition, the majority of GlcNAc in *B. subtilis* is deacetylated as glucosamine (GlcN). MurNAc can also be deacetylated. Another difference is the length of the glycan chains, which varies greatly between *S. aureus* with about 5-10 and *E. coli* with about 20 disaccharides compared to *B. subtilis* with over 50 directly linked disaccharides. The proportion of cross-linked peptide chains also varies between *S. aureus* (about 90 %), *E. coli* (about 50 %) and *B. subtilis* (about 30 %). [16, 23, 24, 25, 26, 27, 28]

This specificity and uniqueness of the PGN in the bacterial cell requires targeted and precise control of synthesis, not only because the production of such macromolecules affects the entire cell, but also because the building blocks are used and act at other important sites.

1.3 Synthesis of peptidoglycan

The *de novo* synthesis of the bacterial cell wall is an energetically costly process for the cell, involving a large number of enzymes catalysing more than 20 reactions. The synthesis of nucleotide precursors of the PGN takes place in the cytoplasm, followed by the production of lipid-bound cell wall precursors, which are assembled in the inner leaflet of the cytoplasmic membrane and then flipped to the outer leaflet. Final polymerisation and assembly of the *de novo* PGN occurs on the outward-facing side of the cytoplasmic membrane. Controlled hydrolysis of the synthesised PGN is necessary and critical for the cell to be able to expand. An overview of both processes, PGN formation and turnover, is shown in Fig.1.

Fructose-6-phosphate is constantly present in the cell as a result of glycolysis or gluconeogenesis and is also an essential building block for *de novo* PGN synthesis. GlmS catalyses the first reaction of amino sugar biosynthesis, the conversion of fructose 6-phosphate to glucosamine 6-phosphate. The latter is processed by GlmM to yield the α -1-phospho-substrate. Which is converted by GlmU to uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc). The subsequent addition of phosphoenolpyruvate (PEP) to UDP-GlcNAc (MurA) and the reduction of this product (MurB) produces UDP-*N*-acetylmuramic acid (UDP-MurNAc), the first committed precursor of PGN (Fig.1). Bacteria-specific amino acid residues are then attached to the lactyl group of UDP-MurNAc via MurC (L-Ala), MurD (D-Glu), MurE (*m*DAP) and MurF (D-Ala-D-Ala), before the sugar peptide portion is transferred to undecaprenyl pyrophosphate (forming lipid I) on the inner membrane by MraY. Finally, MurG links the resulting lipid I to UDP-GlcNAc to form lipid II, which is transported to the outside of the inner membrane by a flippase (MurJ, AmJ). The freshly synthesised lipid II is incorporated into the nascent PGN by various PGN synthases.

Class A PBPs (aPBPs) catalyse polymerisation with the glycan strand via their glycosyltransferase activity (GTase, GT51 type) and the peptide cross-linking via their transpeptidase activity (TPase, Ser type). bPBPs are monofunctional TPases. In parallel, there are two groups of monofunctional GTases, the SEDS (shape, elongation, division, sporulation) PGN synthases and the monofunctional GT51 GTases. This results in the net-like structure described above. The PGN hydrolases are as important as the synthases for the balance of a functioning cell. They ensure the expansion of the PGN by inserting new material and they are therefore important components in cell division, cell shape maintenance and bacterial interactions. The most important classes are *N*-acetylmuramidases (lysozymes and lytic transglycosylases (LT)), *N*-acetylglucosaminidases, amidases, endopeptidases (EPases) and carboxypeptidases (CPases). The enzymes are categorised according to the isomerism of the substrate amino acids.

[29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40 ,41]

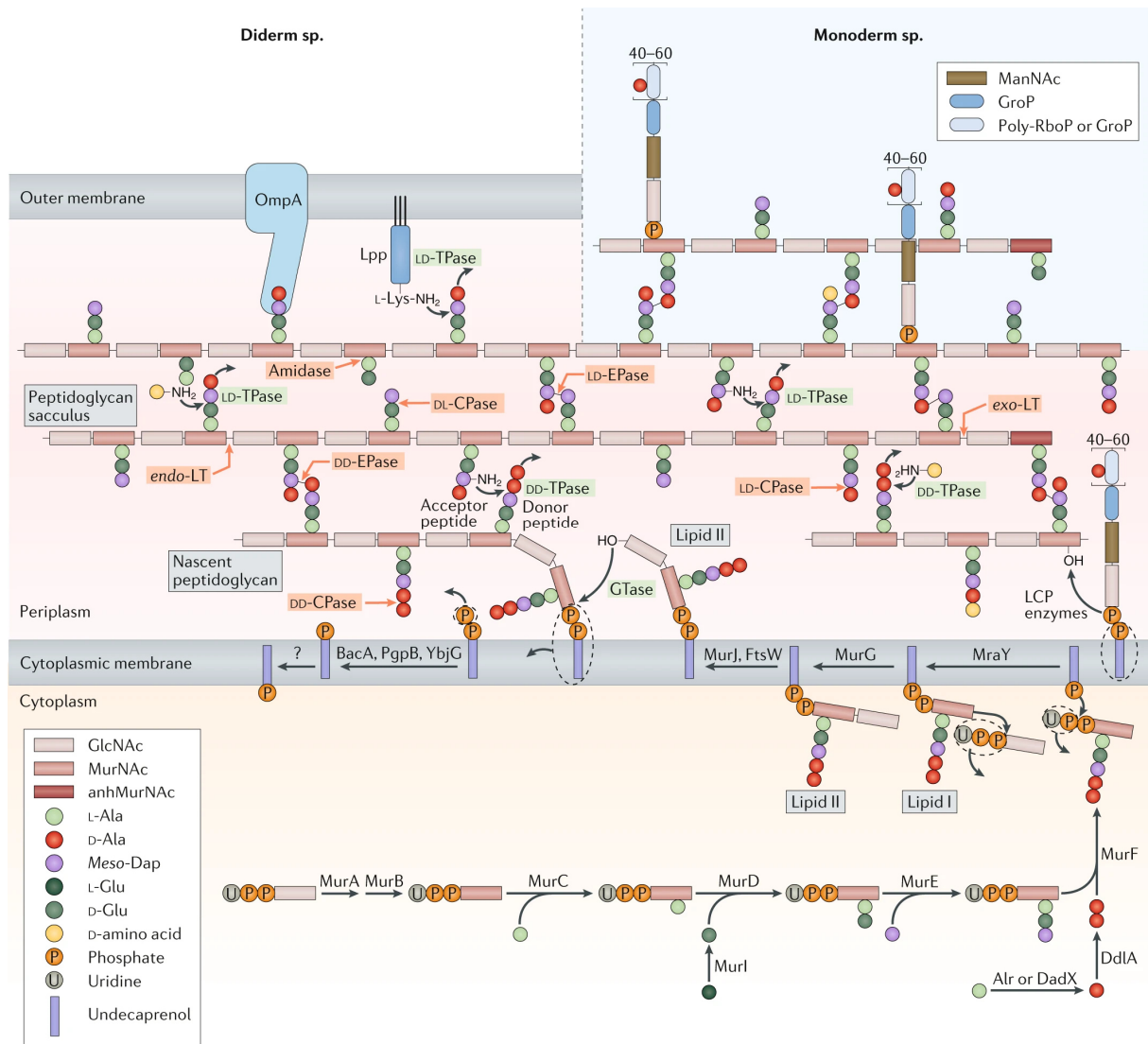


Figure 1: Reactions and enzymes responsible for the synthesis and hydrolysis of peptidoglycan [34]. Alr, Ala racemase, biosynthetic; DadX, Ala racemase, catabolic; DdIA, d-Ala-d-Ala ligase A; GlcNAc, N-acetylglucosamine; GroP, glycerol phosphate; ManNAc, N-acetyl-d-mannosamine; meso-Dap, meso-diaminopimelic acid; MraY, phospho-N-acetylmuramoyl-pentapeptide transferase; MurA, UDP-GlcNAc enolpyruvyl transferase; MurB, UDP-MurNAc dehydrogenase; MurC, UDP-MurNAc L-Ala ligase; MurD, UDP-MurNAc-L-Ala-d-Glu ligase; MurE, UDP-MurNAc-L-Ala-d-Glu-meso-Dap ligase; MurF, UDP-MurNAc-tripeptide-d-alanyl-d-Ala ligase; MurG, undecaprenyldiphospho-muramoylpentapeptide beta-N-acetylglucosaminyltransferase; Murl, Glu racemase; MurJ, MOP (multidrug, oligosaccharide-lipid, polysaccharide) transporter (lipid II flippase); MurNAc, N-acetylmuramic acid; Poly-RboP/GroP, poly-ribitol/glycerol phosphate.

Illustration from: Egan AJF, Errington J, Vollmer W. Regulation of peptidoglycan synthesis and remodelling. *Nat Rev Microbiol.* 2020 Aug;18(8):446-460. doi: 10.1038/s41579-020-0366-3. Epub 2020 May 18. PMID: 32424210. Fig. 1: Peptidoglycan synthesis, cleavage and modification. [34]

1.4 MurA: central enzyme of the peptidoglycan synthesis

The first and the key enzyme in the *de novo* synthesis pathway of PGN is MurA, a UDP-*N*-acetylglucosamine-1-carboxyvinyltransferase (EC 2.5.1.7 (IUBMB)). MurA catalyses the conversion of phosphoenolpyruvate (PEP) and UDP-GlcNAc into UDP-*N*-acetyl-3-*O*-(1-carboxyvinyl)-*D*-glucosamine (UDP-GlcNAc-enolpyruvate) with the release of inorganic phosphate (Fig.2) [42, 43, 44].

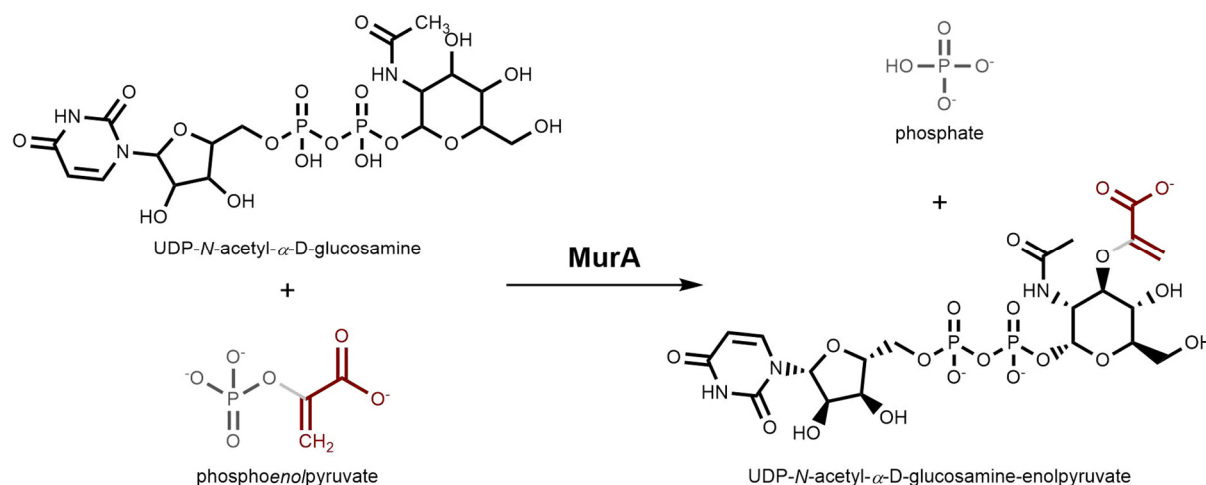


Figure 2: Peptidoglycan precursor biosynthesis: MurA. Schematic illustration of the UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase (E.C: 2.5.1.7) MurA catalysing the reaction of phosphoenolpyruvate and UDP-*N*-acetyl- α -*D*-glucosamine to phosphate and UDP-*N*-acetyl-3-*O*-(1-carboxyvinyl)- α -*D*-glucosamine.

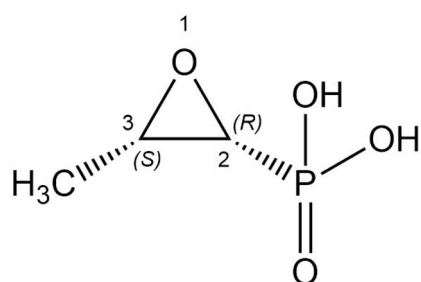
This rather unusual enzymatic reaction involves the cleavage of the stable C-O bond of PEP and not the P-O bond (PEP is mostly used as a high-energy phosphate donor instead of a carboxyvinyl donor). Only one other enolpyruvyltransferase is known to catalyse a reaction with such a specific mechanism: the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase AroA (EC 2.5.1.19, named AroE in some species), a key enzyme of the shikimic acid pathway. Similar overall structures of MurA and AroA revealed an evolutionary relationship between the two enzymes and suggested that they operate by similar reaction mechanisms [45, 46, 47]. MurA consists of two globular domains, I and II. Each domain has six subdomains. Both consist of six helices and three four-stranded β -sheets connected by a double-stranded hinge region. The N- and C-termini and most of the C-terminal part of the sequence are located in domain I, while domain II contains mainly the N-terminal part. The fixed hinge region connecting the two domains consists of two strands of equal length connected by hydrogen bonds and a salt bridge. Each of the six subdomains consists of the same secondary structures in exactly the same order (β 1, α 1, β 2, α 2, β 3, β 4), so that the structure resembles a sixfold repetition of a foldable subunit [48, 49, 50].

A highly flexible loop connecting $\beta 2$ and $\alpha 2$ in subdomain IIc is the active centre. The catalytic centre is located in the space between domains I and II. When no substrate binds to MurA, the flexible loop forms an open conformation, which in turn changes to a closed conformation upon substrate binding, which is spatially separated and in which two hydrophobic surfaces interact with the uridyl ring of UDP-GlcNAc. MurA undergoes a conformational change from the open to the closed conformation through an induced adaptation mechanism [48, 49, 50]. In addition to the interaction with the substrate UDP-GlcNAc, the actual enzymatic catalysis starts with an attack on PEP. This involves interaction with an active site cysteine (Cys), which in the MurA isoforms of some bacterial species can be exchanged to asparagine (Asp), acting as a catalytic acid in the reaction mechanism. The difference between cysteine and other amino acid substitutions with identical properties is that PEP can form a temporary covalent intermediate with cysteine, which can then be further converted. Such a proposed Cys-PEP intermediate may not be necessary for catalysis, as it is not formed (or is less stable) when Cys is replaced by Asp. At the start of the MurA reaction, as described previously, the stable C-O bond of PEP is cleaved and the enolpyruvyl moiety is transferred to the 3'-hydroxyl group of UDP-GlcNAc, while a tetrahedral reaction intermediate proceeds. [51, 52, 53, 54]

Besides the species-specific amino acid substitutions in the active site, bacteria differ in another way with regard to MurA. In many organisms, such as *E. coli*, there is only one essential copy of MurA, so there is no need to explain essentiality. *B. subtilis*, has two homologous versions: MurAA and MurAB. However, in *B. subtilis*, MurAA is essential, but MurAB is not, although both catalyse the identical reaction. In this case, the enzymes appear to be only partially substitutable at certain stages of growth or under certain conditions. In addition, there are species with even more MurA copies whose functions and specific physiological roles are not yet known. [55, 56, 57]

Because of the high energy expenditure required by the cell to synthesise PGN, precise regulation of the pathway is crucial. MurA is predestined for this role because regulation must occur early in *de novo* synthesis. MurA is mainly controlled by feedback regulation in the form of inhibition by UDP-MurNAc (and subsequent cell wall precursors (Fig.1)). Due to its importance, an additional proteolytic regulatory circuit has recently been discovered in *Listeria monocytogenes*, a Firmicutes bacterium related to *B. subtilis*. An adaptor protein, ReoM, was shown to regulate the ClpCP-dependent proteolytic degradation of MurA. The two-component system PrkA, when activated by unknown cell wall cues, possibly related to mucopeptides, phosphorylates and thereby inactivates ReoM, which in turn prevents the proteolytic degradation of MurA. Furthermore, the described conformation of MurA has a major influence on its proteolytic stability. Substrate binding and transition to the closed conformation increases stability, whereas unbound enzyme in the open conformation is easily degraded. This has been demonstrated by trypsin digestion. [43, 58, 59, 60]

1.5 Inhibition of MurA by fosfomycin



[(2*R*,3*S*)-3-methyloxiran-2-yl] phosphonic acid

C₃H₇O₄P

Exact mass: 138.008198 Da

Molecular weight: 138.06 g mol⁻¹

Figure 3: Fosfomycin. Structure and characteristics of fosfomycin (FOS, phosphomycin).

MurA, the key enzyme of PGN *de novo* synthesis, is the primary target of the antibiotic FOS (Fig.3). The inhibition of MurA by FOS is based on an unusual covalent binding of FOS to a sulfhydryl group of a cysteine (*E. coli* MurA: Cys115, *B. subtilis* MurAA: Cys117). As already described, in some species such as *Chlamydia*, *Exiguobacterium* or *Terribacillus* sp., the Cys is substituted, resulting in a natural resistance to FOS. When FOS binds to MurA, the enzyme is permanently and irreversibly inactivated. This leads to a loss of activity, but also to proteolytic stabilisation. It has been shown that the addition of FOS and UDP-GlcNAc reduces the digestibility of MurA by trypsin, thereby maintaining the protein in a closed conformation. [59, 61, 62]

Chemically, FOS (L-cis-1,2-epoxypropylphosphoric acid) is a phosphonic acid that forms its own antibiotic group of epoxides containing a three-membered epoxide ring, which makes the ether compound more reactive due to the ring strength. The antibiotic was first described in 1969 and isolated from cultures of *Streptomyces* sp. It was later discovered that other *Pseudomonas* species can also produce FOS, albeit via a different metabolic pathway. FOS has a long history of use as fosfomycin tromethamine for uncomplicated urinary tract infections (UTI) or gastrointestinal infections caused by *E. coli* and *Enterococcus faecalis*. Because of its broad-spectrum activity, it is increasingly used to treat infections caused by Gram-positive and Gram-negative bacteria, especially multidrug-resistant pathogens such as extended-spectrum (metallo)-beta-lactamase producing *Enterobacteriaceae*, methicillin-resistant *S. aureus* (MRSA) and *Pseudomonas aeruginosa* strains, usually in combination with another antibiotic. FOS has been reported to be synergistic with many antibiotics of different classes, including antibiotics targeting late steps in cell wall biosynthesis (e.g. carbapenems, cephalosporins, glycopeptide antibiotics), rarely shows cross-resistance and has bacteriostatic and bacteriolytic effects depending on the organism. However, the reason for the synergistic effect of FOS with other drugs is unknown. [63, 63, 65, 66, 67, 68, 69, 70, 71]

Due to its low molecular weight ($138.06 \text{ g mol}^{-1}$), FOS has good tissue penetration, even in inflamed tissue, and its good half-life (mean half-life $\pm$ SD, $5.7 \pm 2.8 \text{ h}$) makes it a suitable antibiotic with favourable pharmacological properties. FOS can also be administered intravenously as fosfomycin disodium. [72, 73, 74]

The entry of FOS into bacterial cells is mainly facilitated by two active transporters: the glycerol-3-phosphate transport system (GlpT) and the hexose phosphate transport system (UhpT). Both transport systems are affected by catabolite repression and play a crucial role in the sensitivity of FOS in clinical and in vivo assays due to the different expression patterns of these transporters. In *E. coli* and *S. aureus*, expression of the UhpT transporter is induced by glucose-6-phosphate via a sensor protein in conjunction with a two-component regulatory system. In contrast, *P. aeruginosa* and *B. subtilis* lack the UhpT transporter and associated sensor system and use GlpT as the only FOS uptake system, which is crucial in anaerobically growing cells where its expression is increased [64, 75, 76]. In addition to the primary target responses and the FOS uptake, the occurrence of resistance is a critical factor in understanding the cellular response. In recent years, the rate of resistance development in relevant studies has been relatively stable at around 3 %, which is comparatively promising. The advantage is that resistance to FOS appears to be associated with high biological costs and loss of fitness of the bacterial strain [77, 78]. However, the opposite conclusion was reached in a recent study of uropathogenic strains [79].

As mentioned above, an important factor in the development of resistance at the cellular level is the transport of FOS. Frequently, mutations occur that lead to reduced expression of the transporters UhpT and GlpT or to up-regulation of the transporters, either by affecting GlpR-dependent transcriptional regulation or indirectly via mutations in the catabolite. In addition, resistance is acquired by rare point mutations in MurA (e.g. cysteine to aspartic acid) as described above. Of particular importance is the enzymatic detoxification of FOS, which in Gram-negative bacteria is mainly carried out by glutathione S-transferases of the FosA family and in Firmicutes mainly by the thiol S-transferase FosB. FosB requires the thiol compound bacillithiol (BSH), which is synthesised from UDP-GlcNAc, the substrate of the primary FOS target MurA. BSH and FosB (as well as glutathione and FosA) are normally critical for resistance to oxidative stress. Detoxification of FOS does not seem to be the primary task due to their enzymatic properties (e.g. FosB: K_m : BSH: 4.2 mM FOS: 17.8 mM). [63, 80, 81] Understanding how FOS affects cellular responses globally, beyond the primary target response, requires detailed research at multiple regulatory levels, transcriptomics, proteomics and metabolomics (focus on cell wall metabolomics). Such comprehensive analyses will be of immense value in addressing the problems associated with AMR and in rationalising a better approach to antibiotic (combination) therapy.

1.6 Fundamental research with HPLC and mass spectrometry and its potential

Mass spectrometry (MS), especially in combination with high-performance liquid chromatography (HPLC), plays an important role in validating the cellular effects of antibiotic-target interactions. HPLC-MS has become increasingly important in recent years for rapid clinical diagnostics and is key to confirming that the right antibiotics are having the expected effect [82]. HPLC is generally used to separate components from a mixture. The sample is pumped through the column together with a solution (mobile phase), the components interact with the column material (stationary phase), resulting in a separation over time. The type of separation depends on the column used and its properties. The time-dependent retention of the components is interpreted by a detector into a computer-processable electrical signal. Common HPLC detectors are refractometric (RI), ultraviolet-visible (UV/VIS) and fluorescence (FL) detectors. The most important detector for diagnostics, but also for basic biological research in general, is MS. The main reason for this is that MS allows not only the detection of compounds, but also their identification. This is achieved by analysing the exact mass of a compound. Ions are distinguished according to their mass-to-charge ratio (m/z). This requires two things. First, the sample must be ionised. There are several ways to ionise: a common method is electrospray ionisation (ESI), other examples are inductively coupled plasma (ICP) and matrix-assisted laser desorption ionisation (MALDI). Secondly, the ions need to be measured using a detector. Again, there are many choices, often a quadrupole, time of flight (TOF) or orbitrap analyser is used. [83, 84, 85, 86, 87]

The opportunities and potential of MS extend far beyond clinical diagnostics. Industrially, the technology has become indispensable in pharmaceutical and forensic analysis. Other applications are more relevant to research. Three important examples are metabolomics, proteomics and native MS [88]. MS-based metabolomics allows the identification of many different (small) metabolites in a complex mixture, for example a specific intracellular state of a bacterial cell. This can be used to study metabolic pathways, their regulation and interactions in detail. The combination of MS-based metabolomics with MS-based proteomics adds another powerful dimension. The latter technology allows quantitative analysis of changes in the cellular proteome of complex samples in response to changing growth conditions, such as FOS stress. It provides a more direct view of cellular responses than transcriptomics. In addition, MS-based proteomics allows the assessment of protein interactions, modifications or, through additional pre-processing, the amino acid sequence. Whereas in classical proteomics applications proteins are either digested or denatured during the measurement, MS-based native metabolomics takes a different approach. The focus is on native macromolecules. This includes tertiary and quaternary protein structures as well as protein complexes. This clearly demonstrates the potential to bring research even closer to biological systems. [89, 90]

1.7 Research objective

Fosfomycin (FOS) is a well-known antibiotic with a broad bactericidal spectrum against Gram-positive and Gram-negative bacteria and, interestingly, shows synergistic effects in combination with many antibiotics from different drug families. It covalently blocks the enzyme MurA, which catalyses the reaction of phosphoenolpyruvate (PEP) with uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) to form UDP-GlcNAc-enolpyruvate, the first critical enzymatic step in peptidoglycan biosynthesis.

The reaction of bacteria to FOS, which leads to cell lysis, is still unknown. It is also unclear how prolonged FOS treatment leads to antibiotic tolerance and ultimately bacterial regrowth. Furthermore, the reason for the often observed synergistic effects of FOS in combination with many antibiotics from different drug families is not understood.

The main objective of this work was to analyse the multiple responses of *Bacillus subtilis* to the antibiotic FOS using multi-omics approaches including transcriptomics, proteomics and metabolomics. Apparently, the lytic, tolerance and synergistic effects of FOS cannot be explained solely by MurA targeting and consequent inhibition of cell wall synthesis. Therefore, the goal was to elucidate the responses of *B. subtilis* to FOS and the metabolic adaptations induced by FOS using multi-omics to gain a global view of the multiple physiological and metabolic effects of the antibiotic FOS. One aim was to obtain transcriptomic and proteomic data from the same culture probe under two different conditions to study, firstly, adaptation to FOS and, secondly, cellular effects that cause lytic effects. As there is a lack of methods and standardised workflows, especially in the context of cell wall metabolomics, the aim was to establish a semi-autonomous cell wall metabolomics workflow that significantly facilitates the investigation of the cellular response of *B. subtilis* to FOS.

I also set out to investigate the paralogous proteins MurAA and MurAB, the two main targets of FOS in *B. subtilis*, and to study their reaction mechanism using native protein MS. Although inhibition of both paralogues has been demonstrated, it is unclear how they relate to each other and what different physiological roles they fulfil in the cell, particularly under the influence of FOS. It is not known how the regulation, stability and degradation of MurA and the intracellular concentration of FOS affect *B. subtilis*.

Other objectives were to determine the minimum inhibitory concentrations of FOS in different media and conditions, to study growth behaviour and resistance development, and to quantify the intracellular concentration of FOS using different selective mutants.

Understanding the potential of FOS as an antibiotic required a combination of precise and well-chosen experimental work, together with specific method development and the use of established omics methods.

2 Results and Discussion

The research presented in this thesis is structured in two main topics. The first topic focuses on an in-depth study of the cellular responses of the Gram-positive model bacterium *Bacillus subtilis* to the antibiotic fosfomycin (FOS) (section 2.1). I analysed MurA, the primary target of FOS, using native mass spectrometry (MS) to identify covalent phosphoenolpyruvate (PEP) modifications that render these enzymes resistant to FOS (section 2.1.1, manuscript 5). I also analysed the growth inhibition, MICs, transport, intracellular concentrations and antibiotic tolerance of FOS (section 2.1.2, manuscript 6). Finally, I comprehensively investigated the global cellular responses of *B. subtilis* to FOS using a multi-omics approach (section 2.1.3, manuscript 7).

The second part of my thesis deals with the optimisation of high performance liquid chromatography (HPLC) and mass spectrometry (MS) applications, including LC-MS analyses and the implementation of a cell wall metabolomics workflow. In this methodological part, I established and developed an LC-MS metabolomics workflow for the assessment of cell wall metabolites (section 2.2.1, manuscript 8) and an HPLC method with fluorescence detection for the analysis of amino acids, including chiral cell wall-derived amino acids and other primary amines (section 2.2.2). Optimised LC-MS workflows and analyses supported several projects in which I was mainly involved on the analytical side: identification of carbasugar derivatives in peptidoglycan biosynthesis (section 2.2.3, manuscript 4), the investigation of chitin and chitin-oligosaccharide metabolism (section 2.2.4, manuscript 1), the characterisation of α -phosphohexomutases activity (section 2.2.5, manuscript 2) and the investigation of gluconate metabolism and its secretion (section 2.2.6, manuscript 3).

2.1 FOS mode of action and global cellular responses in *B. subtilis*: target effects, tolerance mechanisms, growth effects and multi-omics analysis

Antibiotic resistance is a rapidly growing challenge, compounded by the lack of new antibiotic discoveries. To overcome this, detailed studies of existing antibiotics can lead to more targeted use and effective combination therapies. FOS is one such antibiotic. How FOS develops its lytic, bactericidal activity and why it exhibits numerous synergies with different classes of antibiotics cannot be explained solely by its primary target, blocking *de novo* peptidoglycan (PGN) synthesis by inhibiting MurA. A detailed and comprehensive understanding requires research on several levels. In addition to analyses of the known target, a comprehensive analysis of the cellular response and growth physiology is required.

2.1.1 Understanding the FOS target MurA: occurrence of covalently modified enzyme species, exploration of its enzymatic properties, investigation of inhibitory effects and deciphering of tolerance mechanisms involved in peptidoglycan synthesis

PGN is essential for the bacterial cell and is important for stability, resistance to turgor pressure and cell shape. Because of this importance, PGN *de novo* synthesis is a natural target for antibiotics, also because the synthesis is tightly regulated due to the high expenditure of the cell and thus has an impact on the entire metabolism of the cell [17, 23]. The main key enzyme of PGN *de novo* synthesis and the catalyst of the first committed step is MurA, a UDP-*N*-acetylglucosamine-1-carboxyvinyltransferase (EC 2.5.1.7 (IUBMB)). MurA catalyses the reaction of phosphoenolpyruvate (PEP) and uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) to form UDP-GlcNAc-enolpyruvate with the release of inorganic phosphate [42, 43]. *B. subtilis*, like many Firmicutes, has two genetically very similar versions of MurA: MurAA and MurAB. Although both catalyse the same reaction, only MurAA is essential. The described unusual covalent binding of the FOS sulfhydryl group to a cysteine in the active site region of MurA, which is crucial for the catalysed reaction, leads to an irreversible inhibition of the catalytic activity. [51, 52]

In manuscript 5 - *Native mass spectrometry reveals that MurA carboxyvinyltransferases are primarily phosphoenolpyruvate-bound and therefore fosfomycin insensitive* - the covalent binding of PEP to *B. subtilis* MurAA and MurAB was demonstrated for the first time using a native MS approach. Native MS allows the mass of an intact protein to be measured in multiple ionisation states, thereby enabling the identification of specific interactions and binding. Using this method, in collaboration with the functional metabolomics lab of Daniel Petras, it was shown that heterologously expressed MurAA and MurAB, which had undergone two chromatographic steps for isolation, still contained a covalently bound PEP molecule (manuscript 5, Fig.3, Fig.4). Trypsin digestion and proteomic measurements confirmed that a cysteine (Cys) in the active site (Cys137 in MurAA and Cys136 in MurAB) carries this modification (manuscript 5, A18, A19). This clearly demonstrated that MurA has a post-translational modification (ptm) with PEP that is strong enough to withstand complete protein purification (manuscript 5, Fig.3, Fig.4). FOS could not react with PEP-bound MurA and was not able to interfere with this ptm [50]. Because FOS and PEP compete for the same Cys, FOS can only covalently bind MurA and thus inactivate it when PEP has been removed from the enzyme. This was observed for the reaction turnover of PEP with UDP-GlcNAc of MurAA (manuscript 5, Fig.3). MurAB behaves slightly differently. MurAB also showed the stable PEP ptm, but when both substrates (with and without FOS) were added, additional products were formed with MurAB that could be identified by native MS (manuscript 5, Fig.4). This may be the intermediate product of UDP-GlcNAc-ep and PEP described in the literature [54].

Differences between the two versions of MurA could also be clearly seen in the inhibition assays with FOS and the kinetic data collected as part of this work (manuscript 5, Fig.2). The performed enzyme assay was based on the measurement of the released free inorganic phosphate by the complexation with malachite green and molybdate. It has been clearly shown that MurAA and MurAB are inhibited to different degrees by FOS. Whereas MurAB retains well over half its activity in the presence of $50 \mu\text{g mL}^{-1}$ FOS, MurAA retains only one tenth of its activity. The FOS sensitivity of both enzymes was significantly increased by a fivefold excess of PEP corresponding to UDP-GlcNAc. Conversely, relatively more UDP-GlcNAc increased the efficacy of FOS (manuscript 5, Fig.2). These two independent experiments confirmed again that only a free cysteine in the active site is a target for FOS. Inhibition of MurA by FOS is therefore only possible with *de novo* synthesised MurA or MurA with active turnover. Similar to FOS, the product of the MurB reaction, UDP-MurNAc, is also able to inhibit MurA. UDP-MurNAc is a very competent inhibitor and almost completely inhibited MurAA at $10 \mu\text{g mL}^{-1}$ (Fig.4). Ten times more FOS was required for the same inhibition (manuscript 5, Fig. 2).

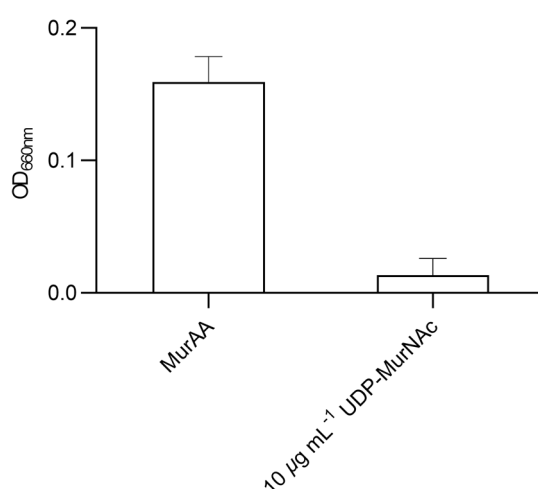


Figure 4: UDP-MurNAc inhibition of *B. subtilis* MurAA. The reaction was carried out at 37°C for 1 h. The inhibition of $1 \mu\text{M}$ *B. subtilis* MurAA was tested with different UDP-MurNAc concentrations ($0 - 100 \mu\text{g mL}^{-1}$) shown are $10 \mu\text{g mL}^{-1}$. Substrate concentrations: 1 mM for both PEP and UDP-GlcNAc. Buffer: 20 mM Tris-HCl, 50 mM NaCl pH 7.5. Phosphate was determined by a colour reaction with malachite green and ammonium molybdate. The OD was determined at 660nm , the mean was plotted with SD from triplicates.

The importance and central role of MurA for *de novo* cell wall synthesis, also from a regulatory point of view, became clear through this strict feedback regulation of the subsequent metabolite UDP-MurNAc. By inhibiting MurA and the resulting accumulation of UDP-GlcNAc (manuscript 8, Fig.6), FOS simulates a feedback regulation in *B. subtilis* in a similar way as UDP-MurNAc, with the difference that cell wall synthesis is not supersaturated and thus influences other metabolic pathways (see following sections 2.1.2 and 2.1.3) in addition to the direct effect on cell wall synthesis. [23, 32, 43, 59]

Further differences can be seen when analysing the determined kinetic parameters. While MurAA has a higher substrate specificity, MurAB has a higher reaction rate and turnover rate at substrate saturation (manuscript 5, Table1, A17).

As mentioned above, the active site Cys plays a crucial role in FOS binding, although this residue is not essential for enzymatic activity. This is evidenced by the existence of MurA variants in certain bacteria that carry a Cys to Asp mutation but retain activity. Apart from MurA, there is one other enzyme with the same specific reaction mechanism that allows the unusual cleavage of a very stable C-O bond (see introduction). This is the 5-enolpyruvylshikimate-3-phosphate synthase (EPSP) AroA (EC 2.5.1.19), a central enzyme in the shikimic acid pathway. Whereas the reaction of MurA and AroA is almost identical, AroA is insensitive to FOS (manuscript 5, Fig.1). The explanation for this is that the central Cys in AroA is replaced by glutamic acid and therefore cannot bind FOS [45]. This effect can also be seen in the MurA relationship and distribution between species. The number of paralogs per species differs significantly, as does the presence of cysteine in the active site, which leads to natural FOS resistance when exchanged for another amino acid.

As previously described, the primary target of FOS analysed here cannot explain the lytic effect of FOS. This requires more detailed analyses of growth under the influence of FOS (section 2.1.2) and the influence on the overall metabolism (section 2.1.3) are required.

2.1.2 Effects of FOS on *B. subtilis* growth: Exploring of inhibition, tolerance development, transport mechanisms, and intracellular concentration

The next logical step after studying the FOS-target interaction was to analyse the global effect of the antibiotic on the growth and metabolism of *B. subtilis*. Firstly, the minimum inhibitory concentration (MIC) of FOS was determined in different media; secondly, the growth behaviour and the development of resistance to interference with FOS inactivation (FosB) and transport (GlpT) were studied; and thirdly, the intracellular concentration of FOS was measured. These studies are described in detail in manuscript 6 - *Investigation of the inhibition of Bacillus subtilis growth by fosfomycin: The role of MurAA and MurAB targets, drug transport, intracellular accumulation and tolerance development*.

The conventional method for MIC determination is the use of MIC test strips, performed according to CLSI guidelines and standardised according to MC-Farland (manuscript 6, Fig.1, A1, A2). A MIC of $18.7 \mu\text{g mL}^{-1}$ for FOS was determined for *B. subtilis* wt. In addition to the growth-free zone (MIC), another area could be identified on the plates (manuscript 6, Fig.1, A1), which appeared like a halo around this inner inhibition zone. This area forms an intermediate zone between growth and non-growth on the plate which, after prolonged incubation, has completely regenerated and formed a uniform bacterial lawn in contrast to the growth-free inner zone.

Cultures in this range survive FOS treatment through reduced growth and metabolism, both of which increase when less FOS is available. The investigation of MIC is meaningful for understanding the response to FOS when different genetic backgrounds are compared. Specific *B. subtilis* strains were used to investigate the most important factors in *B. subtilis* FOS sensitivity. Four major genetic factors were identified: *glpT* (transport: glycerol-3-phosphate permease), *fosB* (FOS detoxification mechanism: bacillithiol-S-transferase), *murAA* and *murAB* (main targets of FOS, only MurAA is essential in *B. subtilis*, interaction and relationship of the paralogs is unclear) and *reoM* (proteolysis of MurAA and possibly MurAB: adaptor protein to initiate proteolytic degradation) [80, 91, 92, 94].

Remarkable was the influence of the thiol S-transferase FosB, which hydrolyses FOS, causing a ring opening and generating an inactive bacillithiol-FOS compound [80]. In the absence of this mechanism (*B. subtilis* $\Delta fosB$ ($\Delta fosB$)), FOS sensitivity increased 10-fold (MIC = 1.8 $\mu\text{g mL}^{-1}$, manuscript 6, Table1, Fig.1, A1). The glycerol-3-phosphate permease GlpT is the only described FOS transporter in *B. subtilis*. Deletion of GlpT significantly affected FOS sensitivity, as shown in this study, with the $\Delta glpT$ mutant (*B. subtilis* $\Delta glpT::erm$ ($\Delta glpT$)) being 10-fold more resistant to FOS (MIC = 117.3 $\mu\text{g mL}^{-1}$, manuscript 6, Table1, Fig.1, A1) [91, 92]. Notably, an additional transporter for FOS has been proposed in *Listeria monocytogenes* [93]. It could not be excluded that the homologue of this oligopeptide transporter, Opp, also imports FOS in *B. subtilis*, but it does not seem to be induced under our conditions. The $\Delta reoM$ mutant (*B. subtilis* $\Delta yrzL::kan$. ($yrzL=reoM$), ($\Delta reoM$)) also increases the resistance to FOS 2-fold compared to wt (MIC = 42.7 $\mu\text{g mL}^{-1}$, manuscript 6, Table1, Fig.1, A1). Recently, ReoM, depending on its phosphorylation state, was found to regulate the ClpCP-dependent proteolytic degradation of MurA, which is the main regulation mechanism of MurA. PrkA senses muropeptides or changes in the PGN pattern and phosphorylates ReoM, which in turn prevents interaction with MurA and leads to proteolytic degradation. In the absence of ReoM, MurAA degradation is not activated, and it is apparent that a greater amount of MurAA requires more FOS for the same inhibitory effect, making the $\Delta reoM$ mutant more resistant to FOS. [94]. Deletion of MurAB (*B. subtilis* $\Delta murAB::kan$ ($\Delta murAB$)), the MurAA paralogue, had no significant effect on sensitivity to FOS compared to the wt (MIC = 24.0 $\mu\text{g mL}^{-1}$, manuscript 6, Table1, Fig.1, A1). So far, the physiological role of MurAB is unclear. The lack of a MIC effect indicates that MurAB does not play a crucial role during vegetative growth on lysogeny broth (LB).

This highlights a key problem with classical MIC endpoint determination in liquid and solid media, which applies to *B. subtilis* wt and all mutants tested. For the purpose of analysing the inhibitory effect of antibiotics on bacterial growth, the MIC endpoint is of very limited value. It is much more useful to monitor growth (OD_{600nm}) over time and analyse how the antibiotic, in this case FOS, affects growth or how the bacteria adapt to the conditions.

This observation also has the advantage and opportunity to identify critical growth phases, e.g. under FOS treatment, for further experiments. Fig.2 in manuscript 6 (additionally manuscript 6, A4) shows a comprehensive experiment with the addition of FOS in the exponential growth phase at different concentrations and a 24-hour monitoring period. Even when looking at the growth curves in the absence of FOS, slight changes in the stationary phase could be seen (such as a decrease in OD_{600nm} in the $\Delta murAB$ mutant and an increase in the $\Delta fosB$ mutant) that would not be seen if only the endpoint value was considered. Differences in the growth behaviour became apparent with FOS treatment, and the erroneous determination of the endpoint was impressively confirmed evident when culture regrowth was examined (manuscript 6, Fig.2). The $\Delta murAB$ mutant showed the least difference in the treatments compared to wt. However, the decrease in growth in the stationary phase was noticeable; the reason for this could be that the lack of MurAB activity only has an effect in the stationary phase, as MurAB may only be active there [43]. While all other strains were only slightly affected by 4 $\mu\text{g mL}^{-1}$ FOS, the $\Delta fosB$ mutant showed a clear lytic effect. In contrast, the $\Delta reoM$ mutant only showed an effect at 1024 $\mu\text{g mL}^{-1}$ FOS, whereas the $\Delta glpT$ mutant was still barely affected.

In addition to FOS treatment, the choice of growth medium also has a significant effect on the growth of *B. subtilis*. When Mueller-Hinton broth 2 (cation-adjusted) (MHII) was used for MIC tests on plates, resistance increased (manuscript 6, A2), the gluconeogenic enhancement of MHII affecting fitness compared to the LB used. In liquid medium, MHII showed no difference to LB medium (manuscript 6, A3). There was no advantage over the used standard LB. The use of various minimal media with glucose as carbon source was not an alternative in either liquid or solid media due to the excessively long growth times (manuscript 6, A3). To address another issue directly related to susceptibility, the development of resistance to FOS in *B. subtilis* was further investigated. According to the literature, the development of resistance is usually caused by mutations in MurA (amino acid exchange, see section 2.1.1) or mutations that directly or indirectly affect the transporter GlpT [63, 75, 91, 92]. Studies in solid media (manuscript 6, A6) and liquid media (manuscript 6, A5) showed no genetically manifested resistance formation in *B. subtilis* wt and $\Delta fosB$ mutant. Although the $\Delta fosB$ mutant occasionally showed slight changes, the level of resistance was never reached; presumably this was also an adaptation rather than a genetically manifested resistance.

Since mutations appear to play a minor role in the effects of FOS, it is even more important to know how much FOS is present in the cell at any given time. In a 24-hour experiment, the intracellular and extracellular concentration of FOS was determined in addition to the OD_{600nm} (manuscript 6, Fig.3). The FOS concentration was determined using LC-MS. For wt, the $\Delta murAB$ and $\Delta reoM$ mutants it was noticeable that the extracellular FOS concentrations of the 128 $\mu\text{g mL}^{-1}$ used hardly decreased.

The amount of FOS available in the medium was therefore never limiting. Confirmation that FOS was indeed being absorbed was obtained by measuring the intracellular concentration of FOS. FOS was absorbed immediately after treatment, the intracellular concentration in the cells dropped rapidly and then stabilised (manuscript 6, Fig.3). The approximate intracellular concentration was 10-20 $\mu\text{g mL}^{-1}$, but it should be noted that only unbound FOS could be determined. The combination of regrowth (manuscript 6, Fig.2, A4) and stabilisation of the intracellular FOS concentration suggests an adaptation of the metabolism, as otherwise the regrowth cannot be explained. The ΔfosB mutant showed an identical profile at lower concentrations. Extracellularly, a change in FOS concentration was measurable due to the lower concentrations used, in contrast to the wt experiments, but intracellularly the change was about 10-fold smaller.

The results obtained show a clear adaptation of *B. subtilis* strains to FOS without developing genetic resistance. The immense influence and importance of FosB and GlpT for *B. subtilis* during FOS treatment was clearly demonstrated. The central role of MurAA was also highlighted. To relate these specific effects to the overall cellular response of *B. subtilis* to FOS, a holistic analysis of the cellular status during FOS treatment is required (see 2.1.3).

2.1.3 Investigation of the cellular responses of *B. subtilis* to FOS using transcriptomics, proteomics and metabolomics

A comprehensive analysis of the responses of *B. subtilis* to FOS using transcriptomics, proteomics and metabolomics is described in manuscript 7 - *Comprehending the responses of Bacillus subtilis to fosfomycin using multi-omics*. In this analysis, two different setups were considered, one investigating cellular responses leading to FOS-induced cell lysis and the other investigating the adaptive cellular response of *B. subtilis* to FOS. The two setups differ in the timing and amount of FOS addition. In both setups, wt and the ΔfosB mutant were examined, allowing the effects of two very different concentrations of FOS to be assessed with two almost identical strains. Due to the presence of the FosB detoxification mechanism in the wt strain, it can withstand much higher concentrations of FOS than the ΔfosB mutant strain. Therefore, concentration-dependent effects could be excluded in this comparison. In summary, the omics results of wt and ΔfosB mutant were largely similar and only slightly different, providing a detailed, accurate and comprehensive picture of the cellular effects of FOS.

Setup 1 (S1) allowed to study the adaptation of *B. subtilis* to FOS. Since there was no growth deficit (manuscript 7, Fig.1, A3), no UDP-GlcNAc accumulation and neither the proteome nor the transcriptome hits were related to cellular stress (manuscript 7, section: *Summary of cellular responses of B. subtilis to FOS revealed by muti-omics*), it could be assumed that all differentially expressed genes and proteins with different abundance were related to an adaptation to the presence of FOS.

On the contrary, setup 2 (S2) allowed the analysis of the lytic effect and thus the stress response of *B. subtilis* to FOS (manuscript 7, Fig. 1, A3) [63, 65, 66]. Individually, both the transcriptome and proteome showed that the differences between individual conditions and treatments were significantly higher in S2 than in S1 (manuscript 7, Fig.2, Fig.3, A5, A6). The key question for understanding the cellular effects of FOS was how FOS, as a cell wall inhibitor of MurA, induces a lytic effect against *B. subtilis* and why FOS often shows synergy with several other antibiotics of different classes. This question could be answered by looking at setup 2 (S2), where a clear lytic effect was present. Many changes in expression levels in the transcriptome and proteome in the S2 analysis were related to cell envelope stress (manuscript 7, Fig.4, Fig.5, Fig.6, Fig.7), and the metabolomics results also showed that S2 FOS treatment induced a cell wall stress response and unbalanced the PGN *de novo* synthesis (manuscript 7, A13; manuscript 8, Fig.6).

Inhibition of MurA, the UDP-*N*-acetylglucosamine-1-carboxyvinyltransferase (EC 2.5.1.7 (IUBMB)), by FOS, is expected to result primarily in the accumulation of the two reaction educts UDP-GlcNAc and PEP, and the depletion of all subsequent cell wall precursors [42, 43, 57]. Exactly these changes (apart from PEP, see below) were indeed the statistically relevant metabolomics results shown for S2. (manuscript 8, Fig.6). Due to the lack of cell wall precursors, D-Ala-D-Ala, the substrate for the formation of the last soluble cell wall precursors UDP-MurNAc-pentapeptide (MurF), accumulated significantly. The expected accumulation of PEP could not be measured. Instead, the concentration of PEP decreased as a result of the FOS treatment (manuscript 8, Fig.6). Presumably, PEP is internalised due to its numerous involvement in metabolic pathways, which further disturbs the metabolic balance in the cell.

Stress responses observed in S2 were mainly categorised under cell envelope stress and are controlled by SigM, Y, W, X, Y. Apart from SigY regulated hits (only upregulated in the transcriptome analysis), a significant amount of genes under the control of these sigma factors were significantly upregulated (manuscript 7, section: *Investigation of the lytic effect on B. subtilis by FOS using a multi-omics approach*). In particular, the SigV regulon was upregulated, which is normally induced by lysozyme stress and therefore leads to resistance to autolytic enzymes. Its upregulation by FOS has not been previously described [95]. The relevance of SigV regulation was confirmed by an additional experiment (Fig.5). If cells treated with FOS (experimental setup: S2) induce a SigV stress response, they should be more resistant to lysozyme digestion than cells in which the SigV regulon is not upregulated. Induction of the SigV regulon leads to O-acetylation of PGN and D-alanylation of teichoic acids, which renders these cells lysozyme resistant [96].

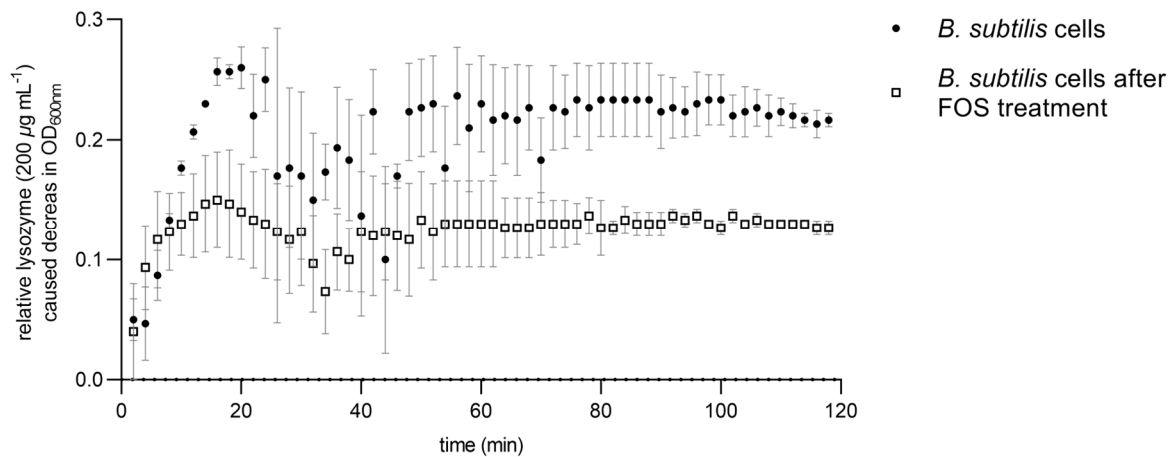


Figure 5: Lysozyme digestion of FOS treated cells. Cells after S2 FOS treatment ($128 \mu\text{g mL}^{-1}$ exponential growth phase, manuscript 8) were harvested (5000 g, 10 min), dissolved in PBS (pH 6.7) and digested with lysozyme (120 min, 37°C) ($n=3$). Control: FOS untreated cells ($n=3$). Lysis was determined by measuring the $\text{OD}_{600\text{nm}}$. As the cells lyse slowly even without lysozyme, parts of the identical sample were incubated without lysozyme for both sets. Shown is the lysozyme induced decrease in $\text{OD}_{600\text{nm}}$ relative to the decrease without lysozyme, of cells with and without FOS treatment.

Fig.5 illustrates that FOS-treated cells are proportionally less affected by lysozyme and lyse less efficiently than the untreated control. PGN modification and teichoic acids are also involved in other non-stress-related FOS responses (see below). Interestingly, under the influence of FOS, PGN *de novo* synthesis was slightly upregulated whereas PGN degrading enzymes such as autolysins, peptidases and amidases were downregulated (manuscript 7, section: *FOS stress leads to differential regulation of various players associated with the PGN*) [32, 34]. Regulation of PGN synthesis, as well as PGN recycling and degradation, played a central role in the cellular response of *B. subtilis* to FOS. This was evident not only from the inhibition of the primary target, MurA, and the resulting shutdown of PGN *de novo* synthesis, with an attempt to compensate by upregulating PGN synthesis and downregulating degradation, but also from the PGN modification just described, mediated by the SigV regulon.

Another outstanding stress-related response of *B. subtilis* to FOS was the upregulation in the proteomics results of the stress regulator Spx (manuscript 8, Fig.5, Fig.7). Spx has been described as a regulator of cell wall and disulfide stress. It is mainly active during heat and oxidative stress and regulates 52 genes [97, 98, 99]. A further understanding of the Spx response, as already described (manuscript 8. section: *B. subtilis regulates the cellular FOS stress response via Spx, thereby initiating an oxidative stress response*), can be gained by looking at Fig.6, which shows the physiological effect of a ΔspxA (Δspx) mutant under the influence of FOS. In the absence of Spx, cells survive FOS treatment better. The stress response induced by Spx that was observed for S2 seems to push the cell further out of energetic balance and exacerbate the problems caused by FOS. It is reasonable to assume that Spx is upregulated by SigM, W or X and enhances the stress response, thus playing an important role in cell lysis in *B. subtilis*.

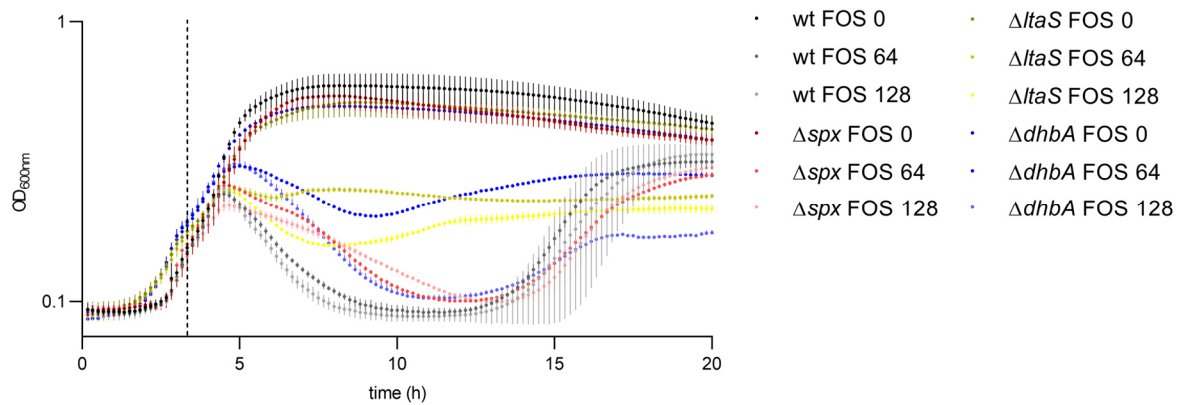


Figure 6: Growth curves of *B. subtilis* mutants under the influence of FOS. Growth curves of the strains: *B. subtilis* 168 (*trpC2*) = wt, *B. subtilis* (*trpC2*) Δ *spxA::kan* = Δ *spx*, *B. subtilis* (*trpC2*) Δ *ltaS::kan* = Δ *ltaS*, *B. subtilis* (*trpC2*) Δ *dhbA::kan* = Δ *dhbA*, all highly upregulated in proteomic analysis (manuscript 8). FOS treatment with 64-128 μ g mL⁻¹ after 3.5 h of incubation. Performed in quadruplicates or duplicate in 24-well plates covered with a semi-permeable sealing membrane (Breathe-Easy, Z380059, Sigma-Aldrich) shaking at 37 °C using the EPOCH2, BioTek Absorbance Microplate Reader, Agilent Technologies, by measuring OD_{600nm} every 10 min.

In order to bring the multitude of analyses and evaluation possibilities of the transcriptome and proteome analyses of S1 and S2 of *B. subtilis* wt and *B. subtilis* Δ *fosB* on a common basis and to work out overlaps, some sample characteristics have to be given: The heatmap and PCA analyses clearly showed that the wt and the Δ *fosB* mutant behave almost identically and can be used as an additional replication in all results (manuscript 7, Fig.2, Fig.3, A5, A6). This and the use of trend analyses between proteome and transcriptome (OmicsTIDE, manuscript 7, Fig.8, A14, A17, A18), Venn comparisons (manuscript 7, A9, A10, A11, A12) and metabolic dependency analyses (manuscript 7, digital appendix Zenodo A19 [32]), confirmed the significance and comparability of the results. This allows the results to be condensed to a common core for both sets and both technologies (manuscript 7, Fig.8, section: *Investigating the adaptation of B. subtilis to FOS using a multi-omics approach*).

These combined analyses showed that FOS treatment of *B. subtilis* resulted in increased lipoteichoic acid (LTA) and teichoic acid (TA) synthesis. Three of the four LTA synthases were upregulated, as were the ABC transporters of teichoic acids. In addition, the enzymes that carry out D-alanine esterification on TA were upregulated, which is related to the SigV operon (see above). Growth monitoring of the deletion mutant of the main LTA synthase LtaS (Fig.6) demonstrated that without LtaS *B. subtilis* was more resistant to FOS and showed reduced lysis compared to the wt. The effect that too much LTA activity increases cell lysis has been shown in other organisms and appears to be the case in *B. subtilis*. In fact, the highly upregulated LTA synthases appear to have a negative effect on the survival of *B. subtilis* under FOS conditions, as deletion reduces cell lysis. [18, 100, 101]

Overall, FOS appears to disrupt the cellular metabolic balance of *B. subtilis*. On the one hand, candidates of fatty acid synthesis were upregulated, whose synthesis consume or generates central cofactors such as NADPH, NADH and CoA, and on the other hand, FOS interacts with iron homeostasis, which also uses NADH as a central cofactor (manuscript 7, section: *Summary of cellular responses of B. subtilis to FOS revealed by multi-omics*) [102]. The ATP/ADP energy charge of the cell does not deteriorate dramatically nor does ppGpp accumulate (results not shown). The disturbance in the energy balance of the cell was also demonstrated by examining the growth behaviour of the $\Delta dhbA$ mutant (Fig.6). DhbA is a central enzyme for the biosynthesis of the siderophore bacillibactin and thus an essential component of iron acquisition and cell homeostasis and was strongly upregulated under FOS treatment (manuscript 7, section: *Comprehensive analysis of transcriptomic and proteomic analyses to investigate the lytic effect of FOS on B. subtilis*). Without this central pathway, *B. subtilis* showed a reduced sensitivity to FOS compared to the wt. Either the increased iron uptake or, in comparison, higher concentrations of NADH when DhbA is very active were unfavourable for *B. subtilis* and enhanced cell lysis [103, 104]. It has been shown that upregulation of the genes that synthesise bacillibactin can produce salicylic acid-like substances that are toxic to the cell [105]. This explained an outstanding hit, the phenolic acid decarboxylase PadC. PadC was also stress-independent (upregulated in both S1 and S2; manuscript 7, section: *Comprehensive analysis of transcriptomic and proteomic analyses to investigate the lytic effect of FOS on B. subtilis*) upregulated and could play an important role in detoxifying the resulting acids and thus the FOS treatment [106, 107].

Another independent proteomics approach showed the influence of FOS on glycolysis, one of the major metabolic pathways which in turn is related to the energy balance of *B. subtilis*. By adapting the proteomics workflow (for details see method section of manuscript 7), proteins that are covalently modified by FOS, such as MurA, could be identified. After considering different approaches and evaluating the binding site, two promising hits were identified (Table1).

Table 1: FOS off targets in *B. subtilis* determined by proteome analyses. Proteomics samples were searched for specific FOS modifications at accessible cysteine residues in proteins. In addition to the main proteomics measurements, 2 additional sets were prepared for this purpose (no reduction in protein isolation). Methodology is described in manuscript 7.

	GapA	MalA
synonyms genes	<i>gapA</i>	<i>malA, glvG, glvA, mdxK</i>
BSU	33940	08180
molecular weight kDa	35.68	50.35
category	catabolic enzyme in glycolysis	maltose utilisation
activity description	glyceraldehyde 3-phosphate dehydrogenase	6-phospho-alpha-glucosidase
E.C.	1.2.1.12	3.2.1.122
FOS-binding site, function in protein AA	152, 151-153 = G3P binding site	171, 171 Mn ²⁺ binding, 172 active site

[108, 109]

GapA, a central enzyme of glycolysis and the transhydrogenation cycle with GapB to balance NADPH, showed a FOS modification at amino acid position 152 (glycerol-3-phosphate binding site) and should therefore lose its activity in the presence of FOS and contribute to the cellular metabolic imbalance in *B. subtilis* (Table1) [108, 109]. Another candidate that bound FOS at a central site of the enzyme was MalA (Table1). As the hit could be attributed to maltose utilisation, it can be assumed that the FOS binding had no additional influence that was not already been determined by proteome and transcriptome analyses. Because metabolism was adapted under FOS conditions, many degradation pathways were downregulated, such as pyrimidine biosynthesis, the utilisation of the amino acids arginine, citrulline and ornithine, alanine/serine, sorbitol/glucitol, inositol, proline, histidine, acetoin or maltodextrin. Central cofactors are also involved (manuscript 7, section: *Summary of cellular responses of B. subtilis to FOS revealed by multi-omics*).

These studies provide a nuanced understanding of the complex cellular responses to FOS, reveal several new hypotheses, shed light on the potential of FOS as a therapeutic agent and provide insights into bacterial adaptation mechanisms.

2.2 Development of methods and experiments for HPLC and LC-MS and associated data evaluation and analysis

The integration of mass spectrometry (MS), especially in combination with high performance liquid chromatography (HPLC), is a key element in the quest for accurate and rapid clinical diagnostics, pharmaceutical or forensic analysis. In addition, this technology can be an important building block for the targeted use of antibiotics by determining the metabolic state of a cell. To exploit the technology and capabilities of HPLC and MS and their powerful combination in these areas, intensive and specialised method development is required. A detailed understanding of the methods and their development for specific research questions is essential to exploit their potential. The focus was on the development and establishment of methods for the separation of complex samples by HPLC and subsequent detection, e.g. by MS, including sample preparation and presentation of the results (2.2.1, 2.2.2). The use of established methods to address specific biological questions was another focus (2.2.3, 2.2.4, 2.2.5, 2.2.6).

2.2.1 Metabolomics workflow for the measurement of cytosolic peptidoglycan precursors in bacterial cell walls

The development of a workflow involves more than just method optimisation. In order to develop a user-friendly and complete workflow, all aspects from the experiment to the presentation of the results must be optimised and harmonised. Fig.7 shows the outline of the workflow developed in manuscript 8 - *A cell wall metabolomics workflow to determine cytosolic peptidoglycan precursor levels in bacteria*.

The *de novo* biosynthetic pathway for PGN is crucial for most bacteria to form a stable cell wall that protects them from environmental stress and osmotic lysis [29, 30, 31, 32]. Due to its central role in bacterial metabolism, PGN synthesis is the target of many antibiotics. Detailed information about these antibiotics can be obtained by metabolomic analysis of PGN precursors. Therefore, an optimised LC-MS-based workflow has been developed for the analysis of all soluble cell wall precursors, from sample preparation to presentation of publication-ready figures. The workflow is based on an expandable in-house library containing all precursors of PGN *de novo* synthesis (including all non-commercially available metabolites, manuscript 8, A3, A4).



Figure 7: Visualisation of a whole LC-MS workflow. Example of the implementation of the individual steps of manuscript 8: A cell wall metabolomics workflow to determine cytosolic peptidoglycan precursor levels in bacteria: 1: cultivation of different bacterial strains and different treatments, 2: fast cooled vacuum fast filtration to harvest the cells and freezing in 60 % ethanol with liquid nitrogen, 3: cells disruption (thawing, shaking, vortexing) centrifugation and speed vac drying, 4: LC separation and MS measurement, 5: bioinformatics evaluation via targeted and untargeted mass search, metabolite identification, statistics and visualisation. Created with BioRender.com.

The development of the workflow started with the establishment of a stable biological experiment. *B. subtilis* wt (with and without FOS) (developed in connection with manuscript 7 setup 2: Fig.1, A3), *B. subtilis* CRISPRi:*murC* (with and without induction) and *E. coli* BW25113 (identical to manuscript 7 S2) were grown in a 30 mL culture in LB medium (see manuscript 8 methods section for details). Various options were tested for subsequent harvesting of the cultures and cell disruption (manuscript 8, Fig.2). The best results were obtained by rapid filtration, quenching the cells with liquid nitrogen, a washing step in saline and cell disruption by freezing and thawing. The extraction solution used was 60 % ethanol [110]. This protocol outperformed methods such as centrifugation or glass bead disruption, both of which are more time-consuming. Glass beads are also more complex to handle experimentally and damage metabolites such as nucleotides (manuscript 8, Fig.2). A fast and standardised LC-MS method was achieved using a specially developed 7 min gradient (after 2.5 min of equilibration with 100 % A (0.1 % formic acid and 0.05 % HCO_2NH_4), a 1 min gradient up to 5 % B (CH_3CN), followed by a 4 min gradient up to 70 % B, after 2 min at 70 % B, a re-equilibration step of 2.5 min at a flow rate of 4 mL min^{-1} on a reversed-phase C18 Gemini column ($3 \mu\text{m}$, $110 \text{ \AA}$, $150 \times 2 \text{ mm ID}$, Phenomenex; 0.2 mL min^{-1} , 37°C) in an ACQUITY ultra-performance liquid chromatography (UPLC) system (Waters Corporation)) (manuscript 8, Fig.3).

Optimised MS settings were selected for the positive ion mode in the relevant mass ranges (LTQ Orbitrap Velos (Thermo Scientific)) (manuscript 8, Fig.3). 5 μ L of each sample was injected (manuscript 8, method section).

A crucial part was data analysis, for which a semi-automated workflow was developed. The method provided automated feature recognition and data analysis but was not fully automated to allow data exploration for specific metabolite discovery. Identification of PGN precursors was based on retention time, accurate mass detection (m/z^{-1} at MS1 level) and quantification using extracted ion chromatograms within defined limits, based on the in-house generated cell wall standard library. The technical implementation was based on OpenMS in combination with UmetaFlow [111, 112, 113]. In parallel, an untargeted metabolomics approach was applied to reveal differences in metabolite expression between experiments and to confirm reproducibility within sample replicates using principal component analysis (PCA) (manuscript 8, Fig.4) and hierarchical clustering coupled with heatmap analyses (manuscript 8, Fig.5) [114, 115].

In addition, the described workflow also allowed statistically relevant analysis and visualisation of changes between different conditions (manuscript 8, Fig.6, Fig.7), such as the effect of FOS on *B. subtilis* or CRISPRi silencing of *B. subtilis* CRISPRi:*murC* metabolically with respect to the cell wall precursor. Although the focus and optimisation were based on the Gram-positive model organism *B. subtilis*, it has been shown that the workflow also works with Gram-negative organisms (see manuscript 8). However, depending on the application, additional optimisation may be required. For other metabolites or pathways, the identification of soluble cell wall precursors is also simple, reliable and reproducible, but a different focus may require different fine-tuning of the workflow. The practical application of the developed workflow was demonstrated by its use in the multi-omics study of the cellular response of *B. subtilis* to FOS (2.1.3, manuscript 7).

2.2.2 HPLC fluorescence detection method for primary amino group analysis

An HPLC fluorescence method offers a simpler alternative to the LC-MS metabolomics workflow described in section 2.2.1 and allows efficient and targeted analysis in a specific area with less complexity. Although the scale and coverage of basic MS detection is challenging, difficulties arise with metabolites of identical mass. By developing a standardised pre-column derivatisation in which primary amino groups react in an alkaline medium with *o*-phthaldialdehyde in the presence of NAC (*N*-acetyl-L-cysteine) to form intensely fluorescent derivatives in less than one minute, in conjunction with an optimised HPLC gradient, it was possible to separate primary amines by chirality and detect them at pmol levels. Derivatisation and detection work identically with general mercaptans such as 2-mercaptoethanol instead of NAC, but separation by chirality is not possible with this reagent. [116, 117, 118, 119, 120]

Using the programmable autosampler (CM5260) of a Chromaster HPLC (Hitachi) and the fluorescence detector (CM5440; extinction 330 nm, emission 440 nm) in combination with a precise gradient (description Fig.8) for a reversed-phase C18 Gemini column (5 μm , 110 $\text{\AA}$, 150 x 4.6 mm ID, Phenomenex; 1 mL min⁻¹, 40 °C), it was possible to separate 18 of the 19 proteinogenic amino acids with primary amino groups. The method worked equally well for amino sugars and dipeptides with primary amino groups. For the derivatisation, 340 μL sample was mixed with 145 μL borate buffer (50 mM boric acid, pH 9.5) and 77 μL OPA reagent (30 mg *o*-phthalaldehyde, 30 mg NAC/2-mercaptoethanol, 0.5 mL borate buffer, 4.5 mL methanol). 100 μL of this mixture was added to 400 μL 40 mM monosodium phosphate (pH 7.8) of which 5 μL was injected onto the column. [116, 117, 118, 119]

To illustrate the functionality, the separation of the different stereoisomers of 2,6-diaminopimelic acid (DAP) and D-Ala is shown in Fig.8. The baseline separation between D,D and L,L as well as the *meso* form was possible with the 10 μmol standards and with complex samples.

The ability to separate isomers using standard HPLC columns provides valuable insight into PGN analysis where differentiation of primary amines and their chirality is essential, particularly for characterising PGN-related enzyme activities.

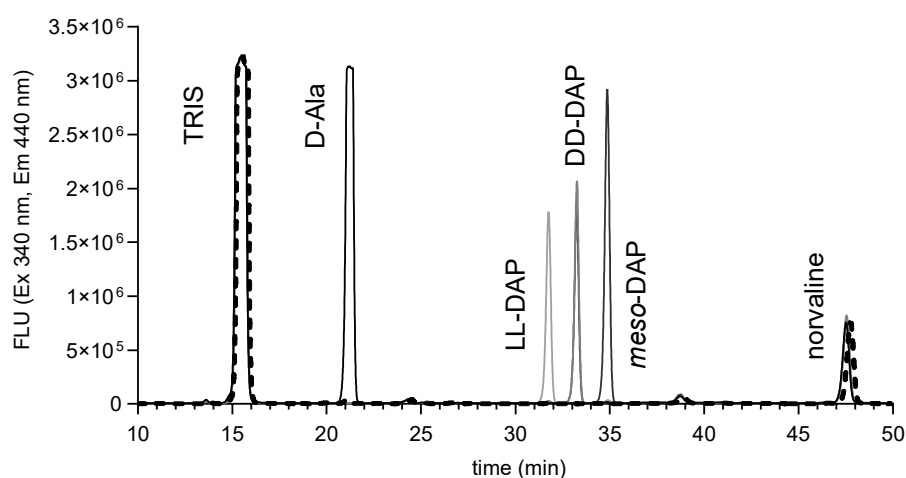


Figure 8: Separation of chiral 2,6-diaminopimelic acid (DAP) isomers using OPA-NAC labelling. The chromatogram of the fluorescence detection after performing the OPA–NAC labelling is shown. Buffer: A: 40 mM monosodium phosphate pH 7.8, B: acetonitrile/methanol/water (45/45/10, v/v). Gradient (% B): 56 min linear 10 % to 33 %, 1 min to 40 %, 23 min to 48 %, 20 min to 100 %. Washing: 5 min 100 % B and A. 0.42 μM norvaline was used as an internal standard. Standards: 1 mM D-Ala, 1 μM DAP. The relevant section of the chromatogram is displayed.

2.2.3 LC-MS analyses of peptidoglycan synthesis in *B. subtilis* using the pseudo-sugar α -D-Carba-glucosamine

In addition to method development, the application of established HPLC and MS analyses was an essential part of this work. The analytical management of a project, from sample planning to measurement, evaluation, analysis and assessment, is of crucial importance in practice and requires experience and expertise in biological experiments and their planning, as well as in the technical aspects of measurement and evaluation.

This is particularly important when non-natural pseudo-metabolites such as α -D-Carba-glucosamine (Carba-GlcN) are used. Carba sugars such as Carba-GlcN are alicyclic carbohydrates that contain a methylene group instead of the usual ring oxygen [121, 122]. Substances that do not occur in nature are ideal candidates for studying intracellular pathways because the origin, and in this case the monoisotopic mass, is unique. Carba-GlcN has been shown to act biologically as a glucosamine (GlcN) analogue, being taken up by GamP and phosphorylated in *B. subtilis*. CGlcN6P formed during transport into the cell cannot be deaminated due to its acetal chemistry but can regulate GlmS expression by activating the *glmS* riboswitch [123].

Specific LC-MS analyses in manuscript 4 - *Metabolization of α -D-carba-glucosamine in vivo generates antimetabolites of cell wall precursors* - revealed that the pseudo-sugar CarbaGlcN was unexpectedly metabolised to at least the UDP-Carba-D-N-acetylmuramic acid pentapeptide (manuscript 4, Fig.4). Methodologically, the isolated cell lysates (for details see manuscript 4, appendix) were analysed using an ESI-TOF mass spectrometer (MicroTOF II; Bruker Daltonics) coupled to an UltiMate 3000 HPLC system (Dionex). A C18 Gemini reversed-phase column (5 μ m, 110 Å, 150 x 4.6 mm ID, Phenomenex; 0.2 mL min⁻¹, 30 °C). The 5 μ L injected sample was separated in a 30 min gradient (5 min wash with 100% buffer A (0.1 % formic acid with 0.05 % ammonium formate), linear gradient over 30 min up to 40 % B (acetonitrile), 5 min gradient delay and 5 min re-equilibration). Analysis was performed using extracted ion chromatograms (accuracy 0.02 Da, baseline 500 cps) with UmetaFlow [113].

By analysing the three benchmark metabolites of PGN synthesis: UDP-D-N-acetylglucosamine (UDP-GlcNAc), UDP-D-N-acetyl-muramic acid (UDP-MurNAc) and UDP-MurNAc-pentapeptide (UDP-MurNAc-5P), the corresponding Carba versions of the metabolites could be identified after the addition of Carba-GlcN (Fig.9). This was the first time that the pseudo-sugar Carba-GlcN was shown to be taken up and metabolised. Furthermore, detailed MS scans showed the structural mass shift of 2 Da, confirming beyond doubt the metabolisation of Carba-GlcN (manuscript 4, Fig.2).

Upon addition of Carba-GlcN, the native forms of the metabolites disappeared and UDP-Carba-GlcNAc and UDP-Carba-MurNAc accumulated, surprisingly a mixture between UDP-MurNAc-5P and UDP-Carba-MurNAc-5P was identified, making this step of the PGN synthesis different from the others (Fig.9). The use of certain antibiotics could further increase the observed accumulations. When Carba-GlcN was administered together with FOS, a strong accumulation of UDP-Carba-GlcNAc was measurable, whereas UDP-GlcNAc was no longer detectable. Treatment of *B. subtilis* with vancomycin resulted in the accumulation of UDP-MurNAc-5P. In the presence of CarbaGlcN, a significant amount of UDP-Carba-MurNAc-5P accumulated in addition to UDP-MurNAc-5P (manuscript 4, Fig.3). The accumulation of the mixture between the Carba version and the natural lipid I precursor UDP-MurNAc-5P indicates that PGN synthesis was blocked at this point and that the subsequent enzyme MraY is unable to convert UDP-Carba-MurNAc-5P.

These results revealed Carba-GlcN not only as a putative antibiotic molecule with previously unknown antimetabolic activity, but also as a tool for studying bacterial cell wall metabolism, in particular for synergy analysis with other antibiotics.

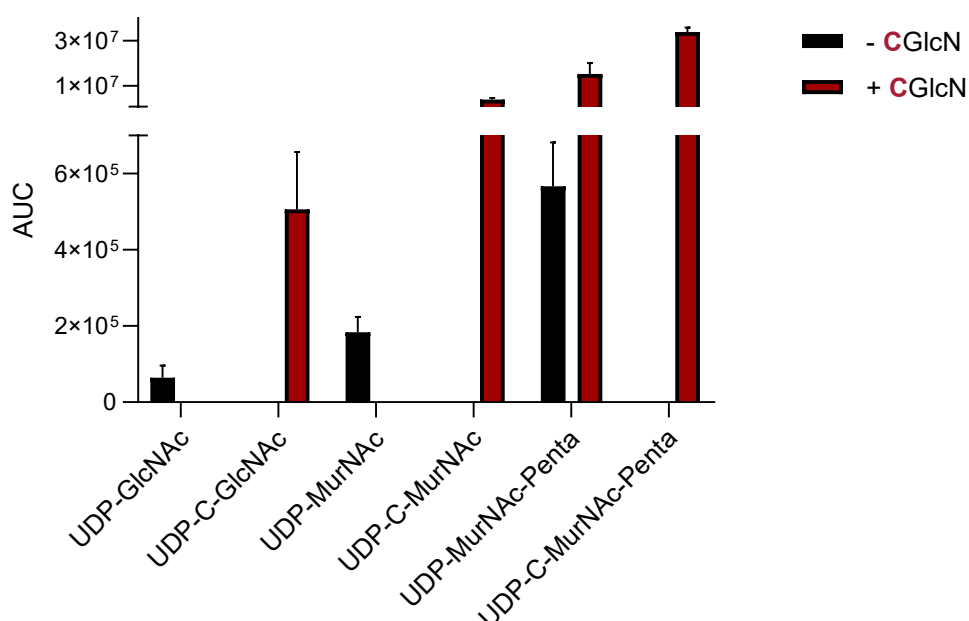


Figure 9: Accumulation of PGN precursors and their carbocyclic variants in Carba-GlcN-treated *B. subtilis* cells. Shown are the relative metabolite concentrations expressed as area under the curve (AUC) values from three independent experiments determined by extracted ion chromatograms with 0.02 Da mass tolerance and baseline adjusted to 500 cps. Carb-aGlcN was added at a concentration of 64 $\mu\text{g mL}^{-1}$ (2x MIC). Black bars: without Carba-GlcN; red bars: with Carba-GlcN.

2.2.4 LC-MS characterisation of the chitin, chitin oligosaccharides and chitin disaccharides metabolism by genetic complementation of parts of the *chb* operon in *E. coli*

By modifying or complementing the natural biological pathways in bacteria, either by removing certain enzymes or by incorporating inducible enzyme activities using inserted plasmids, in combination with LC-MS by analysing the accumulation products, unknown or poorly described metabolic pathways and their enzyme activities can be elucidated. This works particularly well for pathways involving specific metabolites that are not otherwise present in the cell.

E. coli can grow on chitin disaccharides (GlcNAc-GlcNAc; *N,N'*-diacetylchitobiose) or on the chitin trisaccharide (*N,N',N''*-triacetylchitotriose) by expression of the chitobiose operon *chb* (*chbBCARFG*). Chitin is composed of β -1,4-linked *N*-acetylglucosamine (GlcNAc) monomers and is therefore structurally related to PGN. Chitin is the second most abundant natural biopolymer in the world after cellulose and is found in the exoskeletons of insects and crustaceans and in the cell walls of fungi. For *E. coli* to use chitin as a nutrient, the long-chain chitin must be broken down into oligomers, which is achieved by fungi and bacteria that usually secrete the appropriate degradation enzymes. [124, 125, 126]

Publication 1 - *Chitin, Chitin Oligosaccharide, and Chitin Disaccharide Metabolism of Escherichia coli Revisited: Reassignment of the Roles of ChiA, ChbR, ChbF, and ChbG* - has expanded the knowledge of chitin, chitin oligosaccharides, and chitin disaccharide metabolism in *E. coli*. ChbR acts as an activator of the entire operon when it binds the coactivators glucosamine 6P-GlcNAc (GlcN6P-GlcNAc) or GlcN6P-GlcNAc-GlcNAc. These chitin degradation products were formed by ChbG-catalysed deacetylation at the non-reducing end of diacetylchitobiosis and triacetylchitotriosis after these intermediates were transported (and phosphorylated) across the inner membrane by the phosphotransferase system (PTS) ChbBCA (EIIB, EIIC and EIIA) (manuscript 1, Fig.11). [127, 128, 129, 130]

Using deletion mutants, recombinant enzymes and a plasmid allowing IPTG-inducible expression of ChbBCA-PTS (see publication 1 for details), LC-MS analysis (equipment, column and program identical to 2.3.3) was used to determine the accumulation of the corresponding intermediates in comparison to a control, thereby characterising the enzyme activity and elucidating its role in chitin metabolism (manuscript 1, Fig.1, Fig.2, Fig.6). The subsequent steps (publication 1, Fig.11) were elucidated in a similar way. ChbF hydrolyses the coactivators (GlcN6P-...) specifically from the non-reducing end. Contrary to earlier hypotheses, ChbF was not able to cleave GlcNAc-6P (manuscript 1, Fig.3, Fig. 4, Fig.5). Further metabolism involves NagZ and NagK as well as NagB and NagA (publication 1, Fig.11). [129, 131]

New insights were also gained into the steps required for transport (ChiP) into the periplasm and the extracellular hydrolysis of chitin (ChiA) (publication 1, Fig.11).

ChiA, originally thought to have bifunctional chitinase and lysozyme activity, cleaved only chitin and partially deacetylated chitin, but not chitosan (manuscript 1, Fig.7, Fig.8, Fig.9). ChiA is transported into the periplasm by the Sec system, but *E. coli* K12 is unable to secrete the enzyme into the exterior. Complete secretion only occurs in an *hns* mutant that uses the cryptic type 2 secretion system. It is unclear why ChiA is silenced in *E. coli*, but it is probably an effect of adaptation to laboratory conditions. ChiA cleaves chitin into diacetylchitobiose (GlcNAc-GlcNAc) and triacetylchitobiose (GlcNAc-GlcNAc-GlcNAc). Diacetylchitobiose is internalised by non-specific porins, whereas triacetylchitotriose is specifically transported by ChiP. ChiP can also transport short-chain chitin oligomers, which are subsequently hydrolysed by ChiA in the periplasm (publication 1, Fig.11). [132, 133]

This study used LC-MS and genetic modifications to revise the understanding of chitin, chitin oligosaccharide and chitin disaccharide metabolism in *E. coli*.

2.2.5 Elucidation of TonB-dependent pyrroloquinoline quinone uptake and gluconate secretion in *E. coli* K- 1 using LC-MS

E. coli prefers glucose as a carbon source over the chitin fragments described in the previous chapter (2.2.4). Preferably, the transport occurs via a PTS. Alternatively, glucose can be oxidised to gluconolactone in the periplasm by the enzyme glucose dehydrogenase (Gcd). This oxidation reaction is only possible with pyrroloquinoline quinone (PQQ) (publication 3, Fig.1). PQQ is a crucial redox cofactor involved in the enzymatic reactions of many dehydrogenases. Its complex synthesis is carried out by numerous soil bacteria that secrete PQQ. Some Gram-negative bacteria such as *Klebsiella*, *Pseudomonas* and *Acinetobacter* also produce it. Within the microbiome, this benefits bacteria such as *E. coli*, which cannot synthesise PQQ on their own and must obtain it from the environment. The Gcd reaction involving PQQ takes place in the periplasm of *E. coli* and converts glucose to gluconolactone. [134, 135, 136].

In publication 3 - *The TonB-dependent uptake of pyrroloquinoline quinone (PQQ) and secretion of gluconate by Escherichia coli K-12* - the previously unexplained transport of PQQ into the periplasm and the transport and unknown secretion of gluconate in *E. coli* were discovered. In this publication, growth experiments with corresponding deletion mutants showed that PQQ binds to the transporter YncD and is thus transported into the periplasm of *E. coli* via TonB-dependent receptors like comparable substances present at similar low concentrations, such as vitamin B12 or iron siderophores (publication 3, Fig.2, Fig.3) [137, 138, 139]. YncD was renamed PqqU (TonB-ExbBD-dependent) according to its function.

At extracellular PQQ concentrations above 1 nmoL L⁻¹, PqqU enables *E. coli* to activate Gcd. At 30-fold higher concentrations, Gcd became PqqU-independent (publication 3 Fig.2, Fig.3). Further confirmation of the PQQ specificity of PqqU was provided by the observation that PQQ protected the cell from the PqqU-dependent phage Isaak Iselin (Bas10), as both compete for the same receptor protein (publication 3, Fig.4).

In the absence of the glucose PTS system, the cell can only obtain energy from glucose by oxidation to gluconolactone in the periplasm by Gcd. Gluconolactone is then spontaneously or enzymatically (unknown enzyme) hydrolysed to gluconate and can then be taken up into the cell (publication 3, Fig.7).

Using an LC-MS method on an ESI-TOF MS (MicroTOF II; Bruker Daltonics) in negative ion mode connected to an UltiMate 3000 HPLC system (Dionex) equipped with a SeQuant ZIC-pHILIC column (PEEK 150 × 2.1 mm, 5 µm, 110 Å, Merck, operated at 30 °C with a flow rate of 0.2 mL min⁻¹ and a CH₃CN and 100 mM (NH₄)₂CO₃ buffer system (pH 9), with a linear gradient of 25 min CH₃CN from 82 % to 42 % (5 min before and 10 min after the gradient, the column was equilibrated with 82 % CH₃CN)), the exact mass of gluconate (196.058 m z⁻¹) was used to study its phosphate-dependent secretion by analysing the different cell supernatants (injection 5 µL) (publication 3, Fig.5). Under phosphate-limiting conditions, *E. coli* induced Gcd and released gluconate into the medium (publication 3, Fig.5, Fig.6). Gluconate secretion was additionally visualised using acid-indicating Mac Conkey agar plates. (publication 3, Fig.6). Gluconate can dissolve phosphate minerals, e.g. in soil, due to its acidic nature and is therefore important under phosphate-limited conditions to prevent starvation.

The analysis of gluconate secretion by LC-MS was an important contribution to the understanding of the problem and allowed visualisation with e.g. acid-indicating Mac Conkey agar plate, as gluconate was identified unambiguously by the exact mass and a standard.

2.2.6 Unambiguous elucidation of the activity of the α-phosphohexomutases Slr1334 by LC-MS

The advantages of LC-MS analysis are not only demonstrated by the possibility of developing complete metabolomics workflows (2.2.1) or the determination of intracellular accumulating (2.2.3, 2.2.4) or secreted metabolites (2.2.5), but also by the possibility of verifying specific enzyme assays.

In publication 2 - *Glucose-1,6-Bisphosphate, a Key Metabolic Regulator, Is Synthesized by a Distinct Family of α-Phosphohexomutases Widely Distributed in Prokaryotes* - the role of α-D-glucose-1,6-bisphosphate (Glc-1,6-BP) in glycogen metabolism was elucidated with the help of LC-MS. Glc-1,6-BP is known to be an important regulator of glycogen metabolism, amino sugar formation, glycolysis and bacterial cell wall formation.

This importance derives from its properties as an activator of phosphoglucomutase (PGM) and other members of the α -D-phosphohexomutase (α PHM) superfamily, as α PHM is found in all domains of life and all known members catalyse the reversible intracellular phosphoryl transfer to phosphosugar substrates. Across domains, the enzymes share many similarities but differ in substrate specificity. This leads to a classification into four groups: phosphoglucomutases (PGMs), phosphoglucosamine mutases (PNGMs), phosphoacetylglucosamine mutases (PAGMs) and bifunctional phosphoglucomutases/phosphomannomutases (PGM/PMMs). Although much research has been carried out in higher organisms, neither the origin of descent of enzymes in prokaryotes nor the synthesis of Glc-1,6-BP was known prior to this work, as no enzyme producing free Glc-1,6-BP had been identified in prokaryotes. [140, 141]

The unicellular cyanobacterium *Synechocystis* sp. PCC 6803 was chosen as the object of study to increase knowledge in this field. *Synechocystis* has two PGM-like genes: *sll0726* (typical PGM characteristics) and *slr1334* (essential but poorly understood) [142, 143, 144, 145]. Further information on the two proteins was obtained using NCBI RefSeq protein-based phylogenetic analysis (publication 2, Fig.1). Sll0726, which belongs to the group of PGMs catalysing the interconversion of α -D-glucose 1P (Glc-1P) and α -D-glucose 6P (Glc-6P), and Slr1334 differ in their sugar-binding sequence. Enzyme assays showed that Slr1334 had significantly lower PGM activity than Sll0726. As an activator of the PGM reaction, Glc-1,6-BP increased the activity (publication 2, Fig.2). Fructose-1,6-bisphosphate (Frc-1,6-BP), on the other hand, is known to be a PGM inhibitor by competing with Glc-1,6-BP [146]. While Sll0726 was clearly inhibited (publication 2, Table2), the situation with Slr1334 was different. Further investigation led to the hypothesis that Slr1334 forms a product from Frc-1,6-BP and Glc-1P/Glc-6P that activates Sll0726 (publication 2, Fig.3). To ensure that the product was Glc-1,6-BP, LC-MS analysis was required together with a suitable enzyme assay, demonstrating the advantage of accurate mass determination. The LC-MS method (equipment, column and program identical to 2.3.5) identified the product with a monoisotopic mass of 338.988 Da, corresponding to both Glc-1,6-BP and Frc-1,6-BP (publication 2, Fig.4). To separate these isomers, Frc-1,6-BP was enzymatically degraded by a Frc-1,6-BP aldehyde (Fba) and a glycerol-3-phosphate dehydrogenase, leaving only Glc-1,6-BP.

Slr1334 showed phosphotransferase activity to produce free Glc-1,6-BP from Glc-1P/Glc-6P and Frc-1,6-BP and catalyses the reverse reaction (publication 2, Fig.6). Slr1334 is the first bacterial enzyme to produce free Glc-1,6-BP and is therefore an important activator. A homologue of Slr1334 from the human intestinal bacterium *Bacteroides salyersiae* catalyses the same reaction (publication 2, Fig.7), thus identifying the property of an entire phylogenetic group.

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4 Appendix

4.1 Publication 1

Walter A, **Friz S**, Mayer C. Chitin, Chitin Oligosaccharide, and Chitin Disaccharide Metabolism of *Escherichia coli* Revisited: Reassignment of the Roles of ChiA, ChbR, ChbF, and ChbG. *Microb Physiol.* 2021;31(2):178-94.

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Chitin, Chitin Oligosaccharide, and Chitin Disaccharide Metabolism of *Escherichia coli* Revisited: Reassignment of the Roles of ChiA, ChbR, ChbF, and ChbG

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Keywords

Chitin · Peptidoglycan · Chitobiose · *chb* operon · Diacetylchitobiose 6'-phosphate monodeacetylase · 6-Phospho- β -glucosaminidase · Family 4 glycosidase

Abstract

Escherichia coli is unable to grow on polymeric and oligomeric chitin, but grows on chitin disaccharide (GlcNAc-GlcNAc; *N,N'*-diacetylchitobiose) and chitin trisaccharide (GlcNAc-GlcNAc-GlcNAc; *N,N',N''*-triacetylchitotriose) via expression of the *chb* operon (*chbBCARFG*). The phosphotransferase system (PTS) transporter ChbBCA facilitates transport of both saccharides across the inner membrane and their concomitant phosphorylation at the non-reducing end, intracellularly yielding GlcNAc 6-phosphate-GlcNAc (GlcNAc6P-GlcNAc) and GlcNAc6P-GlcNAc-GlcNAc, respectively. We revisited the intracellular catabolism of the PTS products, thereby correcting the reported functions of the 6-phospho-glycosidase ChbF, the monodeacetylase ChbG, and the transcriptional regulator ChbR. Intracellular accumulation of glucosamine 6P-GlcNAc (GlcN6P-GlcNAc) and GlcN6P-GlcNAc-GlcNAc in a *chbF* mutant unraveled a role for ChbG as a monodeacetylase that removes the *N*-acetyl group at the non-reducing end. Consequently, GlcN6P- but not GlcNAc6P-containing saccharides likely function as co-

activators of ChbR. Furthermore, ChbF removed the GlcN6P from the non-reducing terminus of the former saccharides, thereby degrading the inducers of the *chb* operon and facilitating growth on the saccharides. Consequently, ChbF was unable to hydrolyze GlcNAc6P-residues from the non-reducing end, contrary to previous assumptions but in agreement with structural modeling data and with the unusual catalytic mechanism of the family 4 of glycosidases, to which ChbF belongs. We also refuted the assumption that ChiA is a bifunctional endochitinase/lysozyme ChiA, and show that it is unable to degrade peptidoglycans but acts as a bona fide chitinase in vitro and in vivo, enabling growth of *E. coli* on chitin oligosaccharides when ectopically expressed. Overall, this study revises our understanding of the chitin, chitin oligosaccharide, and chitin disaccharide metabolism of *E. coli*.

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Introduction

Chitin is a homopolymer composed of β -1,4 linked *N*-acetylglucosamine (GlcNAc) found in the cell walls of fungi, exoskeletons of crustaceans and insects, as well as the shells of molluscs. It is the most abundant nitrogen-

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containing polysaccharide on earth, with an annual turnover rate of 10^{11} tons, therefore playing a central role in the global nitrogen and carbon cycle [Tharanathan and Kittur, 2003]. Degradation of the recalcitrant chitin biopolymer is achieved by chitinolytic fungi as well as bacteria, e.g., gram-positive bacteria of the genus *Bacillus* or *Streptomyces*, and gram-negative bacteria of the genus *Serratia*, *Aeromonas*, or *Vibrio*, which play an important role in chitin recycling in soil and aquatic (marine) ecosystems [Blokesch, 2012; Hamid et al., 2013; Adams et al., 2019; Wucher et al., 2019]. These organisms secrete various forms of chitinases and *N*-acetylglucosaminidases that fragment polymeric chitin into shorter chitin oligosaccharides, *N,N',N''*-triacylchitotriose (GlcNAc-GlcNAc-GlcNAc, triacyl-chitotriose), *N,N'*-diacylchitobiose (GlcNAc-GlcNAc, diacyl-chitobiose), and GlcNAc [Itoh and Kimoto, 2019]. Furthermore, they possess uptake and catabolic systems to grow on chitin and chitin oligosaccharides [Hamid et al., 2013; Itoh and Kimoto, 2019].

Escherichia coli K12 is unable to grow on chitin and chitin oligosaccharides, despite possessing a functional chitinase (ChiA/YheB) and an outer membrane porin that facilitates chitin oligosaccharide uptake (ChiP) [Francetic et al., 2000a; Soysa and Suginta, 2016]. Under normal laboratory growth conditions, the expression of ChiA and its secretion via the type 2 secretion system is silenced, requiring a mutation in the *hns* gene for activation [Francetic et al., 2000a, b]. As ChiA contains a native signal sequence, in an *hns* background, the chitinase is expressed and partially secreted to the periplasm via the Sec system and to the extracellular space via the type 2 secretome [Francetic et al., 2000a, b]. We became interested in the cryptic ChiA, since it had been described as a bifunctional enzyme with endo-chitinase and lysozyme activity, an assumption that, however, solely relied on the enzyme's ability to cleave the artificial lysozyme substrate ethylene glycol-chitin [Francetic et al., 2000a]. We thus aimed to re-examine the function of ChiA and chitin metabolism in *E. coli*.

Growth of *E. coli* on diacyl-chitobiose and triacyl-chitotriose depends on the *chitobiose* operon *chbBCARFG*. This operon was initially identified as the cryptic *cel* (*cellobiose* utilization) operon, which requires secondary mutations in order to metabolize β -glycosides such as cellobiose, arbutin, and salicin [Parker and Hall, 1990]. Later it was shown that the operon is not cryptic but induced by diacyl-chitobiose, an end product of chitin degradation, and therefore renamed to *chb* [Keyhani and Roseman, 1997]. Encoded by the *chbBCARFG*

operon are components of a phosphotransferase system (PTS) transporter (ChbBCA; EIIB, EIIC, and EIIA) involved in both, the transport of diacyl-chitobiose and triacyl-chitotriose and their simultaneous phosphorylation at the 6-hydroxyl group of the GlcNAc at the non-reducing end [Keyhani et al., 2000a, b, c]. Further encoded by this operon is a transcriptional repressor/activator (ChbR), repressing *chb* transcription by default and acting as an activator upon effector binding [Plumbridge and Pellegrini, 2004]. The *chb* operon is also subject to catabolite repression and is repressed by NagC, the transcriptional regulator of the GlcNAc catabolism, which is derepressed upon GlcNAc6P binding [Plumbridge and Pellegrini, 2004]. The enzymes ChbG and ChbF are involved in the intracellular processing of the phosphorylated diacyl-chitobiose (diacyl-chitobiose-P) and triacyl-chitotriose (triacyl-chitotriose-P). ChbG was identified as a monodeacetylase that is essential for growth on diacyl-chitobiose and triacyl-chitotriose but is dispensable for growth on cellobiose and chitobiose (chitosan dimer; GlcN- β -1,4-GlcN) [Verma and Mahadevan, 2012]. Verma and Mahadevan [2012] further showed that activation of the *chb* promoter by the regulatory protein ChbR is dependent on ChbG, suggesting that deacetylation of diacyl-chitobiose-P and triacyl-chitotriose-P is necessary for their function as co-activators of ChbR. The authors proposed that ChbG removes the *N*-acetyl group from the reducing end, yielding GlcNAc6P-GlcN and GlcNAc6P-GlcNAc-GlcN, which may act as the inducers of the *chb* operon upon ChbR binding [Verma and Mahadevan, 2012]. Subsequent cleavage of these saccharides would yield GlcNAc6P, which upon binding to NagC could relieve repression of the *chb* operon. We questioned these assumptions, because the ChbF protein, which is responsible for the hydrolysis of the ChbG products, monoacyl-chitobiose-P and diacyl-chitotriose-P, was initially characterized as cellobiose-6-phosphate (Glc6P-Glc) hydrolase (named CelF) [Parker and Hall, 1990; Thompson et al., 1999]. Since ChbF cleaves Glc6P-Glc and other 6-phosphoglycosides (e.g., arbutin-P and salicin-P) and as well as chitobiose-P (GlcN6P-GlcN), we suggest that ChbF likely cleaves GlcN6P-glycosides (GlcN6P-GlcNAc and GlcN6P-GlcNAc-GlcNAc), rather than GlcNAc6P-glycosides. ChbF is assigned to the family 4 of glycosidases (GH4). The proposed mechanism for this class of enzymes is very distinct from that of classical glycosidases, which operate via nucleophilic substitution-mechanisms. GH4 glycosidases instead depend on a divalent metal ion and NAD^+ as cofactors and catalyze an oxidation/reduc-

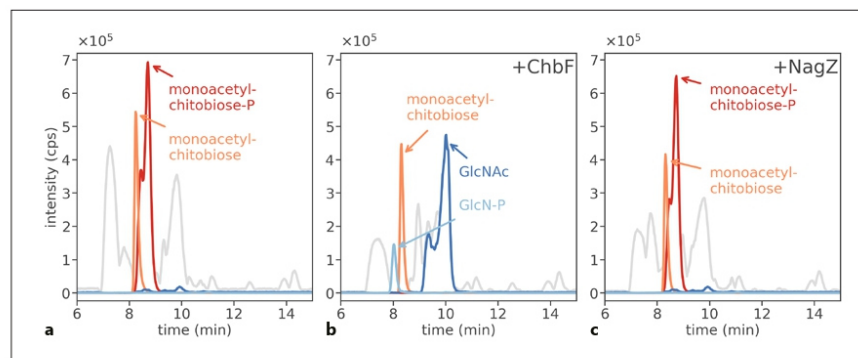


Fig. 1. Accumulation of monoacetyl-chitobiose-P and monoacetyl-chitobiose in $\Delta chbF::kan$ cells and characterization of the ChbF and NagZ products. The cytosolic fraction of *E. coli* $\Delta chbF::kan$ cells, grown in M9 CAA minimal medium supplemented with diacetyl-chitobiose, was extracted and analyzed by LC-MS. **a** Accumulation products with masses (m/z) corresponding to monoacetyl-chitobiose-P ($M+H$)⁺ m/z = 463.0976 (red) and monoacetyl-chitobiose ($M+H$)⁺ m/z = 383.1369 (orange) were detected. **b** Upon treatment with ChbF (+ChbF), the monoacetyl-chitobiose-P ($M+H$)⁺ m/z = 463.0976 (red) completely disappeared, while

masses corresponding to GlcN-P ($M+H$)⁺ m/z = 260.0362 (light blue) and GlcNAc ($M+H$)⁺ m/z = 222.0828 (blue) appeared. The monoacetyl-chitobiose ($M+H$)⁺ m/z = 383.1421 (orange) instead remained intact. **c** Upon treatment with the exo-glucosaminidase NagZ (+NagZ), no GlcNAc was released from either of the accumulation products, indicating that GlcNAc is located at the reducing ends. Shown are the base peak chromatogram (BPC) mass range ($M+H$)⁺ m/z = 120–800 (gray) and the extracted ion chromatograms (EIC) based on the exact masses of displayed compounds (Table 1).

tion of the hydroxyl group at C3 of the sugar at the non-reducing end of a disaccharide. Formation of an intermediate carbonyl at C3 renders the C2 proton more acidic and thereby facilitates the elimination of the glycosidic oxygen, that is, the cleavage of the glycosidic bond [Rajan et al., 2004; Yip et al., 2004, 2007; Yip and Withers, 2006a, b]. Since ChbF operates by a mechanism that involves reactions at C3 and C2, the *N*-acetyl group at C2 likely interferes with this mechanism [Thompson et al., 1999]. Here we revised the function of ChbF as well as of ChbG and unraveled the nature of the effector molecules of the transcriptional regulator ChbR. Furthermore, we characterized ChiA as a bona fide chitinase that cleaves chitin but not chitosan and peptidoglycan, thus disproving its previously suggested role as a bifunctional endochitinase/lysozyme.

Results and Discussion

Growth and Metabolite Accumulation Analyses of *E. coli* Wild Type, *chbG* and *chbF* Mutants

To revisit the functions of ChbG and ChbF encoded by the *chb* operon, we first tested mutants deficient in either

chbG or *chbF* for growth on diacetyl-chitobiose and chitobiose as the sole carbon source in M9 minimal medium, compared to the parental *E. coli* strain (wild type; wt). *E. coli* wt grew overnight to a final OD₆₀₀ of 4.2 in M9 medium supplemented with 2 mM diacetyl-chitobiose, while it failed to grow with chitobiose (data not shown). It has previously been described that *E. coli* wt grows with diacetyl-chitobiose but poorly with chitobiose as the only carbon source, presumably due to the lack of induction of the *chb* operon [Verma and Mahadevan, 2012]. As expected, the $\Delta chbG::kan$ and $\Delta chbF::kan$ strains were unable to grow on either diacetyl-chitobiose and chitobiose (data not shown). To identify accumulation products in $\Delta chbG::kan$ and $\Delta chbF::kan$, we grew the mutants overnight in M9 minimal medium with a mixture of amino acids (casamino acids; CAA) that was supplemented with 2 mM of either diacetyl-chitobiose or triacetyl-chitotriose, isolated the cytosolic fractions, and analyzed them via high-performance liquid chromatography-mass spectrometry (HPLC-MS). In the cytosolic fraction of $\Delta chbF::kan$ cells, we detected large amounts of compounds with masses corresponding to monoacetyl-chitobiose-P ($M+H$)⁺ = 463.1324 m/z , which appears as a double peak due to separation of the α - and β -anomeric

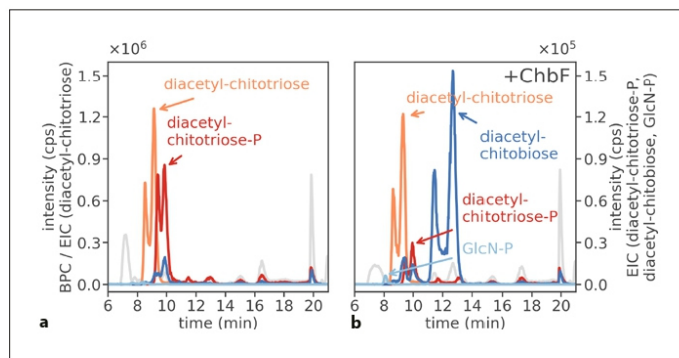


Fig. 2. Accumulation of diacetyl-chitotriose-P and diacetyl-chitotriose in $\Delta chbF::kan$ cells and characterization of the ChbF products. The cytosolic fraction of *E. coli* $\Delta chbF::kan$ cells, grown in M9 CAA minimal medium supplemented with triacetyl-chitotriose, was extracted and analyzed by LC-MS. **a** Accumulation products with masses (m/z) corresponding to diacetyl-chitotriose-P ($M+H$)⁺ m/z = 666.2133 (red) and diacetyl-chitotriose ($M+H$)⁺ m/z = 586.2453 (orange) were detected. The latter appeared as the major accumulation product. **b** Upon treatment with ChbF (+ChbF), the diacetyl-chitotriose-P ($M+H$)⁺ m/z = 666.2122 (red) mostly disappeared, while masses corresponding to diacetyl-chitobiose ($M+H$)⁺

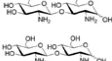
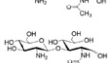
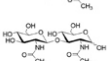
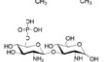
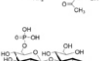
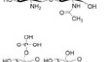
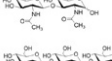
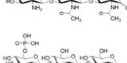
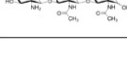
m/z = 425.1790 (blue) and GlcN-P ($M+H$)⁺ m/z = 260.0543 (light blue; a very small peak) appeared. The diacetyl-chitotriose ($M+H$)⁺ m/z = 586.2445 (orange) was not affected by ChbF. A compound appeared with a mass corresponding to diacetyl-chitobiose that elutes at the same retention time as the diacetyl-chitotriose-P (blue), and thus is not diacetyl-chitobiose but an unknown component with the same mass. Shown are the base peak chromatogram (BPC) mass range ($M+H$)⁺ m/z = 180–1,300 (gray) and the extracted ion chromatograms (EIC) based on the exact masses of displayed compounds (Table 1).

forms, and to monoacetyl-chitobiose ($M+H$)⁺ = 383.1660 m/z (Fig. 1a; a list of compounds with their corresponding monoisotopic masses can be found in Table 1). These compounds are likely the products of the activity of the monodeacetylase ChbG, which accumulate due to the lack of ChbF. The additional appearance of a dephosphorylated product indicates that a phosphatase partially cleaves the accumulating monoacetyl-chitobiose-P. To test whether the dephosphorylation event is growth phase-dependent, cytosolic fractions were isolated also from exponential phase-cells (OD_{600} = 1). However, no difference in the relative amount of the phosphorylated and non-phosphorylated accumulation products was found in comparison to the stationary phase cells, indicating the expression of the enigmatic phosphatase is not growth phase-dependent (data not shown). Similarly, when the $\Delta chbF::kan$ strain was grown in CAA-M9 medium supplemented with 2 mM triacetyl-chitotriose, HPLC-MS analysis of cytosolic fractions revealed the presence of compounds with masses corresponding to diacetyl-chitotriose-P ($M+H$)⁺ = 666.2117 m/z (Fig. 2a) and diacetyl-chitotriose ($M+H$)⁺ = 586.2454 m/z , which both appear as double peaks due to separation of the α - and β -anomeric forms. These compounds are the prod-

ucts of the activity of the monodeacetylase ChbG, which apparently deacetylates the trisaccharide-P and possibly also the non-phosphorylated trisaccharide. The appearance of large amounts of diacetyl-chitotriose indicates that the enigmatic phosphatase preferentially cleaves diacetyl-chitotriose-P as compared to monoacetyl-chitobiose-P (cf. Fig. 1a).

Conversely, in the $\Delta chbG::kan$ cytosolic fractions, the expected accumulation products, diacetyl-chitobiose-P and triacetyl-chitotriose-P could not be detected (data not shown). One could speculate that these saccharides do not accumulate because they are metabolized by an unknown, ChbG-independent pathway. If diacetyl-chitobiose is metabolized via a different pathway and thus is used as a source of energy and carbon, growth on this saccharide would be expected. The $\Delta chbG::kan$ mutant, however, failed to grow with diacetyl-chitobiose. It has previously been reported that ChbG is crucial for the generation of monoacetyl-chitobiose, which supposedly acts as the inducer of the *chb* operon [Verma and Mahadevan, 2012]. Thus, the more plausible explanation for the absence of an accumulation product is that no induction occurred and, consequently, the ChbBCA-transporter was not expressed and diacetyl-chitobiose was not taken up.

Table 1. Structures and exact monoisotopic masses of studied metabolites: overview of *chb* metabolites and their exact neutral masses and masses with proton adducts in positive ionization mode

Structure	Name	Formula	Exact monoisotopic mass [M]	Exact monoisotopic mass of the proton adduct [M+H] ⁺
	GlcNAc	C ₈ H ₁₅ NO ₆	221.0899	222.0972
	reduced GlcNAc	C ₈ H ₁₇ NO ₆	223.1056	224.1129
	GlcN6P	C ₆ H ₁₄ NO ₈ P	259.0457	260.0530
	chitobiose (GlcN-GlcN)	C ₁₂ H ₂₄ N ₂ O ₉	340.1482	341.1555
	monoacetyl-chitobiose (GlcN-GlcNAc)	C ₁₄ H ₂₆ N ₂ O ₁₀	382.1587	383.1660
	reduced monoacetyl-chitobiose	C ₁₄ H ₂₈ N ₂ O ₁₀	384.1744	385.1817
	diacetyl-chitobiose (GlcNAc-GlcNAc)	C ₁₆ H ₂₈ N ₂ O ₁₁	424.1693	425.1766
	monoacetyl-chitobiose-P (GlcN6P-GlcNAc)	C ₁₄ H ₂₇ N ₂ O ₁₃ P	462.1251	463.1324
	reduced monoacetyl-chitobiose-P	C ₁₄ H ₂₉ N ₂ O ₁₃ P	464.1407	465.1480
	diacetyl-chitobiose-P (GlcNAc6P-GlcNAc)	C ₁₆ H ₂₉ N ₂ O ₁₄ P	504.1356	505.1429
	diacetyl-chitotriose (GlcN-GlcNAc-GlcNAc)	C ₂₂ H ₃₉ N ₃ O ₁₅	585.2381	586.2454
	diacetyl-chitotriose-P (GlcN6P-GlcNAc-GlcNAc)	C ₂₂ H ₄₀ N ₃ O ₁₈ P	665.2045	666.2117

Deacetylation at the Non-Reducing End by ChbG Is Crucial for ChbF Function

To further characterize the accumulation products of the $\Delta chbF::kan$ strain, the gene encoding the 6-phospho- β -glycosidase ChbF (CelF) was cloned and recombinantly expressed. Recombinant ChbF completely degraded the phosphorylated accumulation product monoacetyl-chitobiose-P and yielded products with masses corresponding to GlcN-P ($M+H$)⁺ m/z = 260.0530 and GlcNAc ($M+H$)⁺ m/z = 222.0972, but was not able to cleave the non-phosphorylated accumulation product (Fig. 1b). It has previously been shown that diacetyl-chitobiose is phosphorylated at the non-reducing end during its transport into the cell by the ChbBCA-PTS system [Keyhani et

al., 2000a]. On this basis, the cleavage of the accumulation product of $\Delta chbF::kan$ into GlcN-P and GlcNAc by ChbF suggests that ChbG deacetylates diacetyl-chitobiose-P at the non-reducing end, yielding GlcN6P-GlcNAc. To unequivocally exclude that the dephosphorylated accumulation product of $\Delta chbF::kan$ is GlcNAc-GlcN, we treated it with *Bacillus subtilis* exo-*N*-acetylglucosaminidase NagZ. This enzyme plays a role in the recycling of peptidoglycan and specifically cleaves off GlcNAc residues from the non-reducing end [Litzinger et al., 2010a, b]. While GlcNAc is released from diacetyl-chitobiose with NagZ (data not shown), no product is observed during digestion of the accumulation products of $\Delta chbF::kan$, indicating the absence of GlcNAc entities at the non-reduc-

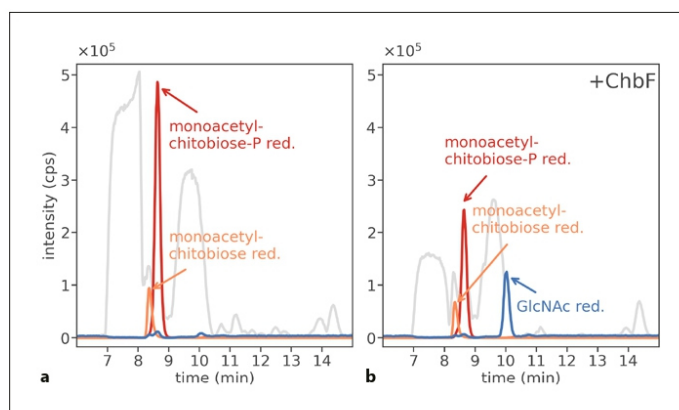


Fig. 3. Identification of the $\Delta chbF::kan$ accumulation product as GlcNP-GlcNAc by reduction and LC-MS analysis of the reduced ChbF products. The cytosolic fraction of *E. coli* $\Delta chbF::kan$ cells containing monoacetyl-chitobiose-P and monoacetyl-chitobiose (see Fig. 1) was reduced with sodium-borohydride, incubated with ChbF and analyzed by LC-MS. **a** Both, the reduced forms of monoacetyl-chitobiose-P ($(M+H)^+$ $m/z = 465.1157$ (red) and monoacetyl-chitobiose ($(M+H)^+$ $m/z = 385.1552$ (orange) were detected. They appear as single peaks, since α and β anomers yield the same product after reduction. **b** Upon treatment with ChbF the peak of

reduced monoacetyl-chitobiose-P ($(M+H)^+$ $m/z = 465.1163$ (red) decreased significantly, but not the reduced monoacetyl-chitobiose ($(M+H)^+$ $m/z = 385.1555$ (orange), and reduced GlcNAc ($(M+H)^+$ $m/z = 224.0974$ (blue) appeared. Notably, the second breakdown product, glucosamine-phosphate (GlcN-P) could not be detected. Shown are the base peak chromatogram (BPC) mass range ($(M+H)^+$ $m/z = 120-800$ (gray) and the extracted ion chromatograms (EIC) based on the exact masses of displayed compounds (Table 1).

ing terminus (Fig. 1c). Similarly, it was shown that ChbF is able to cleave diacetyl-chitotriose-P, which accumulates in *E. coli* $\Delta chbF::kan$ cells grown in the presence of triacetyl-chitotriose, yielding diacetyl-chitobiose and GlcN-P (Fig. 2b). This indicates that triacetyl-chitotriose is also phosphorylated and deacetylated at the non-reducing end by the ChbBCA-PTS and ChbG, respectively, yielding GlcN6P-GlcNAc-GlcNAc, from which GlcN6P at the non-reducing end is cleaved-off by ChbF.

A method to identify the sugar at the reducing end beyond doubt is the reduction with sodium borohydride followed by enzymatic cleavage and/or fragmentation by tandem MS (MS/MS). In these conditions, the reduction of the accumulation products of $\Delta chbF::kan$ was complete and the two reduced products could be detected (Fig. 3a). When ChbF was added, the peak of the phosphorylated, reduced substrate became smaller and a distinct peak emerged, with a mass that corresponds to reduced GlcNAc ($(M+H)^+$ $m/z = 224.1129$ (Fig. 3b). A mass for GlcN-P, which would also be expected, was not detected. GlcN-P does not ionize very well in MS and its ionization could have been worsened in the samples that were reduced with sodium borohydride due to high salt

concentrations. These conditions are also suboptimal for ChbF and, accordingly, the substrate was not completely digested by ChbF. Nevertheless, the detection of reduced GlcNAc ($(M+H)^+$ $m/z = 224.1129$) in significant abundance (83% relative to the reduced monoacetyl-chitobiose-P, which was set to 100%) indicates that there had been GlcNAc at the reducing end of the ChbF substrate (Fig. 4). Both compounds could also be detected in anhydrous forms with lower intensities. The mass difference between reduced monoacetyl-chitobiose-P and reduced GlcNAc corresponds exactly to uncharged GlcN-P- H_2O ($M = 241.0351$ Da. Furthermore, the mass of GlcN-P- H_2O itself could be detected, although in very low abundance of <0.5%. This indicates once more that GlcNAc ionizes readily in MS positive ion mode compared to GlcN-P.

In summary, our results indicate that ChbG deacetylates its substrate diacetyl-chitobiose-P at the non-reducing end. This finding fits well into the previous reports that *E. coli* is able to utilize the β -glucoside sugars cellobiose, arbutin and salicin by expressing the *chb* (formerly designated *cel*) operon [Parker and Hall, 1990]. In each of these sugars, the non-reducing end

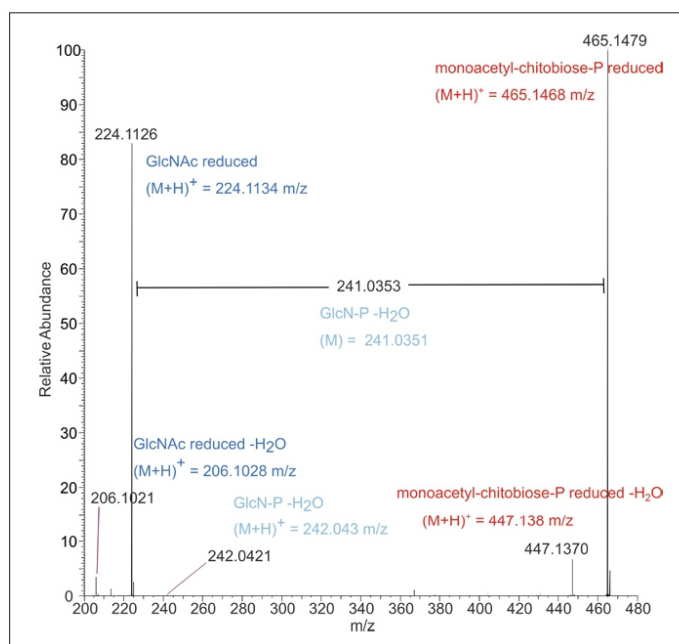


Fig. 4. MS-MS fragmentation analysis of the reduced $\Delta chbF::kan$ accumulation product monoacetyl-chitobiose-P. In order to identify the position of deacetylation in the monoacetyl-chitobiose-P, the $\Delta chbF::kan$ accumulation product was reduced with sodium borohydride (cf. Fig. 3) and analyzed by MS-MS. Reduced monoacetyl-chitobiose-P $(M+H)^+ = 465.1480$ m/z (red) was set to 100% relative abundance. MS-MS fragmentation results in the formation of reduced GlcNAc $(M+H)^+ m/z = 224.1129$ (blue), appearing

with a relative abundance of 83%, which results by loss of uncharged GlcN-P -H₂O ($M = 241.0351$ Da (light blue)). Detected were the mass of GlcN-P -H₂O $(M+H)^+ m/z = 242.0424$ in very low abundance (<0.5%) and of anhydrous forms of reduced monoacetyl-chitobiose-P $(M+H)^+ m/z = 447.1374$ (red) and reduced GlcNAc $(M+H)^+ m/z = 206.1023$ in low relative abundances of 7 and 4.5%, respectively.

consists of glucose, while the residue at the reducing end is variable. These β -glucosides, after phosphorylation, were shown to be substrates for ChbF (previously named CelF) [Thompson et al., 1999]. Our data also fit well into the proposed enzymatic mechanism of GH4 members, which posits that the decisive reaction mechanism takes place at the carbohydrate at the non-reducing end. Indeed, oxidation/reduction of the hydroxyl group at C3 of the sugar at non-reducing end of a disaccharide to a carbonyl renders the C2 proton more acidic and thereby facilitates the elimination of the glycosidic oxygen, that is, the cleavage of the glycosidic bond [Rajan et al., 2004; Yip et al., 2004, 2007]. In the second step of the proposed mechanism, a proton gets released from the non-reducing end sugar's C2 hydrogen to a

catalytic base. This process is favored by a positive dipole on the C2, which is more pronounced with an amino instead of a *N*-acetyl functional group. The binding of Mn^{2+} to C2 and C3 functional groups would also be enhanced with the more negative dipole on the amino group. A well-studied member of GH4 is the 6-phospho- α -glucosidase GlvA from *B. subtilis* [Yip et al., 2007]. The crystal structure of GlvA bound to glucose 6-phosphate (Glc6P) revealed that at C2 there is relatively little space in the binding pocket and steric hindrance could occur with larger side chains. An amino acid sequence alignment among ChbF, GlvA, and other described GH4 enzymes revealed high similarities especially at the site next to the C2 functional group, where a cysteine residue is conserved across all queried enzymes. To

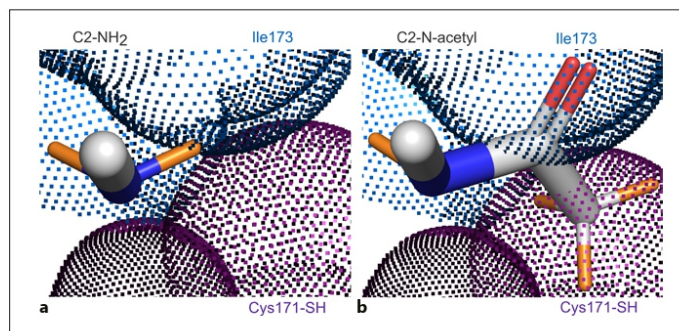


Fig. 5. Structural model of the binding pocket of ChbF in complex with substrates. Based on the crystal structure of the 6-phospho- α -glucosidase GlvA from *B. subtilis* in complex with its substrate glucose-6P (pdb code: 1U8X), the binding pocket of ChbF was modeled using Pymol, identifying steric constraints that discriminate binding of an *N*-acetyl group, but allow binding of an amino or hydroxyl group. **a, b** Shown is the C2 with the functional group shown as sticks (carbon = white, nitrogen = blue, oxygen = red, hydrogen = orange) and the amino acid residues isoleucine (blue) and cysteine (purple) in direct contact with this functional group at C2, shown as dotted spheres. Alignments between the amino

acid sequences of ChbF, GlvA, and other representatives of the GH4 family enzymes show a high degree of similarity and especially a conserved cysteine residue (ChbF Cys172, GlvA Cys171) close to the C2 functional group. Also close are Ile174 of ChbF and Met173 of GlvA. In this structure model, Met173 GlvA was exchanged with Ile to resemble the situation of ChbF. **a** The C2 functional group has been altered to an amino group like in GlcN, which fits well between the Ile and Cys residues. **b** The C2-functional group has been altered to *N*-acetyl group of GlcNAc, which, however, does not fit since the oxygen of the acetyl group collides with the Ile and the methyl group collides with the sulfur of Cys.

further clarify the difference between amino and *N*-acetyl groups at C2 for ChbF we modeled its structure using the GlvA-Glc6P cocrystal structure (pdb code: 1U8X) and exchanging the C2 hydroxyl group of the ligand with an amino or *N*-acetyl group. Two amino acids are in proximity of the functional group, the conserved cysteine and methionine in GlvA, the latter replaced with the isoleucine of ChbF. This modeling suggests that an amino group fits well between the residues (Fig. 5a), whereas the *N*-acetyl group on the other hand does not, as the oxygen collides with the isoleucine and the methyl group collides with the sulfur of cysteine (Fig. 5b).

To test *in vivo* the enzymatic activity of ChbF on diacetyl-chitobiose-P, *E. coli* $\Delta chbG::kan$ was transformed with a plasmid that allows IPTG-inducible expression of the ChbBCA-PTS. Since under these conditions, due to the missing ChbR coactivator, the natural induction of the *chb* operon does not occur, we expected the accumulation of diacetyl-chitobiose-P, when grown overnight in M9 CAA minimal medium supplemented with diacetyl-chitobiose. Accordingly, diacetyl-chitobiose-P (M+H)⁺ $m/z = 505.1429$ accumulated in the cytosolic fraction (Fig. 6a). Intriguingly, ChbF is not able to digest this compound (Fig. 6b), which confirms the inability of ChbF to

take substrates with an *N*-acetyl group at the non-reducing end. Notably, as seen throughout in the accumulation studies, also here phosphorylated and non-phosphorylated saccharide represented the major accumulation product. This further suggests the action of a phosphatase that partially dephosphorylates the accumulation product GlcNAc6P-GlcNAc.

ChiA Cleaves Chitin and Partially Acetylated Chitosan but Not Fully Deacetylated Chitosan

E. coli ChiA was previously characterized as an endo-chitinase, since it cleaved the artificial chitinase substrate 4-methylumbelliferyl (4-MU)-(GlcNAc)₃ but not 4-MU-GlcNAc [Francetic et al., 2000a]. Furthermore, in the latter report, ChiA was described as a bifunctional chitinase/lysozyme, on the basis of its ability to cleave ethyleneglycol chitin (EGC), which is a common lysozyme substrate [Wang and Chang, 1997]. We aimed to re-evaluate the enzyme's specificity using natural substrates, chitin (-oligomers), chitosan (-oligomers), and peptidoglycan, and thus expressed and purified ChiA as a polyhistidine-tagged recombinant protein. Hydrolysis of 4-MU-(GlcNAc)₃ (Sigma-Aldrich) was confirmed with the recombinant protein (data not shown). A preferred substrate of chitinases is colloidal chitin, which was prepared

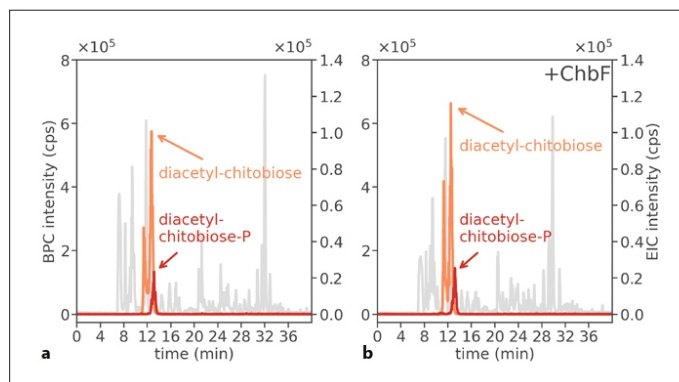


Fig. 6. ChbF is unable to cleave diacetyl-chitobiose-P. **a** Diacetyl-chitobiose ($(M+H)^+$ $m/z = 425.1776$ (orange) and diacetyl-chitobiose-P ($(M+H)^+$ $m/z = 505.1445$ (red) accumulate in cytosolic extracts of strain *E. coli* $\Delta chbG::kan$ carrying pQE32-*chbBCA* and grown in M9 CAA medium in the presence of IPTG and diacetyl-chitobiose. **b** Incubation of the extract in **a** with recombinant ChbF

does not result in a decrease in the intensity of the mass peak of diacetyl-chitobiose ($(M+H)^+$ $m/z = 425.1780$ (orange) and diacetyl-chitobiose-P ($(M+H)^+$ $m/z = 505.1445$ (red)). Shown are the base peak chromatogram (BPC) mass range ($(M+H)^+$ $m/z = 120-800$ (gray) and the extracted ion chromatograms (EIC) based on the exact masses of displayed compounds (Table 1).

by the acetylation of low molecular weight chitosan (<3 kDa in size; obtained from Carbosynth). ChiA was able to cleave colloidal chitin yielding diacetyl-chitotriose and triacetyl-chitotriose (Fig. 7b), whereas an endo-chitinase from *Streptomyces griseus*, in contrast, almost exclusively yielded diacetyl-chitobiose (Fig. 7c). That ChiA releases dimers and trimers in approximately equal amounts could be due to the fact that the enzyme features five chitin-binding type-3 domains which mediate binding to the glycan chains. To understand whether ChiA requires acetylated sugars, we incubated it with chitosan oligomers up to 3 kDa in size. The oligomer mix contained large amounts of chitotriose and chitotetraose and smaller amounts of chitobiose (Fig. 8a). Incubation with ChiA did not result in the release of small chitosan-oligosaccharides, but released significant amounts of diacetyl-chitotriose (Fig. 8b). As the diacetyl-chitotriose product of ChiA could not be further cleaved by the exo-*N*-acetylglucosaminidase NagZ from *B. subtilis*, we suggest the diacetyl-chitotriose product of ChiA is composed of $\text{GlcN}-(\text{GlcNAc})_2$. These data indicate that ChiA is able to cleave particular bonds within polymeric, acetylated chitosan releasing products that have GlcNAc at the reducing end. As a positive control, the chitosanase Csn from *B. subtilis* was cloned, expressed, and purified and the digestion was performed according to the conditions described [Rivas et al., 2000]. After incubation of the chito-

san oligomers with Csn an increased amount of chitobiose could be measured, while chitotetraose was completely digested (Fig. 8c).

ChiA Has No Lysozyme Activity

To investigate the lysozyme activity of ChiA, peptidoglycan was purified from *E. coli* and *B. subtilis*. Unlike the positive controls, Atl (Glc), the glucosaminidase domain of the bifunctional *N*-acetylmuramoyl-L-alanine amidase/endo- β -*N*-acetylglucosaminidase Atl from *Staphylococcus aureus* [Oshida et al., 1995], and mutanolysin, the endo-*N*-acetylmuramidase from *Streptomyces* sp. [Rau et al., 2001], ChiA did not release products from the peptidoglycans of either source. From *E. coli* peptidoglycan, Atl (Glc) released MurNAc-GlcNAc in smaller and $\text{MurNAc-GlcNAc-tetrapeptide}$ ($[\text{MurNAc-GlcNAc}]_4$) in larger quantities (Fig. 9c). From *B. subtilis* peptidoglycan, Atl released disaccharides with and without peptides (online suppl. Fig. S1C; for all online suppl. material, see www.karger.com/doi/10.1159/000515178). To test if ChiA possibly only cleaves denuded peptidoglycan, that is, poly-GlcNAc-MurNAc chains devoid of peptides, the *N*-acetylmuramoyl-L-alanine amidase CwlC from *B. subtilis* [Shida et al., 2000] was used to remove the peptide stems from the peptidoglycan as reported elsewhere [Müller et al., 2021]. CwlC quantitatively released tripeptides (3P) and tetrapeptides (4P) as well as crosslinked

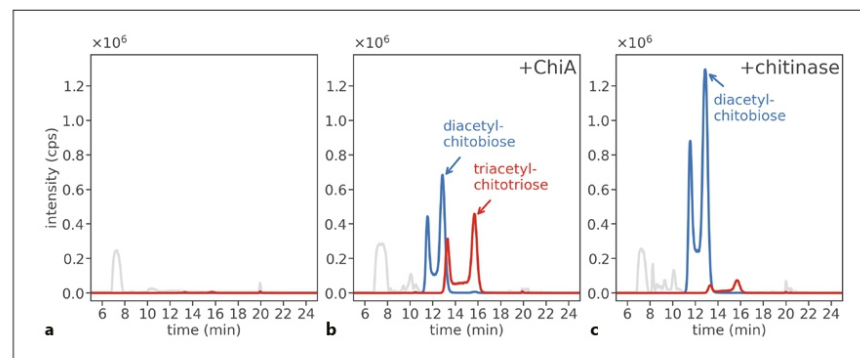


Fig. 7. ChiA cleaves colloidal chitin releasing diacetyl-chitobiose and triacetyl-chitotriose. Colloidal chitin was incubated with ChiA and a chitinase from *S. griseus* and the reaction products were analyzed by LC-MS. **a** Untreated colloidal chitin does not contain soluble chitooligosaccharides, detectable by MS. **b** After a 2-hour digestion of colloidal chitin with ChiA diacetyl-chitobiose ($M+H)^+ m/z = 425.1769$ (blue) and triacetyl-chitotriose ($M+H)^+ m/z = 628.2573$ (red) are formed, but larger oligomers are not visible. **c** A chitinase from *S. griseus* (the positive control) almost exclusively released diacetyl-chitobiose ($M+H)^+ m/z = 425.1765$ (blue) from colloidal chitin. Shown are the base peak chromatogram (BPC) mass range ($M+H)^+ m/z = 199-1,300$ (gray) and the extracted ion chromatograms (EIC) based on the exact masses of displayed compounds (online suppl. Table S2).

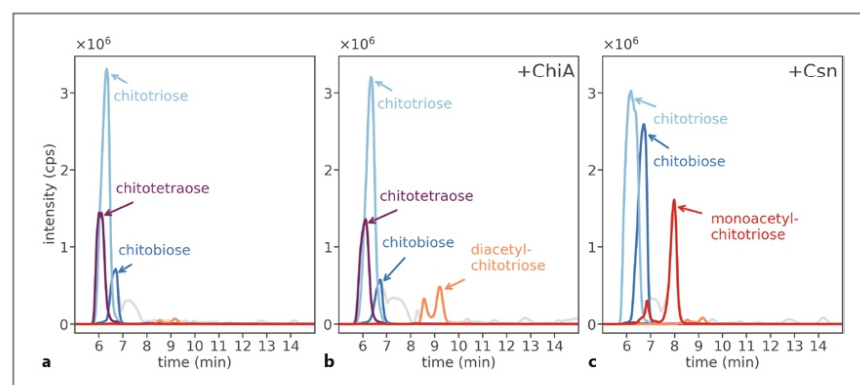
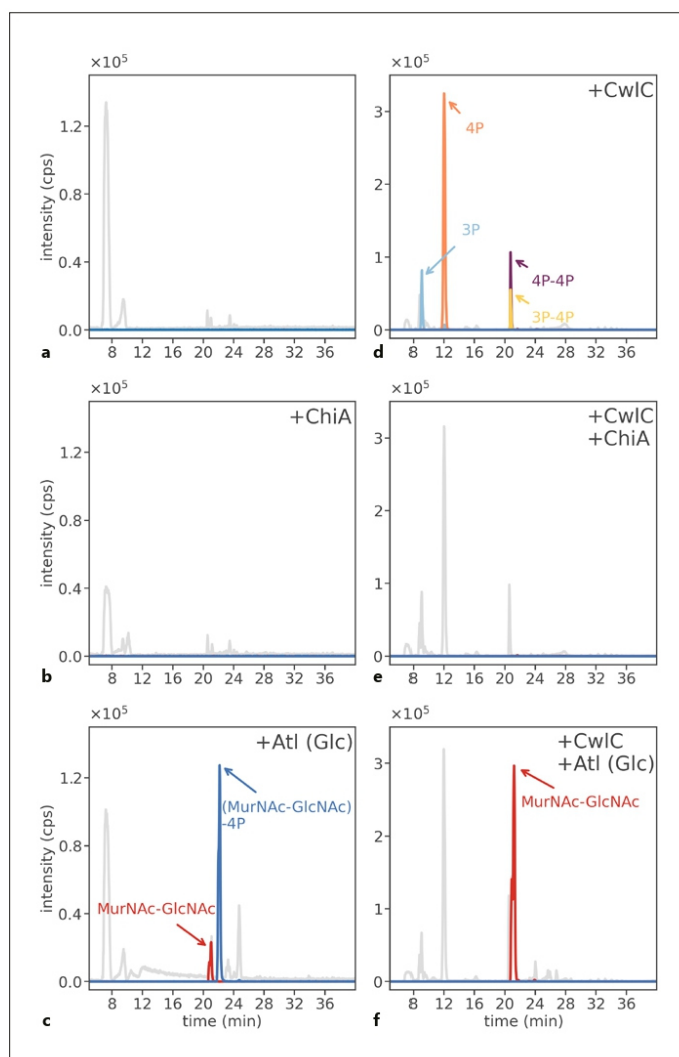


Fig. 8. ChiA cleaves partially acetylated chitosan yielding diacetyl-chitotriose. Chitosan (<3 kDa) was incubated with ChiA and a chitosanase from *B. subtilis* and the reaction products were analyzed by LC-MS. **a** Untreated chitosan (control) already contains small amounts of chitobiose ($M+H)^+ m/z = 341.1576$ (blue), chitotriose ($M+H)^+ m/z = 502.2226$ (light blue), and chitotetraose ($M+H)^+ m/z = 663.2943$ (purple). **b** Upon digestion of partially acetylated chitosan with ChiA (+ChiA), the amounts of deacetylated products were not increased, but the release of diacetyl-chitotriose ($M+H)^+ m/z = 586.2508$ (GlcN-GlcNAc-GlcNAc; orange) was detected. **c** Upon digestion of partially acetylated chitosan with *B. subtilis* chitosanase Csn the amount of chitobiose ($M+H)^+ m/z = 341.1560$ (blue) and also monoacetyl-chitotriose ($M+H)^+ m/z = 544.2386$ (red) were significantly increased, while the chitotetraose was completely digested. Shown are the base peak chromatogram (BPC) mass range ($M+H)^+ m/z = 199-1,300$ (gray) and the extracted ion chromatograms (EIC) based on the exact masses of displayed compounds (online suppl. Table S2).

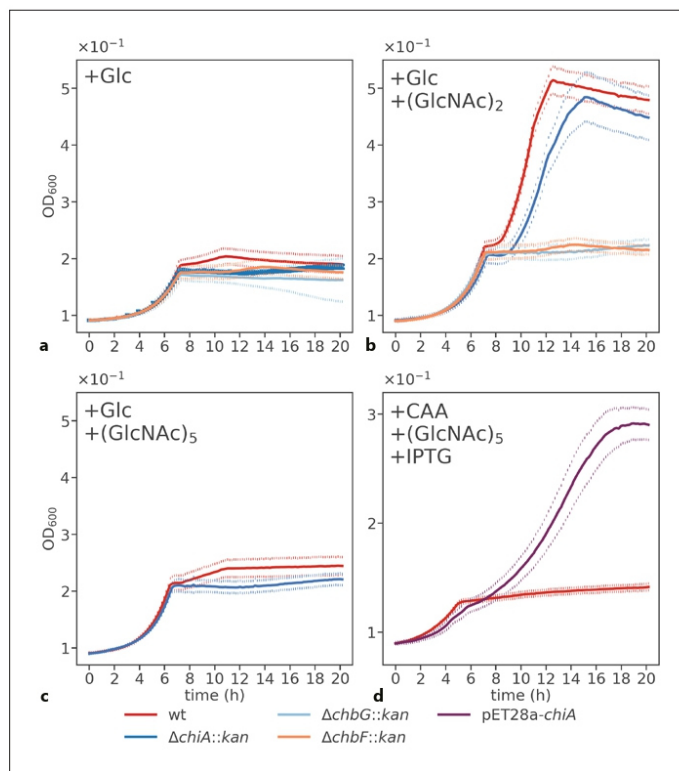
Fig. 9. ChiA does not cleave peptidoglycan. Intact peptidoglycan, derived from *E. coli* (left panels), and peptide-denuded peptidoglycan (right panels), that is, treated with amidase CwlC to remove the peptide stems, were incubated with ChiA or the endo-glucosaminidase Atl (Glc) of *S. aureus*. The formation of reaction products was analyzed by LC-MS. **a** Uncleaved *E. coli* peptidoglycan as a control. **b** Incubation of *E. coli* peptidoglycan with ChiA (+ChiA) does not release any soluble products. **c** Incubation of *E. coli* peptidoglycan with Atl (Glc) (+Atl (Glc)), releases large amounts of (MurNAc-GlcNAc)-tetra-peptide (M+H)⁺ $m/z = 940.3922$ ((MurNAc-GlcNAc)-4P; blue) and to a lesser extent also MurNAc-GlcNAc (M+H)⁺ $m/z = 497.1954$ (red). **d** CwlC (+CwlC) removes tri- (M+H)⁺ $m/z = 391.1650$ (3P, light blue), tetra- (M+H)⁺ $m/z = 462.2006$ (4P, orange), tri-tetra (M+H)⁺ $m/z = 834.3448$ (3P-4P, yellow), and tetra-tetra peptides (M+H)⁺ $m/z = 905.3808$ (4P-4P, purple) from *E. coli* peptidoglycan. **e** From **d**, ChiA (+CwlC+ChiA) is unable to cleave denuded peptidoglycan. **f** From **d**, Atl (Glc) (+CwlC+Atl (Glc)) is able to release large amounts of MurNAc-GlcNAc (M+H)⁺ $m/z = 497.1750$ (red). Intriguing, no MurNAc-GlcNAc-4P could be detected, indicating the complete removal of the stem peptides from the peptidoglycan by CwlC. Shown are the base peak chromatograms (BPC) mass range (M+H)⁺ $m/z = 200\text{--}2,000$ (gray) and the extracted ion chromatograms (EIC) based on the exact masses of displayed compounds (online suppl. Table S2).



4P-4P and 3P-4P peptides (Fig. 9d) from *E. coli* peptidoglycan. From *B. subtilis* peptidoglycan it released 3P*, 3P*-4P*, and 3P-4P*, in which the amino acid meso-diaminopimelic acid is amidated (assigned with *) (online suppl. Fig. S1E). However, ChiA was not able to release any products from denuded peptidoglycan chains (Fig. 9e; online suppl. Fig. S1F). Atl (Glc), on the other hand, re-

leases significant amounts of MurNAc-GlcNAc as the only product in both cases. The fact that no disaccharide with peptides was released using Atl (Glc) indicates that CwlC has completely removed the peptides (Fig. 9f; online suppl. Fig. S1G). Mutanolysin, in contrast, cleaved native peptidoglycan, but was unable to degrade denuded peptidoglycan (online suppl. Fig. S1H). In summary, the

Fig. 10. ChiA is generally not expressed in *E. coli* wt, but periplasmic expression facilitates growth on penta-acetyl-chitopentaose (GlcNAc₅). **a** *E. coli* wild-type (red), $\Delta chiA::kan$ (blue), $\Delta chbG::kan$ (light blue), $\Delta chbF::kan$ (orange), and BL21 (DE3) pET28a-*chiA* (purple) strains were grown in minimal medium supplemented with 0.1% glucose (+Glc) as the only carbon source. wt, $\Delta chbG::kan$, $\Delta chbF::kan$, and $\Delta chiA::kan$ show a similar growth behavior reaching stationary phase after 7 h. **b** In minimal medium supplemented with 0.1% glucose and 0.2% diacetyl-chitobiose (+Glc+(GlcNAc)₂), wt and $\Delta chiA::kan$ showed biphasic growth, whereas $\Delta chbG::kan$ and $\Delta chbF::kan$ grew as in medium with sole glucose. **c** In minimal medium supplemented with 0.1% glucose and 0.2% penta-acetyl-chitopentaose (+Glc+(GlcNAc)₅), wt and $\Delta chiA::kan$ grew as in medium with sole glucose, indicating that penta-acetyl-chitopentaose is not utilized. **d** In minimal medium supplemented with 0.1% CAA and 0.2% penta-acetyl-chitopentaose (+CAA+(GlcNAc)₅+IPTG), again, *E. coli* wt is not able to utilize the penta-acetyl-chitopentaose; however, periplasmic expression of ChiA from a plasmid facilitated growth on penta-acetyl-chitopentaose, resulting in a biphasic growth and a significantly higher final OD₆₀₀ of 0.3.



results show that ChiA can digest chitin but neither chitosan nor peptidoglycan polymers irrespectively of whether they are native or denuded.

ChiA Expression Facilitates Growth on Penta-Acetyl-Chitopentaose

With the sole role of ChiA as chitinase confirmed in vitro, the growth of *E. coli* wt and $\Delta chiA::kan$ in M9 glucose minimal medium was compared in the presence of diacetyl-chitobiose or penta-acetyl-chitopentaose. As expected both strains were able to grow on glucose and diacetyl-chitobiose exhibiting a biphasic growth pattern due to carbon catabolite repression (Fig. 10b). With penta-acetyl-chitopentaose no biphasic growth was observed, as only glucose was utilized (Fig. 10c). However, the wt strain was expected to utilize penta-acetyl-chitopentaose if ChiP and ChiA were active. This was not the case, because *chiA* expression is usually si-

lenced by the nucleotide-structuring protein H-NS [Francetic et al., 2000a]. In contrast, ChiP expression is controlled by the small RNA ChiX but, expression of the *chb* transcript relieves *chiP* inhibition by functioning as trap-RNA [Figuerola-Bossi et al., 2009; Rasmussen et al., 2009]. Thus, expression of ChiA in the periplasm should allow growth on chitooligomers (e.g., penta-acetyl-chitopentaose), which are transported through ChiP into the periplasm. Cleavage by ChiA releases diacetyl-chitobiose and triacetyl-chitotriose, which can be taken up by the ChbCBA-PTS and metabolized by ChbG and ChbF. To test this, ChiA was artificially expressed in *E. coli* BL21 (DE3) from plasmid pET28a-*chiA* in an IPTG-inducible manner. Compared to *E. coli* K12 wt during growth in M9 CAA (to avoid carbon catabolite repression) minimal medium, the expression of ChiA clearly restored the availability to utilize penta-acetyl-chitopentaose (Fig. 10d). To exclude that *E. coli*

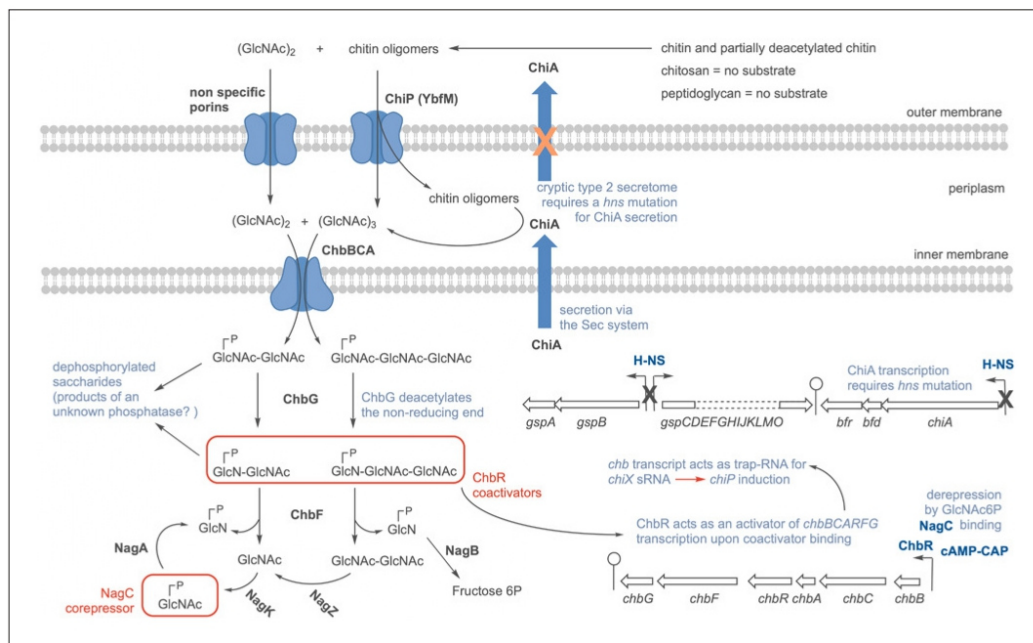


Fig. 11. Overview of the chitin, chitin oligomer, triacetyl-chitotriose, and diacetyl-chitobiose metabolism of *E. coli* and its regulation. Chitin and partially acetylated chitosan is cleaved by the chitinase ChiA, yielding transiently chitin oligomers and as final products triacetyl-chitotriose (GlcNAc₃) and diacetyl-chitobiose (GlcNAc₂). In *E. coli* K12, however, ChiA expression is repressed (silenced) by the nucleoid-structuring protein H-NS [Francetic et al., 2000a]. ChiA is exported into the periplasm via Sec secretion, provided it is expressed either artificially or in a *hns* mutant. In a *hns* strain, ChiA also gets partially secreted into the medium via activation of the cryptic type 2 secretome [Francetic et al., 2000b]. Diacetyl-chitobiose is taken up into the periplasm by nonspecific porins, whereas triacetyl-chitotriose and short-chain chitin oligomers pass through the membrane by a facilitated diffusion process mediated by the chitoporin ChiP [Soysa and Suginta, 2016]. If present in the periplasm, ChiA can cleave the chitin oligomers to diacetyl-chitobiose and triacetyl-chitotriose. *E. coli* grows on diacetyl-chitobiose and triacetyl-chitotriose via the expression of the *chb* operon (*chbBCARFG*). The phosphotransferase system (PTS)

transporter ChbBCA facilitates transport of both saccharides across the inner membrane and their concomitant phosphorylation at the non-reducing end. Subsequently, the phosphorylated saccharides are deacetylated at the non-reducing end by ChbG yielding monoacetyl-chitobiose-P (GlcN6P-GlcNAc) and diacetyl-chitotriose-P (GlcN6P-GlcNAc-GlcNAc). These two products also act as coactivators for ChbR (framed in red), resulting in increased transcription of the *chb* operon. They are cleaved by ChbF and further processed by the exo-glucosaminidase NagZ [Litzinger et al., 2010a, b] and the *N*-acetyl-D-glucosamine kinase NagK [Uehara and Park, 2004]. The *N*-acetylglucosamine repressor NagC, which binds the *chb* promoter thereby inhibiting transcription, is released from the promoter by binding to GlcNAc6P (framed in red) [Plumbridge and Pellegrini, 2004]. Expression of the *chb* operon is also subject to catabolite repression (i.e., activated by cAMP-CAP). GlcNAc6P is degraded by the GlcNAc6P deacetylase NagA and GlcN-6P deaminase NagB to yield fructose 6P thereby entering glycolysis.

BL21 (DE3) expressed endogenous ChiA we monitored its growth on penta-acetyl-chitopentaose and observed the same growth pattern as that of *E. coli* K12 wt (data not shown). This clearly shows that *E. coli* has the potential to utilize chitin oligosaccharides in a ChiA-dependent manner. However, since *E. coli* ChiA expres-

sion and secretion is silenced, the bacterium either relies on scavenging chitinase end products from other organisms or, if there is enough evolutionary pressure, selection against H-NS repression of ChiA and the type 2 secretome might occur.

Conclusion

In this study we refined the understanding of the catabolic pathways of diacetyl-chitobiose (GlcNAc-GlcNAc) and triacetyl-chitotriose (GlcNAc-GlcNAc-GlcNAc) in *E. coli*, as summarized in Figure 11. Catabolism of the saccharides involves phosphorylation via the ChbBCA PTS transporter, as shown previously [Keyhani et al., 2000a, b, c], and subsequent deacetylation via the monodeacetylase ChbG, which specifically removes the *N*-acetyl group from non-reducing end terminal GlcNAc6P entities, as shown in this study. Since activation of the *chb* promoter by the transcriptional regulator ChbR depends on the ChbG activity [Verma and Mahadevan, 2012], the correction of the function of ChbG indirectly identified GlcN6P-GlcNAc and GlcN6P-GlcNAc-GlcNAc as the inducers of the *chb* operon, which upon binding to ChbR activate *chbBCARFG* transcription. These saccharides were found to accumulate in a $\Delta chbF$ strain, thus constituting the natural substrates of ChbF. Intriguingly, ChbF failed to cleave GlcNAc6P-GlcNAc, because the *N*-acetyl group of GlcNAc6P-glycosides sterically interferes with amino acids in the active site pocket and thus prevents binding to ChbF, as shown by structural modeling. Possibly, the unusual redox/elimination-type mechanism of ChbF, belonging to family 4 of Cazy glycosidases [URL: <http://www.cazy.org/GH4.html>] [Henrissat, 1991; Davies and Henrissat, 1995; Yip and Withers, 2012], excludes GlcNAc6P-glycosides as suitable substrates. Accumulation of non-phosphorylated saccharides in the *chbF* mutant indicates that an unidentified phosphatase is active intracellularly that cleaves the phosphorylated 6P-glycosides. We assume that the dephosphorylation occurs due to the accumulation of high concentrations of phosphorylated saccharides and, thus, likely constitutes a detoxification mechanism counteracting sugar phosphate stress. The identity of the phosphatase is so far unclear; however, it could be identical to the phosphatase YigL, which has been reported to relieve glucose-phosphate stress in *E. coli* [Kuznetsova et al., 2006; Sun and Vanderpool, 2013].

The reason for this particular catabolic pathway in *E. coli* is unclear, but may be linked to the function of the chitinase ChiA of *E. coli*. We showed that the chitinase ChiA specifically cleaves chitin as well as acetylated chitosan, yielding diacetyl-chitobiose/triacetyl-chitotriose and GlcN-GlcNAc-GlcNAc, respectively, but does not cleave intact or peptide-free, denuded peptidoglycan as well as chitosan. Chitin in nature barely occurs as a fully acetylated polymer. Since ChiA is able to cleave GlcNAc-beta-1,4-GlcN bonds, it thus generates saccharides that

upon uptake and phosphorylation by the ChbBCA PTS directly act as inducers of the *chb* operon. A still open question is why ChiA expression is silenced in *E. coli*. It is appealing to assume that *E. coli* laboratory strains have adapted to the use of LB- or similar media made up of yeast extract, which contains diacetyl-chitobiose and triacetyl-chitotriose but lacks chitooligomers; thus, ChiA is dispensable under these condition. It could be interesting to screen for undomesticated *E. coli* strains that lack a silent ChiA system and hence retain their ability to grow on polymeric chitin.

Experimental Procedures

Bacterial Strains and Growth Conditions

The bacterial strains used in this study are listed in online supplementary Table S1. *B. subtilis* 168 wild-type and *E. coli* K12 were used for the isolation of peptidoglycan. Bacteria were cultured at 37°C in lysogeny broth (LB broth Lennox, Carl Roth) with continuous shaking at 140 rpm. Overnight cultures (~16 h) were used to inoculate fresh LB medium. Cells were harvested by centrifugation (3,000 × g, 20 min, 4°C). *E. coli* BL21 (DE3) cells (New England Biolabs) used to heterologously express recombinant enzymes were grown in LB medium supplemented with 50 µg/mL kanamycin or 100 µg/mL ampicillin depending on the expression plasmid until OD₆₀₀ 0.7 was reached, followed by induction with 1 mM IPTG and further propagation for 3 h. Cells were harvested by centrifugation (3,000 × g, 20 min, 4°C) and used for the purification of recombinant proteins. For the extraction of accumulation products *E. coli* $\Delta chbG::kan$, $\Delta chbF::kan$, and *E. coli* $\Delta chbG::kan$ pQE32-*chbBCA* were grown at 37°C o/n in 5 mL M9 minimal medium [Harwood and Cutting, 1990] with 6 g/L casamino acids (CAA) instead of glucose and 2 mM diacetyl-chitobiose. The growth curves were conducted in a Tecan plate reader at 37°C in 24-well plates filled with 400 µL M9 minimal medium [Harwood and Cutting, 1990] either with 0.1% glucose or 0.1% CAA and 0.1% of test substrate (diacetyl-chitobiose, triacetyl-chitotriose or penta-acetyl-chitopentaose; obtained from Carbosynth).

Construction of Plasmids and Purification of Recombinant Enzymes

For the overexpression and purification of the enzymes used in this work, *E. coli* K12 ChbF (JW1723) and ChiA (JW3300), *B. subtilis* 168 Csn (BSU26890), NagZ (BSU01660), and CwlC (BSU17410), and *S. aureus* USA300 Atl (Glc) (ABD22514.1), the corresponding genes were amplified by PCR using the respective genomic DNA and primers listed in online supplementary Table S1. Prior to its insertion in pET28a, the *NcoI* site in *cwl* was deleted. The PCR products were purified (GeneJET purification kit and GeneRuler, 1-kb marker, Thermo Fisher Scientific). PCR product and vector (specified in primer name in online suppl. Table S1) were digested with restriction enzymes (specified in primer name in online suppl. Table S1; New England Biolabs) and ligated with T4 DNA ligase (Thermo Fisher Scientific). Chemically competent *E. coli* DH5a cells were transformed using the constructed plasmids and selected for growth on the respective

antibiotic. From positive clones plasmids were purified and checked by sequencing.

The positive plasmids were used to transform *E. coli* BL21 (DE3) cells, which were grown as described above and lysed in a French pressure cell. Recombinant His-tagged enzymes were purified by Ni²⁺-affinity chromatography using a 1-mL HisTrap column (GE Healthcare) followed by size exclusion chromatography on a HiLoad 16/60 Superdex 200 pg column (GE Healthcare) and purity was checked with a 12% SDS-PAGE. From a 1-L culture 3.6 mg ChbF, 1.75 mg CwIC, and 12.5 mg Csn were obtained. The enzymes were stored at -20°C in 20 mM Na₂HPO₄ buffer (500 mM NaCl, pH 7.6). Gibson Assembly was used for the construction of a PTS transporter mutant in *E. coli* Δ chbG::kan. The corresponding gene sections for chbBCA were amplified by PCR from *E. coli* K12 genomic DNA using the appropriate primers (online suppl. Table S1). The purified PCR product was cloned via Gibson Assembly into pQE32, which was linearized via digestion with BseRI and HindIII. Chemically competent *E. coli* Δ chbG::kan cells were transformed with pQE32-chbBCA. Positive clones selected by antibiotics were confirmed by sequencing.

Preparation of Peptidoglycan

For the preparation of *B. subtilis* 168 cell walls 2 L of culture (exponential growth phase, OD₆₀₀ = 1.0) were harvested and resuspended in 30 mL piperazine-acetate buffer (50 mM, pH 6) with 12 U proteinase K and boiled for 1 h. The cytosolic fractions were removed by centrifugation (3,000 × g, 15 min, 4°C). The pellet was resuspended in 6 mL buffer (10 mM Tris, 10 mM NaCl, 320 mM imidazole, adjusted to pH 7.0 with HCl) and 600 µg α-amylase, 250 U RNase A, 120 U DNase I, and 50 mM MgSO₄ were added. The sample was incubated at 37°C for 2 h while shaking, 12 U Proteinase K was added, and the incubation continued for 1 h. 4% SDS solution was added 1:1 and the mixture was boiled for 1 h. SDS was removed by repeated ultracentrifugation steps (20 times at 140,000 × g, 30 min, 40°C) and suspension in H₂O_{bidistilled} as well as dialysis against H₂O_{bidistilled}. The SDS content was controlled with the methylene blue assay described earlier [Hayashi, 1975]. The cell wall preparation was dried in a vacuum concentrator. Wall teichoic acids were removed by incubation with 1 M HCl for 4 h at 37°C.

For the preparation of *E. coli* K12 cell walls 2 L of culture (exponential growth phase, OD₆₀₀ = 1.0) were harvested and resuspended in 90 mL ice-cold H₂O_{bidistilled} (to prevent peptidoglycan autolysis activity). Boiling SDS solution (8%) was added dropwise until a final volume of 100 mL and boiled for 1 h further while stirring constantly. The SDS was removed as described for *B. subtilis* cell wall. After resuspending the pellet again in 20 mL H₂O_{bidistilled}, 300 µL α-amylase stock solution (12 mg α-amylase + 0.5 mL H₂O_{bidistilled} + 0.7 mL 10 mM Tris-HCl pH 7) were added and incubated at 37°C under constant shaking for 2 h. Pronase solution (100 mg pronase + 10 mL 10 mM Tris-HCl buffer pH 7) was pre-incubated at 60°C for 2 h. 500 µL pronase solution was added to the sample and incubation was continued for 2 h further. The sample was boiled for 1 h while stirring followed by one ultracentrifugation step. The cell wall preparation was dried in a vacuum concentrator.

Preparation of Colloidal Chitin

For the preparation of colloidal chitin chitosan (<3 kDa; obtained from Carbosynth) was acetylated with acetic anhydride. 20 mL 1 M sodium bicarbonate (freshly prepared) were added to 500

mg chitosan and mixed by swirling until a brown-colored foam developed. 0.5 mL of >99% acetic anhydride were added dropwise while mixing slowly, followed by incubation for 20 min at room temperature. The solution was precipitated by centrifugation (1,500 × g) and the pellet was resuspended again in 20 mL 1 M sodium bicarbonate with 0.5 mL acetic anhydride and further incubated for 20 min. The reaction was boiled for 10 min and washed with phosphate buffered saline until pH 7 was attained. The colloidal chitin was stored at 4°C.

Enzymatic Reactions

For the digestion of *E. coli* and *B. subtilis* peptidoglycan, colloidal chitin, and chitosan oligomers, 0.2 mg substrate were used. The following buffers were used for the enzymes: 0.1 M potassium phosphate pH 8 for Atl and CwIC; 0.1 M sodium phosphate pH 7.2 for ChiA, chitinase from *S. griseus*, NagZ and ChbF; 0.1 M potassium phosphate pH 6 for mutanolysin; 0.05 M sodium acetate pH 5.7 for Csn. In each case 5 µg of enzyme were used for one preparation and all reactions took place at 37°C. Peptidoglycan preparations were incubated for 1 h, colloidal chitin, chitosan, and cytosolic fractions for 2 h. The digestion volume was 50 µL total, except for the pre-digestion of peptidoglycan with CwIC, which had a volume of 25 µL. After CwIC pre-treatment the pH was adjusted to the corresponding pH value of the subsequent enzyme with 0.1 M potassium dihydrogen phosphate and the volume was adjusted to 50 µL. After each digestion the enzymes were heat-inactivated at 95°C for 10 min.

Generation of Cytosolic Fractions

A 5-mL culture was harvested by centrifugation at 16,000 × g for 10 min and was washed twice in 1 mL Tris-HCl (pH 7.6, 10 mM) buffer. The cells were resuspended in 200 µL buffer and incubated for 10 min at 95°C followed by a further centrifugation step. The supernatant was removed by pipetting and mixed with 800 µL acetone. After a further centrifugation step the supernatant was removed and dried under vacuum at 37°C. Samples were dissolved in 30 µL H₂O_{bidistilled} for LC-MS analysis [Gisin et al., 2013].

Reduction of Samples with NaBH₄

Samples were reduced with sodium borohydride as described earlier [Schaub and Dillard, 2017; Kluj et al., 2018]. To prepare the reducing solution, 500 µL of 0.5 M borate buffer (pH 9) was added to 5 mg sodium borohydride. Samples dissolved in 30 µL H₂O_{bidistilled} were added to 30 µL reducing solution and incubated for 20 min at room temperature. By the addition of 10 µL 8.5% phosphoric acid the pH was adjusted to 3–4 before the samples were dried in a vacuum concentrator at 37°C and dissolved in 30 µL H₂O_{bidistilled} for LC-MS analysis.

Analysis of Reaction Products by LC-MS

Sample analysis was conducted using an electrospray ionization-time of flight (ESI-TOF) mass spectrometer (MicrOTOF II; Bruker Daltonics), operated in positive ion-mode that was connected to an UltiMate 3000 high-performance liquid chromatography (HPLC) system (Dionex). For HPLC-MS analysis 5 µL of the sample supernatant were injected into a Gemini C18 column (150 × 4.6 mm, 5 µm, 110 Å, Phenomenex). A 45-min program at a flow rate of 0.2 mL/min was used to separate the compounds as previously described [Gisin et al., 2013]. An Orbitrap Elite mass spectrometer (Thermo Scientific) was used to record higher energy collisional dissociation (HCD) MS/MS spectra.

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Statement of Ethics

Ethics approval was not required.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

A.W. conducted the experiments. S.F. provided the plasmid pQE32-ChbBCA. C.M. formulated the original problem and provided guidance throughout the study. C.M. and A.W. designed the experiments and developed the methodology. C.M., S.F., and A.W. wrote the manuscript.

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4.2 Publication 2

Neumann N, **Friz S**, Forchhammer K. Glucose-1,6-Bisphosphate, a Key Metabolic Regulator, Is Synthesized by a Distinct Family of alpha-Phosphohexomutases Widely Distributed in Prokaryotes. *mBio*. 2022;13(4):e0146922.

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Glucose-1,6-Bisphosphate, a Key Metabolic Regulator, Is Synthesized by a Distinct Family of α -Phosphohexomutases Widely Distributed in Prokaryotes

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ABSTRACT The reactions of α -d-phosphohexomutases (α PHM) are ubiquitous, key to primary metabolism, and essential for several processes in all domains of life. The functionality of these enzymes relies on an initial phosphorylation step which requires the presence of α -d-glucose-1,6-bisphosphate (Glc-1,6-BP). While well investigated in vertebrates, the origin of this activator compound in bacteria is unknown. Here we show that the Slr1334 protein from the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 is a Glc-1,6-BP-synthase. Biochemical analysis revealed that Slr1334 efficiently converts fructose-1,6-bisphosphate (Frc-1,6-BP) and α -d-glucose-1-phosphate/ α -d-glucose-6-phosphate into Glc-1,6-BP and also catalyzes the reverse reaction. As inferred from phylogenetic analysis, the *slr1334* product belongs to a primordial subfamily of α PHMs that is present especially in deeply branching bacteria and also includes human commensals and pathogens. Remarkably, the homologue of Slr1334 in the human gut bacterium *Bacteroides salyersiae* catalyzes the same reaction, suggesting a conserved and essential role for the members of this α PHM subfamily.

IMPORTANCE Glc-1,6-BP is known as an essential activator of phosphoglucosyltransferase (PGM) and other members of the α PHM superfamily, making it a central regulator in glycogen metabolism, glycolysis, amino sugar formation as well as bacterial cell wall and capsule formation. Despite this essential role in carbon metabolism, its origin in prokaryotes has so far remained elusive. In this study we identify a member of a specific α PHM subfamily as the first bacterial Glc-1,6-BP synthase, forming free Glc-1,6-BP by using Frc-1,6-BP as phosphoryl-donor. PGMs of this subfamily are widely distributed among prokaryotes including human commensals and pathogens. By showing that a distinct subfamily member can also form Glc-1,6-BP, we provide evidence that Glc-1,6-BP synthase activity is a general feature of this group.

KEYWORDS carbon metabolism, glucose-1,6-bisphosphate, glycolysis, phosphoglucosyltransferase

The α -d-phosphohexomutase (α PHM) superfamily is ubiquitous and found in all domains of life. All known members of this superfamily catalyze a reversible intramolecular phosphoryl transfer on their phosphosugar substrates. This reaction requires a bound metal ion (usually Mg^{2+}) together with a conserved phosphorylated seryl residue in the active site and proceeds via a bis-phosphorylated sugar intermediate (1). While showing similar properties in structure and reaction mechanism, these enzymes differ strongly in substrate specificity. Based on their preferred substrate, the α PHMs are traditionally divided into four main groups: (i) Phosphoglucosyltransferases (PGM), (ii) phosphoglucosamine mutases (PNGM), (iii) phosphoacetylglucosamine mutases (PAGM), and (iv) the group of bifunctional phosphoglucosyltransferases/phosphomannosyltransferases (PGM/PMM) (1). The most recent expansion to this classification has been the discovery of the mammalian phosphopentomutase (PGM2) and

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TABLE 1 Subfamilies in the α PHM superfamily with their identifier and proposed main function according to the NCBI conserved domain database (CDD)

CDD group name	CDD identifier	Proposed main function
PGM1	cd03085	Phosphoglucomutase
PGM2	cd05799	Phosphopentomutase (PGM2)/Glc-1,6-BP synthase (PGM2L1)
PGM3(PAGM)	cd03086	Phosphoacetylglucosamine mutase
PMM/PGM	cd03089	Bi-functional phosphomanno-/phosphoglucomutase
PGM like 1	cd03087	Unknown
PGM like 2	cd05800	Unknown
PGM like 3	cd05801	Phosphoglucomutase
PGM like 4	cd05803	Unknown
ManB	cd03088	Phosphomannomutase
GlmM	cd05802	Phosphoglucosamine mutase

glucose-1,6-bisphosphate-synthase (PGM2L1) (2, 3). This classification, however, lacks a more detailed subdivision in between the main groups and also does not take into account α PHMs preferably catalyzing other reactions (e.g., straight phosphomannomutases) (3). The NCBI conserved domain database (CDD), instead, classifies the α PHM superfamily in 11 different subfamilies on a phylogenetic basis (4). This phylogenetic model enables a more detailed classification and includes several groups of bacterial and archaeal enzymes showing typical α PHM properties but being only poorly characterized (Table 1).

The PGM (cd03085) from rabbit and human as well as the bacterial PGM (cd05801) from *Salmonella typhimurium* and the bacterial bifunctional PMM/PGM (cd03089) from *Pseudomonas aeruginosa* are among the best characterized α PHMs with available crystal structures and detailed description of reaction mechanisms (5–9). Catalyzing the interconversion of α -d-glucose-1P (Glc-1P) and α -d-glucose-6P (Glc-6P) makes PGM a key enzyme in glycogen metabolism, glycolysis, and gluconeogenesis. During this reaction, a phosphoserine at the active site of PGM donates the phosphoryl group to the substrate Glc-6P or Glc-1P resulting in the synthesis of the transient intermediate α -d-glucose-1,6-bisphosphate (Glc-1,6-BP). After a reorientation in the catalytic center, the intermediate product re-phosphorylates the active site serine yielding Glc-1P or Glc-6P, respectively. Glc-1,6-BP itself acts as an essential activator of PGM by providing an initial phosphorylation of the active site and was first discovered by Leloir, Trucco (10) in yeast extract (11). While Glc-1,6-BP is not a free metabolite in any metabolic pathway, it is known to be a potent regulator of several enzymes in central carbon metabolism in eukaryotes: in addition to PGMs and other α PHMs, it was shown to regulate hexokinases, 6-phosphogluconate dehydrogenase, phosphofructokinase and pyruvate kinase (12). In mammalian tissues the majority of Glc-1,6-BP is catalyzed by the PGM2L1 enzyme, a member of the cd05799 subfamily exclusive to eukaryotes, utilizing 1,3-bisphosphoglyceric acid (1,3-BPG) as phosphoryl donor for Glc-1P (3). In contrast, despite its essential role in the activity of prokaryotic PGM and other enzymes, the origin of Glc-1,6-BP in prokaryotes has been overlooked and no enzyme producing free Glc-1,6-BP has been identified so far (13, 14).

The unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) expresses two enzymes that are encoded by the genes *slr0726* and *slr1334* and annotated as PGM-like (4, 15). While *Slr0726* exhibits typical properties of PGMs (16–18), little is known about *Slr1334*. The only study addressing this issue reported that purified *Synechocystis* *Slr0726* has about a 10-fold higher activity *in vitro* than *Slr1334* and that a *slr0726* knockout mutant only shows around 3% PGM activity compared to the wild-type (19). Despite its low contribution to overall PGM activity, the *Slr1334* product appeared to be essential as it was not possible to acquire fully segregated knockout mutants of the *slr1334* gene, which raised the question of the role of *Slr1334* in *Synechocystis*.

In this work, we show that *Slr1334* has a phosphotransferase activity catalyzing the production of free Glc-1,6-BP from Glc-1P/Glc-6P and fructose-1,6-bisphosphate (Frc-1,6-BP). Thereby, *Slr1334* is responsible for the formation of the key activator of PGM,

Glc-1,6-BP, making it a major regulator in glycolysis and other carbon pathways. Our finding makes Slr1334 the first bacterial enzyme specific for the production of free Glc-1,6-BP. Importantly, we show that the homologue of Slr1334 in the human gut bacterium *Bacteroides salyersiae* catalyzes the same reaction.

RESULTS

Classification of the two PGM isoenzymes from *Synechocystis*. *Synechocystis* possesses two genes annotated as PGMs, the genes *slI0726* and *slr1334*. To classify them within the α PHM superfamily, we performed a phylogenetic analysis by multi-level alignment of all α PHM subfamilies that are considered PGM or "PGM_like." For this, we collected the entry sequences from the NCBI Conserved Domains Database (CDD) (4) and retrieved further sequences by performing BLAST searches in the NCBI RefSeq_protein database. To focus on glucose-specific α PHMs, the groups of enzymes specific for other sugars like PMMs, PNGM and PAGMs (cd03086 cd03088, cd05802, cd05805) were omitted from the analysis (Fig. 1). In general, the α PHMs can be divided into four conserved regions which are from N- to C-terminus: (i) the active site domain, (ii) the metal binding domain, (iii) the sugar binding, and (iv) the phosphate binding domain. While the domains 1, 2, and 4 are usually highly conserved between the different subfamilies, the sugar binding domain shows a greater variance and can assist in the categorization of α PHMs (Table S1 in the supplemental material) (2).

Slr1334 is part of the cd05800 (PGM_like2) subfamily present in many bacterial groups. In agreement with the CDD, our phylogenetic analysis shows that this subfamily forms a distinct cluster of PGMs which clearly separates from other PGM and PGM like enzymes. The SlI0726 PGM, by contrast, is a member of the cd05801 subfamily, to which the well characterized PGM from *S. typhimurium* belongs. With the chosen parameters (see methods part) we detected a total of 2140 hits for Slr1334 homologues.

Slr1334 shows low PGM activity compared to SlI0726 but behaves differently in the presence of fructose-1,6-BP. To gain deeper insights into the role of the enigmatic Slr1334, we overexpressed SlI0726 along with Slr1334 in *Escherichia coli* and assessed the PGM enzymatic activity of the purified proteins by measuring the inter-conversion of Glc-1-P to Glc-6-P in an assay that couples Glc-6-P formation to its subsequent oxidation using glucose-6-phosphate-dehydrogenase (G6PDH) and NADP⁺. We could show that Slr1334 only displays about 5% of catalytic efficiency compared to SlI0726 when both PGMs were tested with saturating amounts of the activator Glc-1,6-BP (60 μ M) (Fig. 2, Table 2; see also Fig. 3). Interestingly, the PGM activity of both enzymes was also measurable without addition of Glc-1,6-BP, which is considered to be an essential activator. However, in the absence of Glc-1,6-BP, the catalytic efficiency was about 70 times lower due to a significantly increased Michaelis-Menten constant (K_m) for SlI0726 and Slr1334 alike.

Fructose-1,6- biphosphate is an endogenous intermediate of the glycolytic pathway and is known to be an inhibitor of PGM most likely by competing with the reaction intermediate Glc-1,6-BP (20, 21). In a preliminary experiment we tested the effect of different concentrations of Frc-1,6-BP on SlI0726 and observed major inhibition already at concentrations of 100 μ M (data not shown). When testing PGM activity by replacing 60 μ M Glc-1,6-BP with 100 μ M Frc-1,6-BP, we detected a decrease in catalytic efficiency to 19% as a consequence of a strongly increased K_m (Fig. 2, Table 2). Strikingly, however, for Slr1334 we detected a different effect: while the catalytic rate constant (k_{cat}) decreased by about 2-fold, we measured an approximately 100-fold reduced K_m , resulting in 50 times increased catalytic efficiency. This raised the question of how Frc-1,6-BP affected the PGM-activity of Slr1334. We speculated that Frc-1,6-BP might either function as a direct activator similar to Glc-1,6-BP or be required for the synthesis of the activating compound Glc-1,6-BP during the reaction of Slr1334. As we will show below, the activating compound turned out to be Glc-1,6-BP and not Frc-1,6-BP.

Slr1334 forms a product out of Frc-1,6-BP and Glc-1P/Glc-6P that strongly activates PGM reaction of SlI0726. To determine the amount of Glc-1,6-BP needed to achieve activation of the SlI0726 PGM reaction at 1 mM Glc-1P we first tested PGM activity

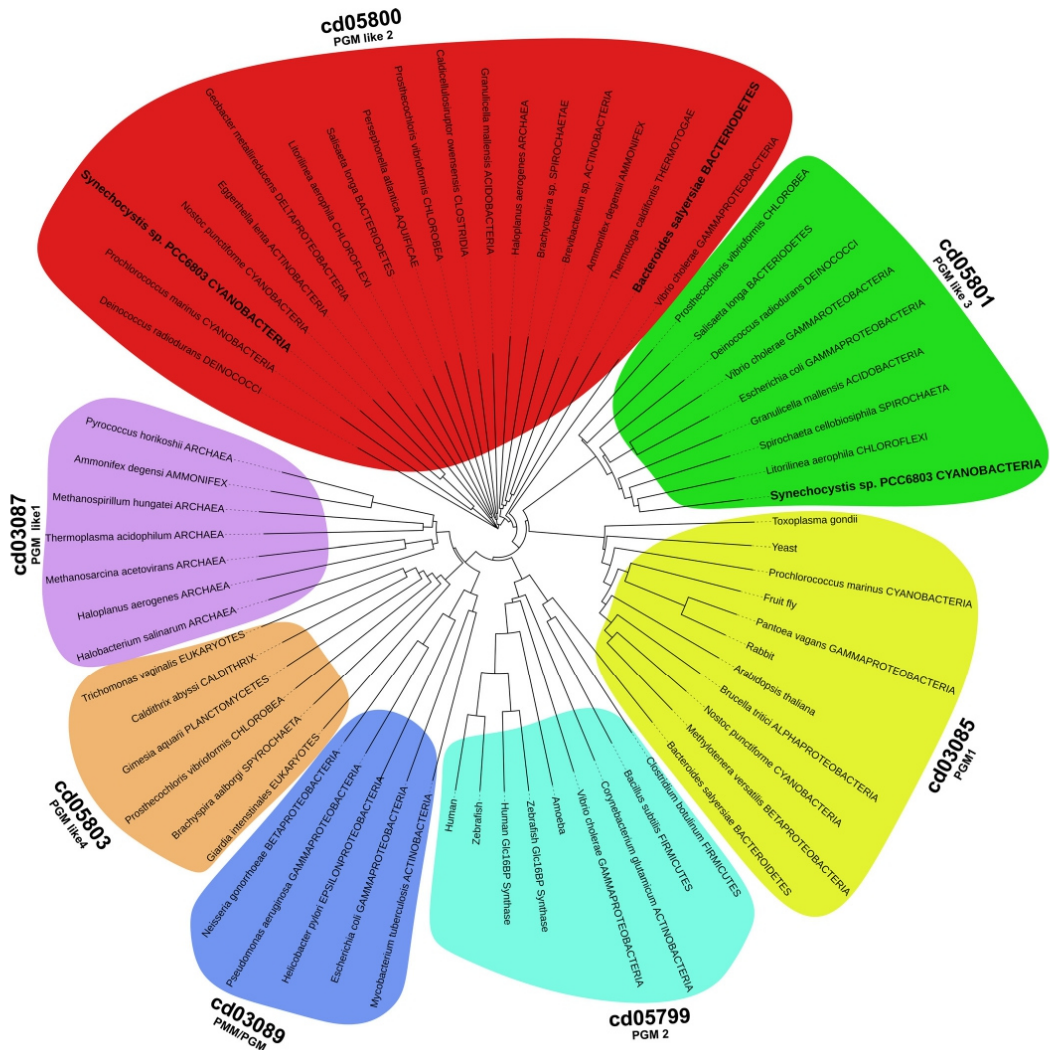


FIG 1 Phylogenetic tree of the α PHM subfamilies that are considered to be “PGM-like” according to CDD. The tree was built on the taxonomy based on the protein sequences present in the NCBI RefSeq_protein database. The CDD subfamilies cd03086, cd03088, cd05802 and cd05805, which are known to catalyze distinct reactions, were omitted from the analysis. The organisms investigated in this study are highlighted in bold letters.

of Sli0726 at different Glc-1,6-BP concentrations. Maximum activation was achieved at Glc-1,6-BP concentrations between 30 and 60 μ M resulting in a 5-fold increased activity compared to a control reaction without any effectors (Fig. 3). Next, we incubated Slr1334 (0.5 μ M) with the phosphorylated sugars Frc-1,6-BP, Glc-1P and Glc-6P (1 mM each) in different combinations and subsequently tested whether the Sli0726 PGM reaction could be activated by the corresponding reaction products (Fig. 3). As a control, we incubated the same phosphosugars with Sli0726 (0.5 μ M) instead of Slr1334 before testing activation of the Sli0726 PGM reaction. After incubating Slr1334 and the control reaction with the phosphosugars for 1.5 h, the reaction was stopped by heat inactivation and the supernatant was added in a dilution of 1:10 to the reaction mixture of the Sli0726 PGM reaction assay,

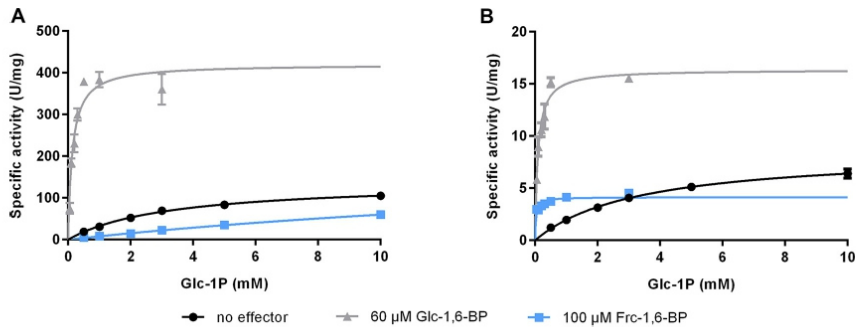


FIG 2 Slr1334 shows lower PGM activity than Slr1334 and behaves differently in the presence of Frc-1,6-BP. Shown are the Michaelis-Menten kinetics of Slr1334 (A) and Slr1334 (B): in black, without addition of effectors; in light gray in the presence of 60 μ M Glc-1,6-BP; in blue, in the presence of 100 μ M Frc-1,6-BP. Three replicates were measured for each data-point. Error bars represent the SD.

which was then started by adding 1 mM Glc-1P. Only after adding the supernatant of the Slr1334 reaction containing Frc-1,6-BP and Glc-1P (or Glc-6P) as substrates, the velocity of the Slr1334 PGM reaction was increased by 5-fold in comparison to the control reaction without any effector (Fig. 3). When the Slr1334 PGM reaction was performed with the supernatant of the Slr1334 control reaction or the reaction products of Slr1334 using Frc-1,6-BP as sole substrate, the enzyme velocity was lower than the control without effectors. This reduction can be explained by the inhibitory effect of Frc-1,6-BP on Slr1334 as shown in Fig. 2.

In addition to Frc-1,6-BP, we also tested whether Slr1334 can utilize 1,3-BPG instead of Frc-1,6-BP for Glc-1,6-BP formation as described for the mammalian PGM2L1. Since 1,3-BPG is not commercially available, we established an assay whereby we continuously produced 1,3-BPG by a phosphoglycerate kinase (PGK) reaction using a His-tagged purified PGK in combination with 1 mM 3PG and 1 mM ATP (see methods part). This reaction was directly coupled to the reaction of Slr1334 with Glc-1P or Glc-6P or no substrate. As a control the same reaction was performed with Slr1334 instead of Slr1334. To test the possible formation of Glc-1,6-BP, the supernatants of these reactions were added to the Slr1334 PGM reaction before performing the enzymatic assay as described above.

We detected an increase in the Slr1334 PGM activity by approximately 2.5-fold compared to the control without effectors when the supernatant of the Slr1334 reaction contained 1,3-BPG and either Glc-1P or Glc-6P. No increase in activity was detected when the supernatant solely contained 1,3-BPG. The supernatant of the control reaction with Slr1334 instead of Slr1334 resulted in no marked change in activity.

By comparing the activation of the Slr1334 PGM reaction by the Slr1334 reaction products to the activation at different concentration of Glc-1,6-BP we attempted to estimate the efficiency of Glc-1,6-BP formation from either Frc-1,6-BP or 1,3-BPG. In this experiment, the Slr1334-catalyzed reaction using 1,3-BPG and G1P as substrates produced an estimated 1–5 μ M the activating compound, while the F-1,6-BP + G1P

TABLE 2 Kinetic parameters for the conversion of Glc-1P to Glc-6P by Slr1334 and Slr1334 in the presence of different effector molecules as indicated^a

Effector	Slr1334			Slr1334			Catalytic efficiency ratio (%) (Slr1334/Slr1334)
	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($M^{-1}s^{-1}$)	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($M^{-1}s^{-1}$)	
No effector	3.27 ± 0.21	138 ± 3.7	42.2×10^3	3.31 ± 0.14	7.5 ± 0.1	2.3×10^3	5.5
Glc-1,6-BP	0.19 ± 0.04	415 ± 30.6	3038.9×10^3	0.09 ± 0.02	14.4 ± 0.7	158.1×10^3	5.2
Frc-1,6-BP	26.86 ± 3.35	218 ± 21.3	8.1×10^3	0.03 ± 0.01	3.6 ± 0.2	117.5×10^3	1,450

^a K_m and K_{cat} values are means of triplicates with $\pm$ SD. Catalytic efficiency ratio is given in percentage and was calculated by the ratio of K_{cat}/K_m of Slr1334 and Slr1334.

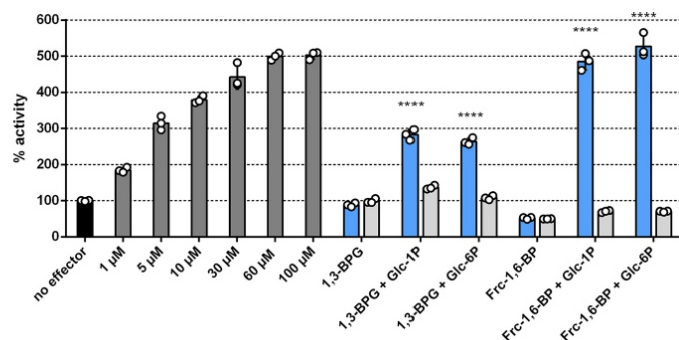


FIG 3 Activation of Slr1334 PGM reaction by Glc-1,6-BP or by the reaction products of Slr1334. Shown is the Slr1334 PGM activity in the presence of different effectors relative to the activity in the absence of effector molecules (set as 100%, black bar). The different bars represent the following reactions: Dark gray bars: Relative PGM activity in the presence of different concentrations of activator molecule Glc-1,6-BP, as indicated. Blue bars: Relative PGM activity in the presence of the supernatant of the Slr1334 reaction carried out with different combinations of substrates, as indicated. Light gray bars: Same conditions as adjacent blue bars, except that Slr1334 was replaced by Slr10726, as a control. Three biological replicates were measured for each datapoint. Error bars represent the SD; asterisks represent the statistical significance in comparison to the reaction without effectors.

reaction produced an estimated 30–60 μ M the activating compound Glc-1,6-BP (Fig. 3). This implies that Frc-1,6-BP as phosphorylating sugar is about 10-fold more effective than 1,3-BPG for the Slr1334 reaction.

The product of the Slr1334 reaction is not degradable by the Frc-1,6-BP-aldolase. To confirm that the activating compound formed from Glc-1P/Glc-6P and Frc-1,6-BP through the Slr1334 reaction is in fact Glc-1,6-BP, we analyzed the reaction product via liquid chromatography-mass spectrometry (LC/MS). Therefore, we incubated Slr1334 together with 500 μ M Frc-1,6-BP and 1 mM Glc-1P for 1.5 h. As a control, the same substrates were incubated without enzyme. Since LC/MS analysis does not allow discriminating Glc-1,6-BP from Frc-1,6-BP, we performed an additional reaction step to specifically degrade Frc-1,6-BP. Therefore, after heat inactivation, the reaction mixture was incubated with Frc-1,6-BP-aldolase (Fba) and glycerol-3-phosphate-dehydrogenase (GPDH) as well as 1 mM NADH:Fba specifically cleaves Frc-1,6-BP into dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3P while Glc-1,6-BP is unaffected. GPDH subsequently forms glycerol-3-phosphate (glycerol-3P) and NAD⁺ out of DHAP and NADH. This is necessary to enable full degradation of Frc-1,6-BP.

After this treatment, LC/MS analysis revealed that a compound with a mass corresponding to either Glc-1,6-BP or Frc-1,6-BP was present in the Slr1334 reaction while this mass was not detectable in the control reaction where no Slr1334 was present (Fig. 4). This indicates that Frc-1,6-BP in the control reaction was completely degraded in the subsequent reaction by Fba as expected, while Frc-1,6-BP in the Slr1334 reaction was metabolized into a molecule of the same mass that was not degraded by Fba. This is precisely what is expected when Glc-1,6-BP is produced from Frc-1,6-BP turn-over. Furthermore, the reaction products from the coupled GPDH reaction, NAD⁺ and glycerol-3P were more abundant in the control reaction without Slr1334 compared to the reaction with Slr1334. This indicated that more Frc-1,6-BP was degraded out of the control reaction than out of the reaction containing Slr1334 due to partial conversion of Frc-1,6-BP into Glc-1,6-BP by Slr1334 (Fig. 4). Altogether, this experiment strongly indicated that in the presence of Slr1334, Frc-1,6-BP was partially utilized to form Glc-1,6-BP. The fact that only a part of Frc-1,6-BP was consumed could be either due to a too short reaction time or because the reaction reached an equilibrium state.

Slr1334 keeps Frc-1,6-BP and Glc-1,6-BP in an equilibrium. For investigating whether the formation of Glc-1,6-BP out of Glc-1P/Glc-6P and Frc-1,6-BP is an equilibrium reaction, we performed an enzymatic assay to determine the amount of the

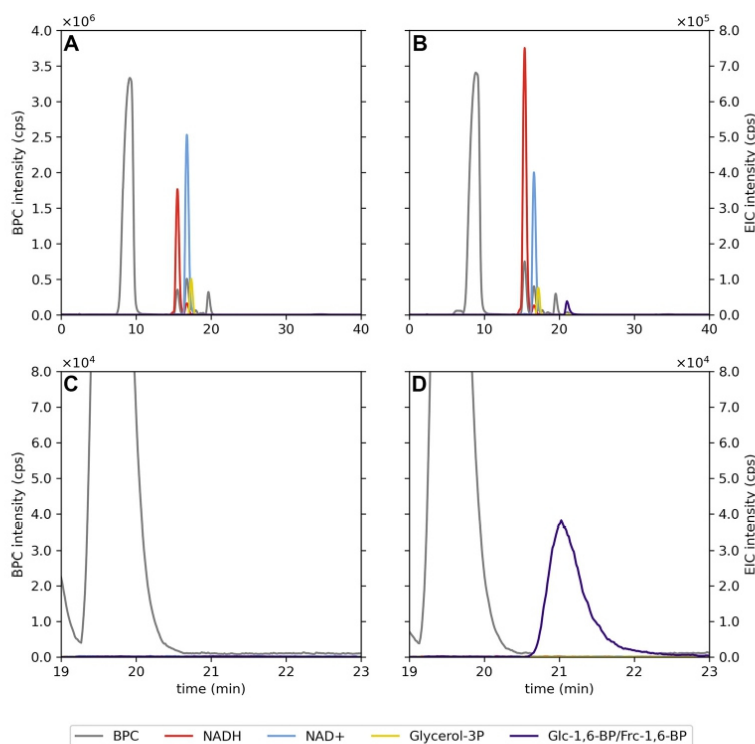


FIG 4 LC-MS analysis reveals that Slr1334 metabolizes Frc-1,6-BP into a molecule of identical mass that is not degraded by Frc-1,6-BP-aldolase. Analysis of the products of the reaction without (A) and with Slr1334 (B) via LC/MS. Shown are the base peak chromatograms (BPC) in the mass range ($M-H$) $m/z = 79-1,601$ (gray) and the extracted ion chromatograms (EIC) based on the exact masses of NAD^+ ($M-H$) $m/z = 662.101$ (light blue), $NADH$ ($M-H$) $m/z = 664.117$ (red), $Glycerol\ 3-P$ ($M-H$) $m/z = 171.006$ (yellow), $Glc-1,6-BP/Frc-1,6-BP$ ($M-H$) $m/z = 338.988$ (purple). The presence of the NAD^+ (blue) and $Glycerol-3-P$ (yellow) peaks in (B) shows that Frc-1,6-BP was not fully metabolized into Glc-1,6-BP. Magnification and adjustment of the graphs in (A) and (B) is clear evidence of the absence (C) or formation (D) of Glc-1,6-BP.

remaining Frc-1,6-BP. Therefore, Slr1334 was incubated with 500 μM Frc-1,6-BP and 1 mM Glc-1P for 1.5 h. As controls, the same assays were performed with Slr0726 or without any enzyme. After the reaction was stopped by heat inactivation, the supernatant was used to run the coupled Fba/GPDH assay in a dilution of 1:10, which would correspond to a final concentration of 50 μM Frc-1,6-BP if no Frc-1,6-BP was consumed. As further controls, standards of 50 μM and 25 μM Frc-1,6-BP were used in the Fba/GPDH assay. This experiment showed that the concentration of Frc-1,6-BP decreased by about 50% when in the first reaction Slr1334 was present, while apparently no Frc-1,6-BP was utilized in the control reactions (Fig. 5). This result agreed with the assumption that the reaction reaches an equilibrium at 50% product formation, either because of the reversibility of the reaction or product inhibition.

The Slr1334 reaction is reversible. To reveal whether Slr1334 can catalyze the proposed reverse reaction and synthesize Frc-1,6-BP from Glc-1,6-BP and fructose-1P (Frc-1P)/fructose-6P (Frc-6P), formation of Frc-1,6-BP was coupled to the Fba and GPDH reaction. For the assay, 50 μM Glc-1,6-BP was used and the reaction was started by the addition of 5 mM Frc-6-P. As control reaction, *Synechocystis* Slr0726 was used. In addition, we also tested PGM1 from rabbit since this type of PGM was stated to possibly catalyze this reaction albeit at a very low efficiency (22, 23). The results clearly showed

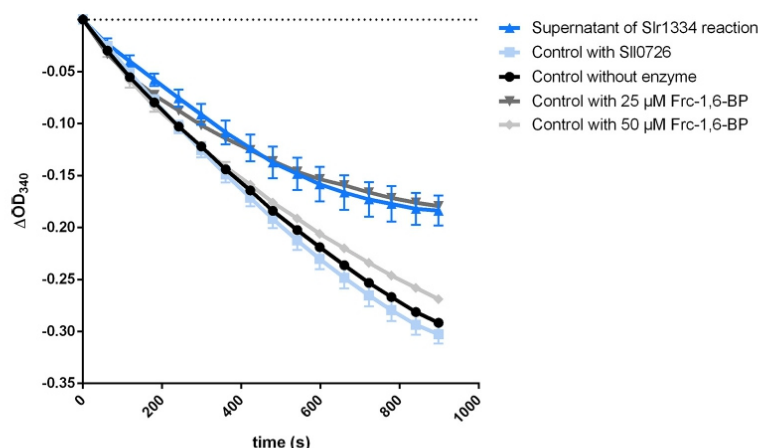


FIG 5 Slr1334 produces Glc-1,6-BP out of Frc-1,6-BP in an equilibrium reaction. Shown is the Frc-1,6-BP-aldolase activity: in dark blue, after addition of the supernatant of the Slr1334 reaction; in light blue, after addition of the supernatant of the SI0726 reaction as a control; in black, after addition of the supernatant without any enzyme. In dark gray and light gray, the aldolase activity measured at Frc-1,6-BP concentrations of 25 μ M and 50 μ M, respectively, as further control. Frc-1,6-BP was used at concentrations of 500 μ M in the Slr1334 reaction and respective control reactions. Therefore, its final concentration in the SI0726 assay after 1:10 dilution was expected to be 50 μ M assuming no consumption of Frc-1,6-BP in the Slr1334 reaction. Three replicates were measured for each datapoint. Error bars represent the SD.

that Slr1334 could produce Frc-1,6-BP, resulting in measurable aldolase activity while the SI0726 and rabbit PGM controls showed no marked activity (Fig. 6a). Using this assay, we tried to determine the kinetic constants for the two substrates Frc-6-P and Glc-1,6-BP. For Frc-6-P, a K_m of 1 mM and a K_{cat} of 1.2 s^{-1} could be determined (Fig. 6b, Table 3). By contrast, the affinity of Slr1334 for Glc-1,6-BP was so high that, even at the lowest measurable substrate concentrations (2 μ M), the reaction proceeded with maximal velocity (Fig. 6c). Altogether these experiments unequivocally demonstrate that Slr1334 is the first characterized bacterial Glc-1,6-BP synthase. In contrast to the eukaryotic Glc-1,6-BP synthase, the prokaryotic enzyme uses Frc-1,6-BP as major phosphoryl donor for Glc-1,6-BP production.

The Glc-1,6-BP synthase reaction is also performed by other members of the cd05800 group. To determine whether the reaction catalyzed by Slr1334 is representative for α PHM enzymes of the cd05800 family, we decided to analyze the corresponding homologue from the human microbiome associated bacterium *Bacteroides salyersiae*. Therefore, we tested formation of Glc-1,6-BP under the same conditions as described above for Slr1334 by incubating the recombinant cd05800 enzyme from *B. salyersiae* with Frc-1,6-BP and Glc-1P or Glc-6P and subsequently adding the supernatant of this reaction to the SI0726 assay. As a positive control we used the reaction of Slr1334.

The addition of the supernatant from the *B. salyersiae* cd05800 PGM increased the velocity of SI0726 in a comparable manner as the supernatant of the Slr1334 reaction (Fig. 7). This indicates that similar amounts of Glc-1,6-BP have been formed in both reactions, hence revealing that *B. salyersiae* cd05800 PGM catalyzes the same reaction as Slr1334.

DISCUSSION

Among the key reactions in carbon metabolism are the phosphoryl transfer reactions on different phosphosugars, which are catalyzed by the members of the α PHM superfamily. These reactions are essential for glycogen metabolism, amino sugar metabolism as well as cell wall synthesis and are conserved throughout all domains of life. The interconversion of Glc-1P to Glc-6P and vice versa by PGM is by far the best

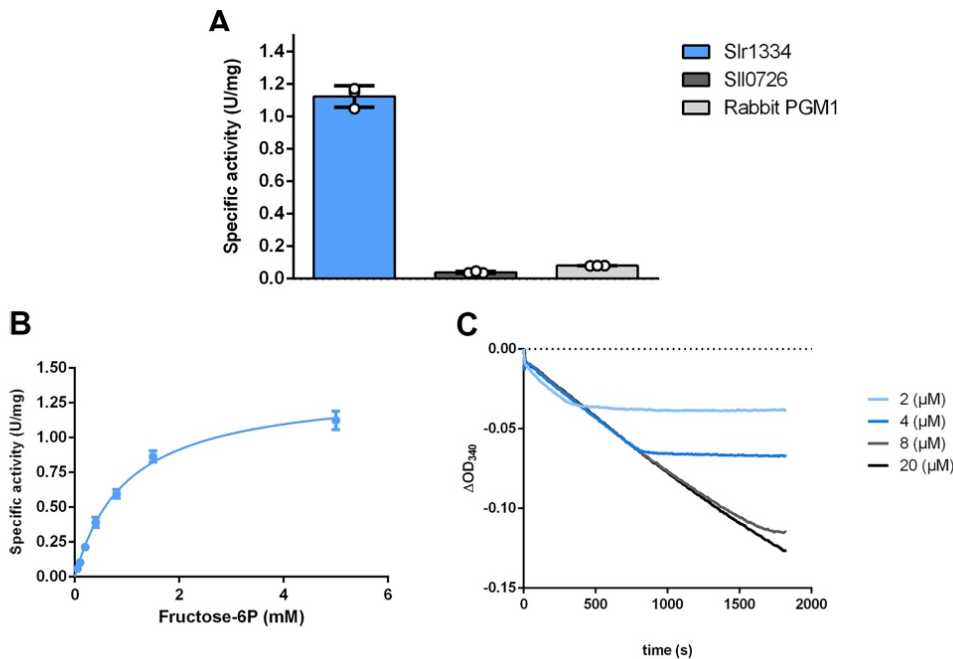


FIG 6 Slr1334 catalyzes the reverse reaction to form Frc-1,6-BP out of Glc-1,6-BP and Frc-6P. (A) Activity of Slr1334 (blue), Sli0726 (gray), and rabbit PGM1 (black) using 50 μ M Glc-1,6-BP and 5 mM Frc-6P. (B) Michaelis-Menten kinetics of Slr1334 using different concentrations of Frc-6P and 50 μ M Glc-1,6-BP. (C) Slr1334 reaction at different concentrations of Glc-1,6-BP and 0.5 mM Frc-6P. Three replicates were measured for each datapoint. Error bars represent the SD; asterisks represent the statistical significance.

studied of these reactions and investigation of PGM goes back to the 1940s. It has long been known that PGM requires Glc-1,6-BP as an activator (10). While in mammals free Glc-1,6-BP has been shown to derive from the reaction of the PGM2L1 enzyme utilizing Glc-1P and 1,3-BPG as substrates, its origin in prokaryotes has remained unknown (3).

In this study, we used the unicellular cyanobacterium *Synechocystis* sp. PCC6803 as a model organism to identify the first bacterial glucose-1,6-BP synthase. This reversible reaction is a novel function of a member of a distinct α PHM subfamily producing free α -D-Glc-1,6-BP and Frc-6P from Frc-1,6-BP and Glc-1P/Glc-6P.

Glc-1,6-BP is essential for PGM activity due to the requirement for phosphorylation of the serine residue at the active site for enzyme activation. The activating reaction converts Glc-1,6-BP into either Glc-1P or Glc-6P. After this initial activation, the interconversion of Glc-1P to Glc-6P is mediated by a transfer of the phosphate group from the serine residue at the active site to the 1' or 6' position of the substrate respectively, forming a Glc-1,6-BP intermediate. This intermediate has to reconfigure inside the enzyme and transfer the phosphate of its 6' or 1' position back to the active site serine leading to the release of Glc-6P or Glc-1P, respectively. As a result of this mechanism, the active site remains phosphorylated, and the enzyme is ready for a new cycle. However, it has been shown that the phosphorylation of PGM at the active site is lost after an aver-

TABLE 3 Kinetic parameters of Frc-1,6-BP synthesis by Slr1334^a

Substrate	K_m (mM)	K_{cat} (s ⁻¹)	K_{cat}/K_m (M ⁻¹ s ⁻¹)
Glc-1,6-BP	<0.002	1.2 ± 0.03	600,000
Frc-6P	1.0 ± 0.07		>1,200

^a K_m and K_{cat} values are means of triplicates with $\pm$ SD.

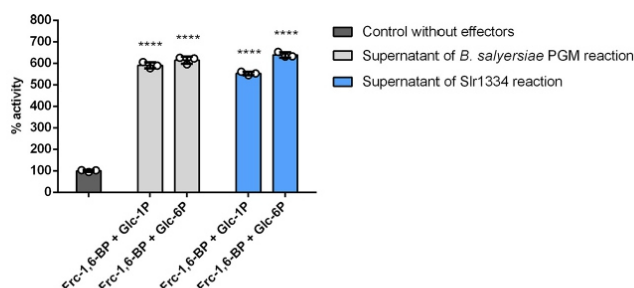


FIG 7 Slr1334 homologue from *B. salyersiae* also forms Glc-1,6-BP out of Frc-1,6-BP. Shown is the SI0726 PGM activity in the presence of different effectors relative to the activity in the absence of effector molecules (set as 100%, dark gray bar). The different bars represent the following reactions: Light gray bars: Relative PGM activity in the presence of the supernatant of the *B. salyersiae* PGM reaction carried out with different combinations of substrates, as indicated. Blue bars: Same conditions as adjacent blue bars, except that *B. salyersiae* PGM was replaced by Slr1334, as a positive control. Error bars represent the SD; asterisks represent the statistical significance in comparison to the reaction without effectors.

age of 15–20 reaction cycles by a spontaneous dissociation of the intermediate Glc-1,6-BP leaving behind an inactive dephosphoenzyme. Therefore, a constant level of Glc-1,6-BP is necessary to ensure that PGM is kept in an active state (24, 25). While the loss of the intermediate might be regarded as an unfavorable event, it can also be considered as a regulatory mechanism that enables regulation of PGM activity based on Glc-1,6-BP levels. For other α PHMs like PMM and PNGM it is known that the corresponding intermediates (e.g., α -D-mannose-1,6-BP and α -D-glucosamine-1,6-BP) are formed during the reaction of the respective enzymes in the same way as for PGM but no source of those intermediates is known that would enable an initial activation of these α PHMs or would keep them in an active state overtime when phosphorylation is lost. However, because many members of the α PHM family show at least some residual PGM activity, Glc-1,6-BP was suggested to be the activating compound for these enzymes as well. This has in fact been shown for PNGM from *E. coli* which is considered to be an essential enzyme (26). This makes the role of Glc-1,6-BP in carbon metabolism even more relevant and would explain the failed attempts to delete the *slr1334* gene (19).

In mammals, it was shown that Glc-1,6-BP can also regulate central enzymes of carbon pathways by inhibiting hexokinases, 6-phosphogluconate dehydrogenase and fructose-1,6-bisphosphatase and by activating phosphofructokinase and pyruvate kinase (12). Further studies in mammals showed that Glc-1,6-BP is especially elevated in brain tissue to a level that highly exceeds the regulatory concentrations needed for PGM activity, and that reduced levels of Glc-1,6-BP cause severe neurological problems (27). This indicates that at least in mammals there are likely more functions of Glc-1,6-BP to be discovered. Given the important role of Glc-1,6-BP in eukaryotic metabolism, it is surprising that very little is known on the role of Glc-1,6-BP in bacterial physiology. The only study about the origin of α -D-Glc-1,6-BP production in bacteria proposed a phosphodismutase reaction with Glc-1P as the sole substrate in *E. coli* (28). Moreover, the levels of Glc-1,6-BP in eukaryotes are affected by a specific Glc-1,6-BP phosphatase, PMM1, which is a phosphomannomutase that is not part of the α PHM superfamily. Whether a homologue exists in bacteria is currently unknown (29).

In the absence of Glc-1,6-BP, both Slr1334 and SI0726 showed low PGM activity. This residual activity can be explained by the fact that a phosphorylation of the active site serine can also be achieved at high concentrations of Glc-1P but at a much lower efficiency, leading to high K_m values and overall strongly reduced activity (11). In the presence of Frc-1,6-BP, SI0726 was strongly inhibited, which agrees with the inhibitory effect of Frc-1,6-BP on other PGMs (20, 21). By contrast, Frc-1,6-BP enhanced the overall

catalytic activity of Slr1334, primarily because of a strongly decreased K_m . This effect can now be attributed to the formation of the activator Glc-1,6-BP.

Like mammalian PGM2L1, Slr1334 can utilize 1,3-BPG as a phosphorylating compound for Glc-1,6-BP formation, however with a much lower efficiency than Frc-1,6-BP. Although it cannot be excluded that 1,3-BPG could play a role under certain conditions, Frc-1,6-BP seems to be more important as it can accumulate to high levels in bacteria (30).

For the mammalian PGM2L1 a reverse reaction (formation of 1,3-BPG and Glc-1P from Glc-1,6-BP and 3-PG) has not been shown. For Slr1334, we could clearly show reversibility of the reaction, producing Frc-1,6-BP from Glc-1,6-BP and Frc-6P. Provided that both directions of the reaction have similar kinetic properties, Slr1334 appears to be a *bona-fide* Glc-1,6-BP synthase, which is prone to activate the main PGM Slr0726.

In contrast to Glc-1,6-BP, Frc-1,6-BP is an obligatory intermediate in glycolysis, gluconeogenesis and Calvin-Benson-Bassham cycle. In contrast to mammalian PGM2L1, *Synechocystis* Slr1334 uses this sugar as the major substrate for Glc-1,6-BP synthesis. This links the synthesis of Glc-1,6-BP to the levels of Frc-1,6-BP. Its steady state level depends on the activity of the opposing reactions of phosphofructokinase (Pfk) and fructose-1,6-bisphosphatase (FBPase), both of which are regulated in an opposite manner: While PFK is usually activated by the low energy state metabolites ADP and AMP and downregulated by the high energy state metabolites ATP and citrate, FBPase is downregulated by AMP while unaffected by high energy compounds. The level of Frc-1,6-BP is further determined by the Frc-1,6-BP-aldolase activity. Frc-1,6-BP is also known to be an allosteric activator of pyruvate kinase (PK), the final step in glycolysis (31).

Altogether, in the cyanobacterium *Synechocystis* PCC6803, Frc-1,6-BP appears to have two counteracting effects on PGM activity. On the one hand, it acts as a direct inhibitor of PGM; on the other hand, it gives rise to the synthesis of the PGM activator Glc-1,6-BP through the activity of Slr1334. Furthermore, since these double phosphorylated sugars may affect further enzyme reactions, by connecting these two metabolites, Slr1334 might represent a crucial regulatory point in glycolysis (Fig. 8). This conclusion would further underline the essential role of *slr1334* (19).

Interestingly, our phylogenetic analysis revealed the cd05800 subfamily as the most primordial group of phosphoglucomutases. This group appears to be especially present in bacterial classes that are considered deeply branching bacteria meaning they are relatively close to the last common universal ancestor. Examples for this are the classes of Deinococci, Aquificae, Thermotogae, Bacteroidetes, and Cyanobacteria, where this PGM subfamily is widely distributed. This finding is supported by the fact that cd05800 members can also be found in archaea. In agreement with this, the first characterized representative of this group, Slr1334 shows both PGM and Glc-1,6-BP activity. This suggests that in primordial systems these PGMs with broad functionality could have performed PGM reactions without the requirement for specific Glc-1,6-BP synthase enzymes. During subsequent functional diversification, the PGMs may have adopted unique functions, with specialization toward efficient conversion of Glc-6-P into Glc-1P on the one hand. On the other hand, they may have lost the ability to sufficiently catalyze other reactions like Glc-1,6-BP synthesis. This assumption agrees with our observation that in some bacteria the primordial, more universal cd05800 PGM is the only subfamily found. In contrast, the more specialized and highly efficient cd03085 and cd05801 PGMs are always accompanied by other subfamily members (mainly cd05800). Conversely, the cd03089 PGM/PMM, often associated with pathogenic bacteria, is never accompanied with a cd05800 representative which raises the question how those strains produce Glc-1,6-BP (Table S2). To clarify these issues, further investigations on α PHM members from various bacterial groups are required.

We were able to show that the cd05800 PGM from the human gut commensal bacterium *B. salyersiae* also catalyzes the synthesis of Glc-1,6-BP out of Frc-1,6-BP like Slr1334 (Fig. 7). In the case of *B. salyersiae*, the enzyme appears to have an even higher catalytic efficiency for the Glc-1,6-BP synthase reaction than for the standard PGM reaction (Fig. S1 in the supplemental material). Therefore, we conclude that the formation

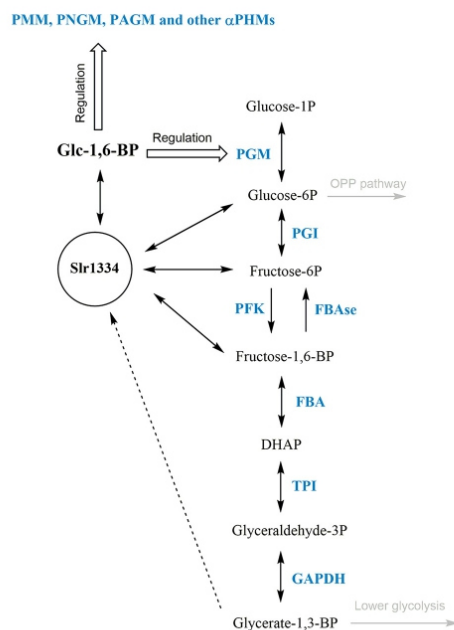


FIG 8 Potential role of Slr1334 in glycolysis. Shown is the potential role of Slr1334 in regulating activity of α PHMs and the concentration of key metabolites in glycolysis. PGM: Phosphoglucosyltransferase; PGI: Glc-6P-Isomerase; PFK: Phosphofructokinase; FBAse: fructose-6P phosphatase; FBA: Frc-1,6-BP-aldolase; TPI: Triosephosphate isomerase; GAPDH: Glyceraldehyde-3P dehydrogenase; DHAP: Dihydroxyacetone phosphate.

of Glc-1,6-BP might be a general feature of this PHM subfamily. However, further studies on members of this group are required to support this hypothesis.

Interestingly, members of the cd05800 group are also present in emerging human pathogenic species like *Vibrio*, *Eggerthella*, and *Brachyospyra* (Fig. 1) and are likely to play a key role in the metabolism of these pathogens. In fact, due to their crucial role in capsule and cell wall synthesis PGMs have been suggested as novel targets for the development of anti-infectives in prophylactic and therapeutic interventions (5, 32). It is tempting to speculate that Glc-1,6-BP synthases of the cd05800 family, in virtue of their absence in eukaryotes, may also be interesting candidates for the development of novel drugs modulating the microbiota with little collateral damage to the host.

MATERIALS AND METHODS

Isothermal, single-reaction DNA assembly (Gibson cloning). Cloning was performed as described by Gibson, Young (33) using *E. coli* NEB10 β cells (details in Table S5). All primers and plasmids used are shown in Table S3 and Table S4 in the supplemental material, respectively.

Cultivation of *Escherichia coli*. If not otherwise stated *E. coli* was grown in Luria-Bertani medium at 37°C (34). For growth on plates, 1.5% (wt/vol) agar-agar was added. For cells containing plasmids, the appropriate concentration of antibiotics was used. All *E. coli* strains used in this study are listed in Table S5 in the supplemental material.

Protein overexpression and purification. The plasmids used for protein overexpression are shown in Table S4 in the supplemental material. *Escherichia coli* Rosetta-gami (DE3) (details Table S5) was used for the overexpression of all proteins. For this, cells were cultivated in 2xYT (3.5% tryptone, 2% yeast extract, 0.5% NaCl; 1L of culture in 5L flasks) at 37°C until reaching exponential growth (OD_{600} 0.6–0.8). Protein overexpression was induced by adding 75 μ g/L anhydrotetracycline, followed by incubation at 20°C for 16 h. Cells were harvested by centrifugation at 4000 g for 10 min at 4°C. Cell disruption was performed by sonication in 40 mL of lysis buffer (100 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 mM MgCl₂, 20 mM Imidazole for His-tagged proteins and 100 mM Tris-HCl pH 8, 150 mM NaCl, 10 mM MgCl₂ for Strep-tagged proteins); lysis buffers were supplemented with DNase I and cOmpleteTM protease inhibitor cocktail (Roche, Basel).

The cell lysate was centrifuged at 40,000 g for 45 min at 4°C and the supernatant was filtered with a 0.22 μ m filter.

For the purification of His-tagged proteins, 5 mL Ni-NTA HisTrap columns (GE Healthcare, IL, USA) were used. The cell extracts were loaded onto the columns, washed with wash buffer (50 mM Tris-HCl pH 7.4, 500 mM NaCl, and 50 mM Imidazole) and eluted with elution buffer (50 mM Tris-HCl pH 7.4, 500 mM NaCl, and 500 mM Imidazole).

Measurement of PGM activity of Slr1334 and Slr1334 *in vitro*. Buffer for enzymatic reactions was composed of 50 mM HEPES-KOH pH 7.5, 150 mM KCl, 10 mM MgCl₂, 1 mM NADP⁺, 1 mM DTT, and 1 U/mL G6PDH from *Saccharomyces cerevisiae* (G6378, Sigma-Aldrich). For Slr1334 activity, 60 ng of Strep-tagged purified protein was added to each reaction. For Slr1334 activity, 650 ng of purified protein was added. For tests with saturating Glc-1,6-BP concentrations, 60 μ M Glc-1,6-BP have been used. Reaction was started by the addition of glucose-1-P. Reactions were carried out in a total of 300 μ L in a 96-well microplate. Absorption change at 340 nm was continuously measured for 15 min at 30°C in a TECAN Spark Multiplate reader (Tecan Group AG, Männedorf, Switzerland). At least three replicates were measured.

Measurement of Glc-1,6-BP formation. The reaction buffer was composed of 50 mM HEPES-KOH pH 7.5, 150 mM KCl and 10 mM MgCl₂. To test the formation of Glc-1,6-BP out of Frc-1,6-BP 1 mM Frc-1,6-BP, 1 mM Glc-1P or 1 mM Glc-6P were added in different combinations. To test the formation of Glc-1,6-BP out of 1,3-BPG instead of Frc-1,6-BP, 1 mM ATP, 1 mM 3-PG and 60 μ g PGK have been used. Slr1334 concentration were 30 μ g/mL. The reactions were performed for 1.5 h at 30°C followed by heat inactivation at 90°C for 10 min with subsequent centrifugation at 25000 g for 10 min at 4°C. The supernatants of the various reactions were used at 1:10 dilution in the Slr1334 activity assay.

Measurement of Frc16BP formation by coupling to Frc-1,6-BP aldolase assay. To measure the formation of Frc-1,6-BP by Slr1334, the reaction buffer was composed of 50 mM HEPES-KOH pH 7.5, 150 mM KCl, 10 mM MgCl₂, 0.2 mM NAD, 7 μ g Slr1334, 1U/mL FBP-aldolase from rabbit muscle and 1U/mL GDH from rabbit muscle. Concentrations of Glc-1,6-BP and Frc-6P were varying depending on the experiment. Reaction was started by the addition of Glc-1,6-BP or Frc-6P depending on the experiment.

To measure residual Frc-1,6-BP after running the Slr1334 reaction, the buffer was composed of 50 mM HEPES-KOH pH 7.5, 150 mM KCl, 10 mM MgCl₂, 0.2 mM NAD, 0.02U/mL aldolase from rabbit muscle and 1U/mL GDH from rabbit muscle.

All reactions were carried out in a total of 300 μ L in a 96-well microplate. Absorption change at 340 nm was continuously measured for 15 min at 30°C in a TECAN Spark Multiplate reader (Tecan Group AG, Männedorf, Switzerland). The enzymatic activity was then calculated. At least three replicates were measured.

LC-MS measurement. Sample preparation. Reaction buffer was composed of 50 mM HEPES-KOH pH 7.5, 150 mM KCl and 10 mM MgCl₂. For the reaction without enzyme 500 μ M Frc-1,6-BP and 1 mM Glc-1-P were added. For the Slr1334 reaction, 30 μ g of Slr1334 have been used additionally. Reaction was carried out in a total volume of 1 mL at 30°C for 1h. Afterwards, reaction was stopped by heat inactivation at 90°C followed by centrifugation at 25000 g for 10 min at 4°C.

For degradation of Frc-1,6-BP, 1 mM NADH, 2U/mL of aldolase from rabbit muscle and 2U/mL of GDH from rabbit muscle were added. Reaction was carried out followed by inactivation at the same conditions as before.

LC/MS analysis. LC/MS analysis was performed using an electrospray ionization time of flight (ESI-TOF) mass spectrometer (MicroTOF II; Bruker Daltonics), operated in negative ion-mode connected to an UltiMate 3000 high-performance liquid chromatography (HPLC) system (Dionex). The separation in the HPLC was carried out using a SeQuant ZIC-pHILIC column (PEEK 150 $\times$ 2.1 mm, 5 μ m, 110 Å, Merck) at 30°C with an CH₃CN (buffer A) and 100 mM (NH₄)₂CO₃ pH 9 (buffer B) buffer system. A single run (injection volume of 5 μ L) was performed with a flow rate of 0.2 mL/min and a linear gradient of 25 min, reducing the concentration of buffer A from 82% to 42%. Before (5 min) and after (10 min) the gradient, the column was equilibrated with 82% buffer A.

Phylogenetic analysis of α PHM subfamilies. The occurrence of members of the α PHM subfamilies was investigated in organisms included in the NCBI RefSeq_protein database using the default settings of blastp. The search returned 2140 hits for Slr1334 homologues and >5000 hits for Slr1334 homologues by using the following threshold:

E-value $\leq 10^{-40}$; sequence identity $\geq 30\%$; Query coverage $\geq 95\%$

Phylogenetic tree data were created using the ClustalW implementation from the European Bioinformatics Institute and the phylogenetic tree was created using the Interactive Tree of Life (iTOL) v5 online tool (35, 36).

Statistical analysis. Statistical details for each experiment can be found in the figure legends. GraphPad PRISM was used to perform one-sided ANOVA to determine the statistical significance. Asterisks (*) in the figures symbolize the P-value: one asterisk represents $P \leq 0.05$, two asterisks $P \leq 0.01$, three asterisks $P \leq 0.001$, and four asterisks $P \leq 0.0001$.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 0.1 MB.

TABLE S1, DOCX file, 0.02 MB.

TABLE S2, DOCX file, 0.02 MB.

TABLE S3, DOCX file, 0.01 MB.

TABLE S4, DOCX file, 0.01 MB.

TABLE S5, DOCX file, 0.02 MB.

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N.N. performed cloning, protein purification, enzymatic assays phylogenetic analysis and wrote manuscript. S.F. performed LC/MS analysis. K.F. conceived study, interpreted data, and edited manuscript.

We declare no competing interest.

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4.3 Publication 3

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The TonB-dependent uptake of pyrroloquinoline-quinone (PQQ) and secretion of gluconate by *Escherichia coli* K-12

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Abstract

Glucose is taken up by *Escherichia coli* through the phosphotransferase system (PTS) as the preferred carbon source. PTS mutants grow with glucose as a carbon source only in the presence of pyrroloquinoline quinone (PQQ), which is needed as a redox cofactor for the glucose dehydrogenase Gcd. The membrane-anchored Gcd enzyme oxidises glucose to gluconolactone in the periplasm. For this reaction to occur, external supply of PQQ is required as *E. coli* is unable to produce PQQ de novo. Growth experiments show that PqqU (previously YncD) is the TonB-ExbBD-dependent transporter for PQQ through the outer membrane. PQQ protected the cells from the PqqU-dependent phage Isaakselin (Bas10) by competition for the receptor protein. As a high affinity uptake system, PqqU allows *E. coli* to activate Gcd even at surrounding PQQ concentrations of about 1 nmoL/L. At about 30-fold higher PQQ concentrations, the activation of Gcd gets PqqU independent. Due to its small size, Pqq may also pass the outer membrane through porins. The PQQ-dependent production of gluconate has been demonstrated in many plant growth-promoting bacteria that solubilise phosphate minerals in the soil by secreting this acid. Under phosphate limiting conditions also *E. coli* induces the glucose dehydrogenase and secretes gluconate, even in absence of PTS, that is, even when the bacterium is unable to grow on glucose without PQQ.

KEYWORDS

phosphate limitation, PqqU (previously)pyrroloquinoline-quinoneTonB, YncD, YncDgluconate secretion

1 | INTRODUCTION

Enterobacteria like *Escherichia coli* use glucose as the preferred carbon source. The uptake depends on the phosphotransferase (PTS) system, which is required for translocation of the carbohydrate across the inner membrane concomitant with its phosphorylation. However, glucose may also be oxidised in the periplasm

to gluconolactone by the glucose dehydrogenase Gcd (Van Schie et al., 1985). In addition, the periplasmic soluble aldose sugar dehydrogenase Asd (Ylii), which has a very broad substrate specificity, may contribute to glucose oxidation, leading to formation of gluconate (Southall et al., 2006). Gluconate may then be transported into the cell by one of the four sugar acid transporters GntU, GntT, GntP, or IdnT (=GntW) (Peekhaus et al., 1997) for further catabolism.

Abbreviations: AUC, area under the curve in a mass spec; Pi, inorganic phosphate; PQQ, pyrroloquinoline quinone; PTS, phosphotransferase sugar transport system.

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However, gluconate that is produced in the periplasm may not always be taken up directly into the cytoplasm. It is also found in the medium as reported previously (Bouvet & Grimont, 1988). Both dehydrogenases Gcd and Asd require pyrroloquinoline-quinone (PQQ) (Figure 1) as a redox cofactor for their activity. Since *E. coli* is unable to produce PQQ (Matsushita et al., 1997), this sugar oxidation is not observed without exogenous addition of PQQ. However, in the context of a microbiome where PQQ may be synthesised by other Gram negative bacteria like *Klebsiella*, *Pseudomonas*, *Acinetobacter*, this kind of sugar utilisation may be important for *E. coli*. The secretion of large ($\mu\text{mol/L}$) amounts of PQQ into the growth medium has been demonstrated for more than 15 different soil bacteria (McIntire, 1998).

The complicated biosynthesis of PQQ has been elucidated in the recent years. In a ribosomally synthesised peptide, a glutamate residue is linked to a tyrosine residue and further shaped to PQQ (Zhu & Klinman, 2020). It may be expected that PQQ is present in very low amounts in the natural environment of *E. coli*. Rare substances like vitamin B12 and iron siderophores are generally imported across the *E. coli* outer membrane by TonB-dependent receptors. These uptake processes are energised by the membrane potential via the TonB-ExbBD complex which allows high affinity uptake (Braun, 1995). Of the predicted nine TonB-dependent receptors in *E. coli* K-12, six are known to mediate the uptake of iron siderophores (enterochelin and its degradation products, ferrichrome, coprogen and iron-citrate) (Braun & Hantke, 2007). The TonB-dependent protein BtuB is the receptor for vitamin B12 (Bradbeer, 1993), which is used as a cofactor for methylgroup transfer reactions and methionine biosynthesis. The structure of the two other predicted TonB-dependent proteins PqqU (previously YncD) and YddB has been elucidated, but their function in *E. coli* is still unknown (Grinter et al., 2019; Grinter & Lithgow, 2020). Upstream of *pqqU* (previously *yncD*) on the complementary strand the gene *yncE* encodes a periplasmic protein that has been shown to bind DNA and whose structure has been determined to be a seven bladed propeller (Kagawa et al., 2011). In quinoproteins,

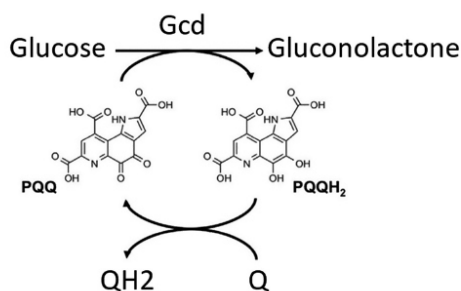


FIGURE 1 Overview of the PQQ/Gcd-dependent oxidation of glucose in gluconolactone. Pyrroloquinoline quinone (PQQ, 4,5-dihydro-4,5-dioxo-1H-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid, methoxatin) is a redox cofactor for quinoproteins like glucosedehydrogenase (Gcd), PQQH₂ may be oxidised by ubiquinone (Q) (Cordell & Daley, 2022).

PQQ is often bound to bladed propeller structures. Therefore, this protein is predicted in some databases as a PQQ-binding protein (Keseler et al., 2021), although its function is unknown.

Here, we tested whether PQQ is a substrate of the outer membrane protein YncD. Since we found evidence that YncD mediates uptake of PQQ, we propose to name this receptor PqqU based on its function.

2 | RESULTS

2.1 | YncD is the receptor for PQQ

Glucose is mainly taken up by the PTS system for further metabolism. In a PTS-deficient mutant glucose is oxidised in the periplasm to gluconolactone by the glucose dehydrogenase Gcd (Cleton-Jansen et al., 1990) and the sugar aldose dehydrogenase YliI (Southall et al., 2006). Gluconolactone may hydrolyse spontaneously or, more likely, is hydrolysed to gluconate by lactonase. The product of the hydrolysis, gluconate, is then taken up and further metabolised. PQQ-dependent growth of *E. coli* 6281 $\Delta(\text{ptsHI } \text{cr})$ (abbreviated Δpts) was demonstrated in liquid minimal medium MOPS or M63, containing 0.1 mmol/L or 100 mmol/L Pi, respectively, supplemented with 0.1% glucose and different concentrations of PQQ (Figure 2). Even at a concentration of 1 nmol/L PQQ stimulated rapid growth of 6281 Δpts in both minimal media (Figure 2a,c). In contrast, at low PQQ concentrations the *E. coli pts pqqU* deletion mutant displayed a delayed growth start in the MOPS medium, an effect that was concentration dependent and not observed at high PQQ concentrations (Figure 2b). In the M63 medium, the $\Delta(\text{ptsHI } \text{cr}) \Delta\text{pqqU}$ deletion mutant showed a long lag phase, and at lower PQQ concentrations no growth was observed in the time given (2D). When instead of glucose, gluconate was used as carbon source, both the PTS-deficient strain and the PTS and PqqU-deficient strain were able to grow without addition of PQQ (Figure 2). This shows that only the oxidation of glucose to gluconate is affected in the absence of PqqU activity.

Hydrophilic molecules with a molecular weight below 600 Da are expected to pass through the outer membrane by diffusion across porins (Nikaido, 2003). Growth of the PqqU-deficient mutant at high PQQ concentrations may be explained by the small size of PQQ which has a molecular weight of 330 Da and can freely diffuse across porins, and thus fulfil the demands of the glucose dehydrogenase Gcd as a cofactor. At low concentrations, the high affinity uptake system for PQQ is necessary.

As a TonB-dependent transport is predicted from the structure of PqqU, we tested the growth of a PTS and TonB-deficient mutant, *E. coli* 6284 $\Delta(\text{ptsHI } \text{cr}) \text{tonB}$. In the presence of PQQ, a growth response to 3000 nmol/L PQQ was observed, but no response to 100 nmol/L PQQ (Figure 3a). Since the energisation of these receptors is dependent on the TonB/ExbB/ExbD complex an *exbB::Tn10* mutant was also tested. In minimal medium with gluconate, the ExbB mutant grew as well as the parent strain. Growth with glucose was

FIGURE 2 PQQ-dependent growth of a PTS deficient *E. coli* strain. Cells were pregrown in minimal medium M63 0.6% glycerol over night. Strains 6281 $\Delta(ptsHI\ cr)$ (abbreviated *pts*) (a, c) and 6289 $\Delta(ptsHI\ cr)\Delta pqqU$ (abbreviated *pts pqqU*) (b, d) were grown in minimal medium MOPS 0.1mmol/L Pi or M63 100mmol/L Pi as indicated in the chart title in a 96 well plate. Carbon source was 0.2% gluconate (GlcN, black) or 0.1% glucose. The different concentrations of PQQ, 3000 to 0 nmol/L, are indicated in the bottom line of the graph.

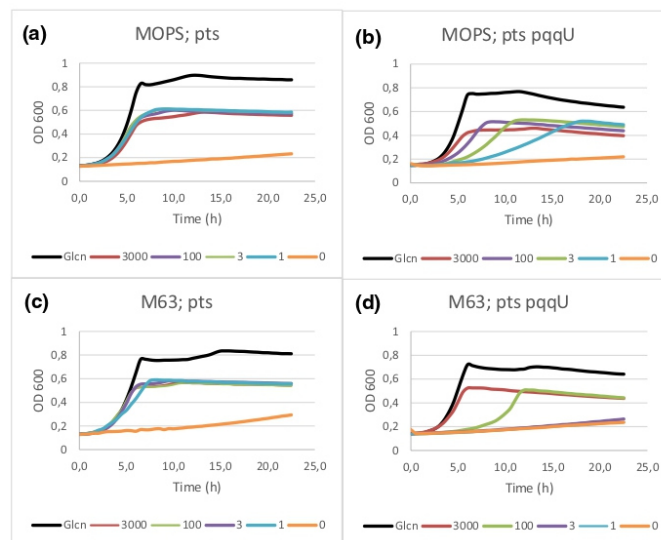
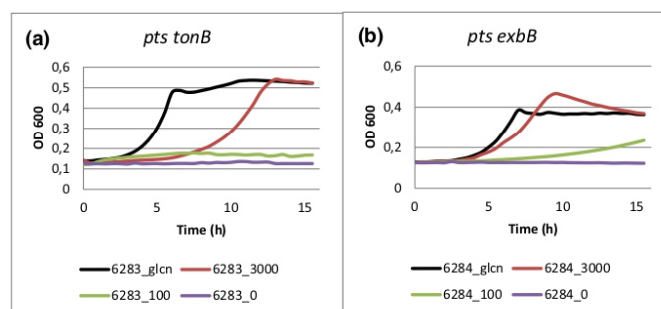


FIGURE 3 Growth of the *E. coli* *pts* deletion mutant in the presence of low concentrations of PQQ depends on the TonB/ExbBD system. Growth of TonB and ExbB mutants on M63 minimal medium with 0.1% gluconate (glcN) or 0.1% glucose supplemented with 3000, 300, 30, 10 or 0 nmol/L PQQ. Strains: (a) 6284 $\Delta(ptsHI\ cr)\ tonB$, (b) 6283 $\Delta(ptsHI\ cr)\ exbB$.



stimulated by high PQQ concentrations but delayed at lower ones (Figure 3b), indicating a dependence of PQQ uptake on ExbB. In summary, at low concentrations *E. coli* appears to require the concerted activity of PqqU and TonB/ExbBD for PQQ uptake.

2.2 | Inhibition of phage infection by PQQ

The phages T5 and BF23 are known to use the TonB-dependent outer membrane proteins FhuA and BtuB, respectively, as receptors for infection. It was shown that the substrates of these receptors, ferrichrome and vitamin B12, respectively, could protect the cells from infection (Bradbeer et al., 1976; Hantke & Braun, 1978). Since there are also phages known to use PqqU as a receptor, such as phage IsaakIselin (Bas10) (Maffei et al., 2021), we asked whether there could be competition between the substrate PQQ and phage for binding to PqqU. Plating efficiency of Bas10 was the same on the parental strain BW15113 and the TonB-deficient mutant JW5195

while the PqqU mutants JW1446 and 6289 were resistant. On agar plates, the application of filter paper discs soaked with PQQ inhibited plaque formation by Bas10 (Figure 4). Interestingly, the single *tonB* and the double *tonB pts* mutants were protected more efficiently than the parental strains as a roughly 1000-fold higher concentration of PQQ was necessary to protect the latter. These findings are in line with what has been observed for the ferrichrome and vitamin B12 uptake systems: due to the lack of TonB PQQ, which remains bound to the receptor and efficiently blocks phage adsorption.

2.3 | Influence of phosphate on PQQ-dependent growth and secretion of the oxidation product

As it can be seen in Figure 2, there is a dramatic difference in the growth response of the PqqU mutant between a phosphate-rich and phosphate poor medium. Adamowicz et al. (1991) studied two glucose-negative mutants of *E. coli*, *ptsM ptsG* and *pgi zwf*

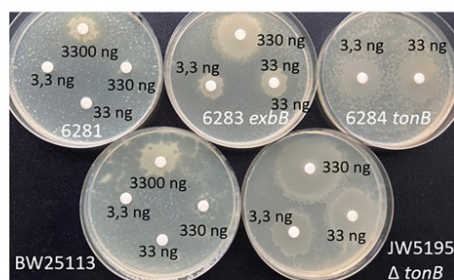


FIGURE 4 Inhibition of phage infection by PQQ. An LB agar plate was overlaid with 3 ml LB soft agar containing about 5×10^8 cells of the indicated bacterial strains and 10^4 Bas10 phages. Filter paper discs with the indicated amount of PQQ were applied. After incubation at 37°C, bacteria protected from the phage induced lysis were able to grow in the PQQ diffusion zone around the paper discs. In the TonB mutants, the substrate is not taken up, remains bound to the receptor and inhibits adsorption of the PqqU-dependent phage Bas10. The ExbB mutant is less well protected due to the partial complementation by *tolQR*.

respectively, which were unable to grow on glucose in a minimal medium. Only after addition of PQQ growth was observed. Both strains had a rather long lag phase when they were pre-cultured in a phosphate-rich medium. It was suggested that the porin PhoE, with its known preference for anionic substrates, could be the gate for PQQ to get access to the inner membrane-anchored glucose dehydrogenase Gcd facing the periplasm (Figure 7). Pre-growth of *E. coli* in a phosphate-rich medium would repress PhoE expression and lack of PhoE would slow down PQQ uptake. However, this hypothesis was not experimentally verified.

Phosphate limitation leads to a general nutrient limitation with major changes in gene expression, which are mediated by the regulatory protein DksA (Gray, 2020). Using an RNA sequencing screen, Gray (2020) analysed those changes after a downshift from LB medium to a MOPS 0.1 mmol/L Pi medium. Interestingly, *gcd* expression increased about 10-fold (Gray, 2020). This could lead to a different interpretation of the data of Adamowicz et al. (1991). Cells grown in a phosphate-rich medium may have phosphate reserves and may need more time to adapt to the stress imposed by low phosphate levels. In addition, we did not observe an influence of PhoE on PQQ-dependent growth (data not shown).

Among plant growth-promoting bacteria like *Pseudomonas*, PQQ-dependent glucose oxidation and secretion of gluconate is widespread (Miller et al., 2010; Raymond et al., 2021). Gluconate solubilises phosphate minerals and in doing so furthers plant growth. Not only *Pseudomonas* but also Enterobacteria such as *Serratia* (Ludueno et al., 2017) and *Klebsiella* (Hommes et al., 1989) strains are described to produce PQQ and secrete gluconate.

We investigated whether *E. coli* K-12 also secretes the oxidation product of Gcd into the medium. Strain BW25113 was grown with PQQ in MOPS (0.1 mmol/L Pi) medium with 0.1% glucose. The supernatant was analysed by liquid chromatography-mass

spectrometry (LC-MS). About 3 mmol/L gluconate were found during the late logarithmic growth phase in the medium (Figure 5). This was consumed in the late logarithmic growth phase. Instead, much less gluconate (less than 0.2 mmol/L) was secreted in the phosphate-rich M63 medium. Interestingly, also the *pts* deletion mutant H2681 secreted gluconate into the medium, although one would have expected gluconate to be directly transported from the periplasm to the cytoplasm. In MOPS medium with 0.1% glucose, about 0.9 mmol/L gluconate was found in the late log phase. In M63, a similar amount of about 0.7 mmol/L gluconate was determined. It remains to be clarified how much gluconolactone/gluconate is directly taken up from the periplasm.

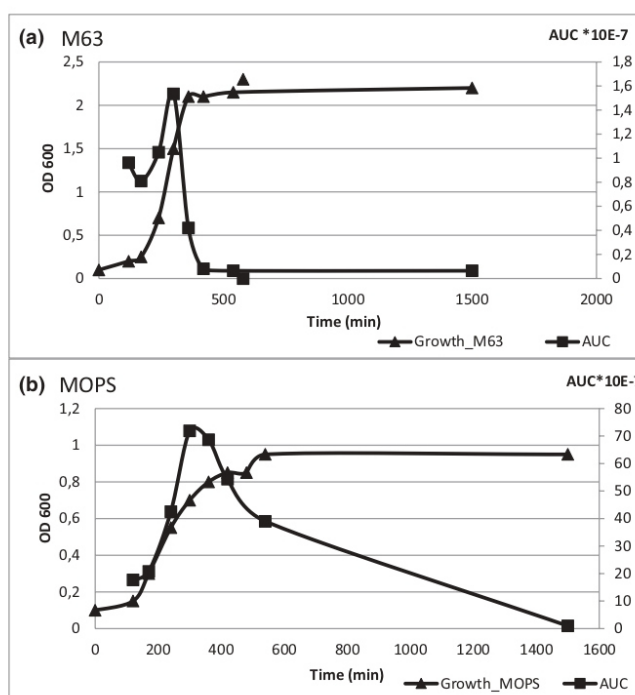
2.4 | Visualisation of gluconate secretion

Secretion of acids may be visualised on MacConkey agar plates. *E. coli* forms red colonies on glucose-containing solid medium because glucose overflow metabolism causes secretion of organic acids such as acetic acid. The acidification leads to a colour change of the pH indicator neutral red from yellow to red, and to the precipitation of bile acids which makes the agar turbid. PTS mutants cannot utilise glucose and form pale whitish colonies. Application of filter paper discs soaked with PQQ allowed *E. coli* Δpts to utilise glucose. This resulted in red colonies and a red turbid halo in the diffusion zone. The size of the red and turbid zones was dependent on the PQQ concentration (Figure 6a). However, inoculum size and incubation time had a large influence on the size of the halo. While for the *E. coli* Δpts application of about 10 nmol/L PQQ in the agar led to the formation of red colonies with a turbid halo, for strain 6289 $\Delta pts \Delta pqqU$, an approximately 30 to 100 fold higher PQQ concentration was necessary to observe red colonies with a turbid halo (Figure 6a). This again indicates that the *pqqU* mutant lacks a high affinity uptake system for PQQ.

A similar difference in colony colour development was observed when MacConkey plates contained defined PQQ concentrations (Figure 6b). Colonies of 6289 *pqqU* null mutant were only red with 1000 nmol/L PQQ while the parent strain 6281 produced even at 3 nmol/L PQQ red colonies. In addition, an *YncE*-deficient mutant was tested, which in both, this plate assay and in growth experiments (data not shown) behaved like the parent strain.

The addition of 10 μ mol/L iron to the MacConkey agar plates was necessary to suppress the acid production of strain 6284 $\Delta pts \Delta tonB$ with and without glucose (Figures 6a and S1). This indicates that acid is produced from peptones. After addition of iron, pale white colonies appeared (Figure S1). The glucose-independent acid production is a consequence of the *tonB* mutation. The strain is unable to utilise siderophore bound iron and secretes the high affinity iron chelator enterochelin to overcome the iron deficit. This aggravates the situation as more iron is chelated and less iron is available for low affinity uptake systems. It seems that acids are secreted due to the internal iron deficit. In chemostat cultures upon iron limitation, an overflow metabolism with secretion of acetate and lactate has been observed in *E. coli* (Folsom et al., 2014).

FIGURE 5 Gluconate production of BW25113. *E. coli* K-12 BW25113 was grown in minimal medium M63 (a) or MOPS (b) 0.1 mmol/L pi with 0.1% glucose and 0.6 μ mol/L PQQ. Gluconate in the supernatant was determined by LC-MS; 70×10^7 AUC (area under the curve in a mass spec) are about 3 mmol/L gluconate; 1.5×10^7 AUC are below 0.2 mmol/L gluconate. Note the different scales of the abscissa (AUC). Cells grown in M63 medium achieved twice the cell density compared with cells grown in MOPS.



Interestingly, a similar observation has been made in *Pseudomonas* (Sasnow et al., 2016).

On iron-supplemented Mac Conkey plates with glucose (Figure 6a), the TonB mutant secretes acids only at relatively high PQQ concentrations demonstrating that PQQ is not taken up by the high affinity uptake system.

The ExbB-deficient mutant showed even larger red turbid zones around the PQQ discs in comparison with the parent strain. This may be explained by the partial complementation of ExbBD by TolQR which is observed in several TonB-dependent uptake systems (Bradbeer, 1993; Braun & Herrmann, 1993). The lower velocity of PQQ uptake may allow further diffusion of PQQ, especially at low cell densities and could explain the larger response zone.

2.5 | PQQ has no direct influence on the *E. coli* iron metabolism

The crystal structure of PqqU (previously YncD) has been solved (Grinter & Lithgow, 2020) and a certain similarity to the di-iron-di-citrate siderophore receptor FecA was observed. The predicted binding site contains several positively charged residues. This argues for a negatively charged substrate, but an iron-citrate

complex did not fit. PQQ (Figure 1) fulfils the postulate of the authors "...the substrate of YncD is likely to be relatively small and negatively charged" (Grinter & Lithgow, 2020). The carboxylate groups of PQQ are known to interact with metals like Ca²⁺, Mg²⁺, and lanthanoids (Wehrmann et al., 2020) in the binding pockets of quinoproteins. Therefore, we tested whether the supply of PQQ in an iron poor environment would influence the iron balance in the cells. In an iron-rich medium, the *fhuF-lacZ* fusion of strain H1717 is repressed by the iron uptake regulator Fur (Stojiljkovic et al., 1994). Supply of PQQ did not change the expression of the *fhuF-lacZ* fusion neither under iron-rich nor under iron poor conditions (data not shown). This indicated that PQQ neither increased nor decreased the internal iron level. These results are in line with the findings of different groups that PqqU is not regulated by Fur and does not transport iron in relevant amounts (Grinter & Lithgow, 2020).

3 | DISCUSSION

Vitamin B12 and PQQ are needed as cofactors for certain enzymes. Since *E. coli* cannot synthesise them de novo, the bacterium depends on an external supply. In the environment, both substances may be present only in low concentrations. Accordingly, uptake of

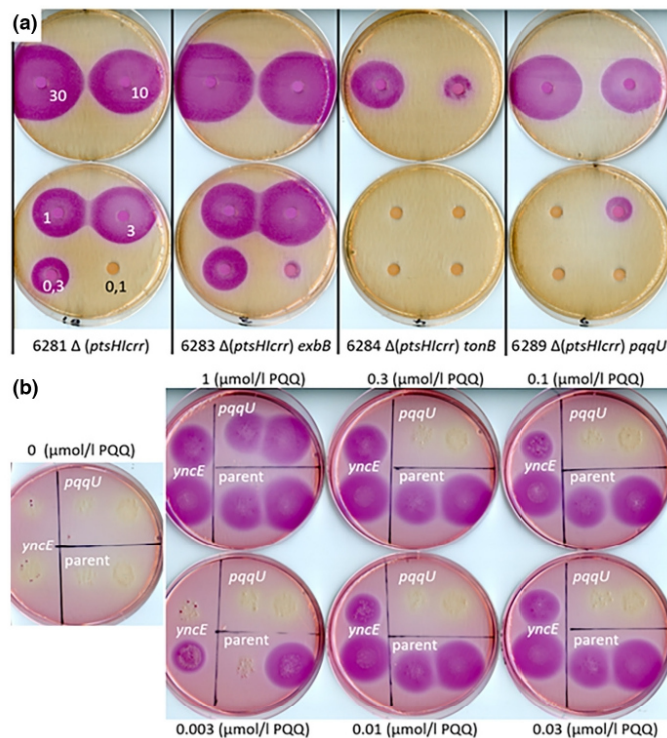


FIGURE 6 The PqqU/TonB/ExbB uptake system is required for PQQ-dependent production and secretion of gluconate at low PQQ concentrations. (a) Approximately 10^4 cells of the indicated strains were spread on MacConkey agar plates (0.1% glucose 10 $\mu\text{mol/L}$ Fe(III)Na(EDTA)) and filter paper discs containing 30 ng to 0.1 ng PQQ were applied. Plates were incubated at 37°C for about 14 h. (b) MacConkey glucose agar plates were prepared containing 0 to 1 $\mu\text{mol/L}$ PQQ as indicated. Twenty microlitre containing about 30 to 300 colony forming units of the strains 6281 Δpts , 6289 $\Delta pts \Delta pqqU$, and 6280 $\Delta pts \Delta yncE$ were applied. After about 18 h of incubation at 37°C, pictures were taken.

vitamin B12 requires the high affinity, TonB-dependent receptor BtuB (Bradbeer, 1993). It is not surprising that also PQQ is taken up by a TonB-dependent receptor, namely PqqU. Unlike vitamin B12, PQQ is a small molecule and may also pass the outer membrane without PqqU. However, this diffusion-dependent uptake process is rather inefficient, which may be a disadvantage when only low amounts of PQQ are present.

As a "common good", PQQ like vitamin B12 may promote interspecies interactions in the microbiome (Sokolovskaya et al., 2020). PQQ-dependent gluconate production is the characteristic for numerous plant growth-promoting bacteria (Meyer et al., 2011; Shen et al., 2012; Wu et al., 2022). Organic acids like gluconate mobilise phosphate from insoluble calcium phosphates and thereby further bacterial and plant growth. There is evidence that phosphate limitation induces PQQ synthesis in *Pseudomonas putida* (An & Moe, 2016) and *Serratia marcescens* (Ludueno et al., 2017). Interestingly, iron limitation also led to a high secretion of gluconate (44% of the supplied glucose) in *Pseudomonas putida* (Sasnow et al., 2016). However, under the growth conditions used, the additionally synthesised siderophore mobilised most of the iron mineral supplied as goethite. Gluconate did not contribute significantly to the iron supply of the cells (Sasnow et al., 2016).

In *E. coli*, gluconate secretion has been described for a *pgl* mutant, which is unable to produce phospho-gluconolactonase in the

cytoplasm (Kupor & Fraenkel, 1969). For the product of Gcd, gluconolactonase, it is unknown whether it is enzymatically hydrolysed. Under physiological conditions gluconolactone is rather unstable and may hydrolyse spontaneously before or after it is secreted. A gluconolactonase in the periplasm was identified in a gluconate secreting *Pseudomonas* (Tarighi et al., 2008). Since gluconolactones are toxic due to their acylating activity, a lactonase may be expected for "house cleaning" purposes (Galperin et al., 2006). A possible candidate could be YncE, which is encoded upstream of *pqqU* on the complementary strand. The bladed propeller structure of YncE (Kagawa et al., 2011) is often found in quinoproteins. Therefore, in the beginning of our studies we had the hypothesis that it could be a PQQ-binding protein involved in transport. However, PQQ-dependent growth of the *yncE* deletion strain did not differ from that of the parent strain (Figure 6b and data not shown). This would not necessarily be expected for a gluconolactonase mutant, since a *pgl* mutant shows only minor growth defects with glucose as carbon source (Kupor & Fraenkel, 1972). Sequence comparison did not allow assignment of a function for *yncE*. In this context, it is interesting to mention that YncE has been found to be a protective antigen in *Escherichia* (Moriel et al., 2016) and a diagnostically interesting antigen in *Salmonella* (Franklin et al., 2020). Also PqqU (previously YncD) from *Salmonella* has been demonstrated in mice to be a protective antigen (Xiong et al., 2012).

TABLE 1 List of *E. coli* strains

Strain	Description	Source
BW25133	F ⁻ , $\Delta(\text{araD-araB})567$, $\Delta(\text{lacZ4787}::\text{rrnB-3})$, λ^- , <i>rph-1</i> , $\Delta(\text{rhaD-rhaB})568$, <i>hsdR514</i>	Laboratory strain collection
#1233	MC4100 $\Delta(\text{ptsHI cr})::\text{kan}$ $\Delta(\text{murP})::\text{kan}$	C. Mayer (Tübingen) (Kluj et al., 2018)
H1388	$\Delta(\text{pro lac})$ <i>tsx aroB exbB::Tn10</i>	(Hantke & Zimmermann, 1981)
H5073	<i>aroB tsx malT tonB trpB114::Tn10</i>	This institute
JW1446-1	BW25113 $\Delta(\text{pqqU})::\text{kan}$ ($=\text{yncD}::\text{kan}$)	(Baba et al., 2006)
JW1447-2	BW25113 $\Delta(\text{yncE-744})::\text{kan}$	(Baba et al., 2006)
JW0231-1	BW25113 $\Delta(\text{phoE759})::\text{kan}$	(Baba et al., 2006)
6281	BW25113 $\Delta(\text{ptsHI cr})::\text{kan}$	This work
6280	JW1447-2 $\Delta(\text{yncE-744})::\text{kan}$ $\Delta(\text{ptsHI cr})::\text{kan}$	This work
6283	6281 <i>exbB::Tn10</i>	This work
6284	6281 <i>tonB</i>	This work
6289	6281 $\Delta(\text{pqqU})::\text{scar}$ ($=\text{yncD}$)	This work
6296	6281 $\Delta(\text{phoE})::\text{scar}$	This work

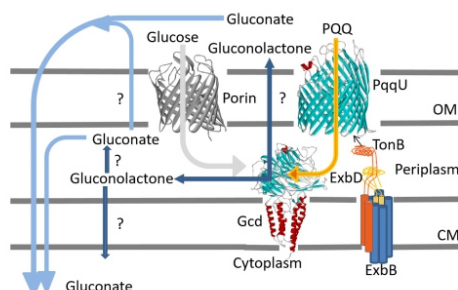


FIGURE 7 PQQ uptake and gluconate secretion in *E. coli*: The question marks indicate that it is unknown where and how gluconolactone hydrolises or is enzymatically hydrolised.

The importance of PQQ for *E. coli* has also been demonstrated by chemotaxis towards PQQ (De Jonge et al., 1996). However, it may not be the molecule PQQ that is detected in this case, but the redox state of the respiratory chain (Samanta et al., 2016) which may determine the direction during swimming.

4 | MATERIALS AND METHODS

4.1 | Bacterial growth conditions and strains

Bacteria were grown in LB or Difco Nutrient Broth at 37°C, with kanamycin (50 µg/ml) or tetracycline (15 µg/ml) if necessary. Difco MacConkey agar base with 10 g/L glucose was used and supplemented where indicated with 10 µmol/L Fe(III)Na-EDTA. Approximately, 10,000 bacteria per plate were applied to demonstrate PQQ-dependent sugar utilisation. The diameter of the red zone was determined after 18 h.

The minimal medium M63 (containing per litre: 5.3 g KH_2PO_4 , 13.9 g, $\text{K}_2\text{HPO}_4 \times 3\text{H}_2\text{O}$, 2 g $(\text{NH}_4)_2\text{SO}_4$, 1 mM MgSO_4 was used for growth. Carbon sources were added as indicated. $\text{OD}_{600\text{nm}}$ was measured in a TECAN SPARK 10 M reader. The MOPS minimal medium contained 40 mM MOPS, 86 mM NaCl, 9.5 mM NH_4Cl , and 13.3 mM KCl; pH was adjusted to 7.2 with KOH. The Pi-limiting MOPS minimal medium contained 0.1 mM Pi by adding 1 ml of M63 salts/L. Carbon sources were added as indicated.

The bacterial strains used are listed in Table 1. Strains of the Keio collection (Baba et al., 2006) were kindly supplied by Heike Brötz-Oesterhelt. The kanamycin resistance gene was removed with pCP20 as described previously (Datsenko & Wanner, 2000) and further strain constructions were done by P1 transductions. Strains 6281, 6289, 6296 were constructed by P1 transduction of $\Delta(\text{ptsHI cr})::\text{kan}$ (#1233) into the respective parent strains by selecting for kanamycin resistance. In the case of 6289 and 6296, the kanamycin resistance was removed with pCP20 from strains JW1446-1 and JW0231-1 respectively. Strain 6277 was constructed by P1 transduction of $\Delta(\text{ptsHI cr})::\text{kan}$ (#1233) into JW1447-2. The PTS mutants were enriched with streptomycin, and a white colony from MacConkey glucose agar plates was selected. Strain 6284 was constructed by transduction of *trp::Tn10 tonB* into 6281 under selection for tetracycline resistance. In a second transduction, it was selected for *trp*⁺ on a minimal medium.

Phage IsaakIselin (Bas10) was obtained from A. Harms (Basel) (Maffei et al., 2021) and propagated on LB agar plates with BW25113 as host.

4.2 | LC-MS analysis

Bacteria were grown in baffled 100 ml Erlenmeyer flasks with 15 ml medium under constant shaking at 37°C. Samples were prepared with Costar® Spin-X® centrifuge filters immediately after drawing 0.5 ml from the culture supernatant. Extracellular gluconate was analysed

using an electrospray ionisation time of flight (ESI-TOF) mass spectrometer (MicrOTOF II; Bruker Daltonics), connected to an UltiMate 3000 high-performance liquid chromatography (HPLC) system (Dionex). Separation in the HPLC was carried out using a SeQuant ZIC-pHILIC column (PEEK 150 × 2.1 mm, 5 µm, 110 Å, Merck) at 30°C with an CH₃CN (buffer A) and 100 mM (NH₄)₂CO₃, pH 9 (buffer B) buffer system: 5 µl of the culture supernatant were injected and separated on the HPLC column using a previously published protocol (Geraci et al., 2022). Briefly, for separation of gluconate, a flow rate of 0.2 ml/min and a linear gradient of 25 min, reducing the concentration of buffer A from 82% to 42% was used. Before (5 min) and after (10 min) the gradient, the column was equilibrated with 82% buffer A.

The mass-to-charge (*m/z*) ratios of the separated samples were analysed by MS operated in negative ion-mode with a mass range of 85–900 *m/z*. An extracted ion chromatogram of gluconate based on the exact mass of (M-H)[−] = 195.05 *m/z* (±0.02) was generated and used to calculate the areas under the curve (AUC). The estimation of the amounts of gluconate contained in the samples was ensured by an appropriate calibration.

5 | CONCLUSION

At low PQQ concentrations, it is taken up with the help of the TonB-ExbBD energised outer membrane receptor PqqU (previously YncD) (Figure 7). In the presence of PQQ, the quinoprotein Gcd oxidises glucose to gluconolactone. Where and how gluconolactone is hydrolysed, enzymatically or spontaneously, is unknown. Appreciable amounts of gluconate appear in the medium and are completely metabolised in a minimal medium when the stationary growth phase is reached. FIGURE 7 PQQ uptake and gluconate secretion in *E. coli*: The question marks indicate that it is unknown where and how gluconolactone hydrolyses or is enzymatically hydrolysed.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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4.4 Manuscript 4

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Metabolization of α -D-carba-glucosamine in vivo generates antimetabolites of cell wall precursors

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ABSTRACT

α -D-Carba-glucosamine (CGlcN) is a carbocyclic analog of α -D-glucosamine that inhibits growth of *Bacillus subtilis* and *Staphylococcus aureus*. CGlcN is internalized and concomitantly phosphorylated via the phosphotransferase system yielding α -D-carba-glucosamine-6-phosphate (CGlcN6P), which interferes with expression of the glutamine-fructose-6-phosphate amidotransferase (GlnS; glucosamine synthase) by activating the *glmS* riboswitch. Herein, we report that CGlcN6P is efficiently metabolized to carbasugar nucleotides along the peptidoglycan biosynthetic route. Mass spectrometric analysis confirmed the occurrence of carbocyclic peptidoglycan nucleotides UDP-carba-D-N-acetylglucosamine (UDP-CGlcNAc) and UDP-carba-D-N-acetylmuramic acid-pentapeptide (UDP-CMurNAc-5P) in the presence of CGlcN and revealed accumulation of these carba-metabolites upon antibiotic treatment interfering with biosynthetic enzyme functions. Thus, carbocyclic carbohydrates and nucleotide analogs are generated by the promiscuous bacterial cell wall biosynthetic enzymes and act as antimetabolites, causing bacterial growth inhibition by interference with cell wall synthesis. Our findings reveal CGlcN not only as putative antibiotic molecule with previously unknown antimetabolite mode of action, but also as tool to study the bacterial cell wall metabolism, e.g., in synergy with other antibiotics.

Carbasugars are alicyclic carbohydrate analogs that contain methylene in place of the ring oxygen. They structurally resemble the parental sugars, but are unable to perform the acetal chemistry of their carbohydrate analogues. This increases stability and makes carbasugars useful probes as competitive inhibitors of enzymes such as the glycosidases and glycosyltransferase.¹ Recently, an efficient, high-yielding synthesis of α -D-carba-glucosamine (CGlcN) was reported that allowed the biological evaluations of its mode of action as an glucosamine analog.²⁻⁵ In *B. subtilis*, CGlcN is taken up and concomitantly phosphorylated, like the natural metabolite, via the glucosamine-specific PTS system GamP, gaining intracellularly CGlcN-6-phosphate (CGlcN6P).³ Due to impaired acetal chemistry CGlcN6P cannot be deamidated via the glucosamine deamidase (NagB) such as the carbohydrate counterpart glucosamine-6-phosphate (GlcN6P). However, due to a feedback mechanism, it regulates the expression of GlmS, an essential enzyme that catalyzes GlcN6P synthesis.³ This regulation is mediated by the *glmS* ribozyme, located in the 5'UTR of the *glmS* mRNA. Self-cleavage of the *glmS* ribozyme is activated by GlcN6P binding, which ultimately leads to a hydrolysis of the mRNA.⁶⁻⁹ CGlcN6P also is able to activate the *glmS* ribozyme in vitro and in vivo, resulting in a loss of *glmS* mRNA and bacterial growth inhibition.³ We previously demonstrated that (5aR)-Fluoro- α -D-carba-glucosamine-6-phosphate (F-CGlcN6P), another synthetic carba-mimic of GlcN6P, also activates the *glmS* riboswitch in vitro.¹⁰ This activation, however, was found to be ~32fold less efficient when compared to CGlcN6P (EC₅₀-values are 192 μ M vs. 6 μ M). Treating *B. subtilis* with (5aR)-Fluoro- α -D-carba-glucosamine (F-CGlcN), a precursor of F-CGlcN6P, revealed inhibition of bacterial growth and we determined a minimal inhibitory concentration (MIC) of ~40 μ g/mL (**Fig. 1a,b**). Interestingly, this value is in the same range as the one found for CGlcN (32 μ g/mL) although the activation of the *glmS* riboswitch in vitro was much less effective with F-CGlcN6P. We further demonstrate that antibacterial activity of F-CGlcN depends on uptake by GamP and induces cell envelope stress (**Fig. 1c,d**), measured by experiments using reporter genes under control of the global stress signals promoters.¹¹⁻¹⁴ These two features are also observed from treating *B. subtilis* with CGlcN.³ These contradicting data suggest a secondary antibacterial effect of CGlcN (and F-CGlcN) which might be independent of *glmS* riboswitch activation. For an in-depth mode-of-action analysis of CGlcN and to address these potential secondary effects of CGlcN treatment on bacterial cell wall metabolites, we performed targeted metabolomics. To this end, we treated *B. subtilis* at exponential growth phases with a 2-fold MIC (64 μ g/mL) of CGlcN for two hours. A detailed description of the experimental procedures can be found in the Supporting Information. In brief, the cells were then harvested by centrifugation and the pellets were resuspended in water and disrupted by sonication. Cell debris was separated from the cytosolic fraction by centrifugation and proteins were removed by acetone precipitation. The acetone was evaporated and samples dissolved in water. The isolated cytosolic fractions were analyzed by LC-MS.

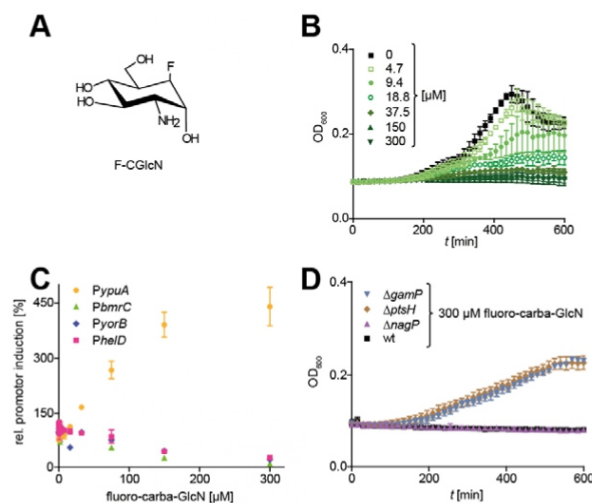


Figure 1. Impact of (5aR)-Fluoro-α-D-carba-glucosamine (F-CGlcN) on growth of wild-type *B. subtilis* and on mutant strains as well as stress response in reporter strains. (A) Chemical structure of Structure of F-CGlcN. (B) Growth curves of *B. subtilis* 168 in the presence of F-CGlcN (0–300 μM) were obtained from optical density at 600 nm. (C) Cell envelope stress response after F-CGlcN treatment shown by firefly luciferase expression under the control of stress-inducible promoter *ypuA*. No induction of *bmrC* promoter (protein synthesis inhibition), *hcd* promoter (RNA biosynthesis inhibition) and *yorB* promoter (DNA synthesis inhibition/damage) were observed. (D) Impact of deletions of *gamP* (blue; encoding the glucosamine PTS), *ptsH* (brown; encoding the general PTS kinase HPr) and *nagP* (purple; encoding the *N*-acetylglucosamine PTS) on the antibacterial activity of F-CGlcN compared to wt *B. subtilis* 168 strain.^{15–17} Growth curves were measured in the presence of 300 μM F-CGlcN as described in (B).

The results of these experiments demonstrate metabolization of CGlcN, thereby yielding the carba-variants of the central cell wall precursor molecule UDP-GlcNAc and the lipid/II progenitor UDP-MurNAc-5P, namely UDP-CGlcNAc and UDP-CMurNAc-5P (Fig. 2a,b). We detected metabolites with a mass difference of 2 Da in the CGlcN treated bacterial cell lysates, reflected by the replacement of the ring oxygen atom with a methylene group (Fig. 2a,b). Of note, CGlcN6P was not detectable, indicating a rapid turnover of the compound once taken up through the PTS.

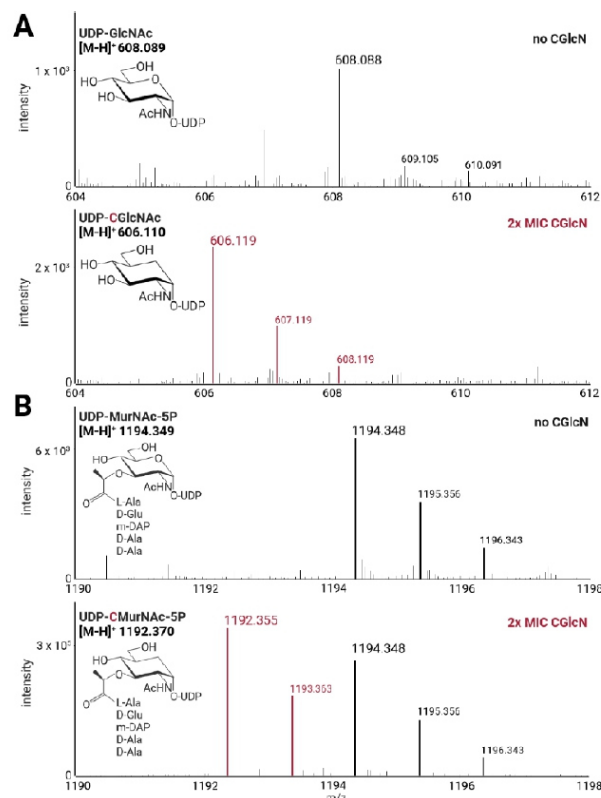


Figure 2. Occurrence of UDP-CGlcNAc and UDP-CMurNAc-5P in a soluble extract of CGlcN-treated *B. subtilis* cells. (A) In the presence of CGlcN, UDP-GlcNAc was not identified but a compound with a 2 Da lower mass. Identical MS scans are shown for the control (no CGlcN) and the treated samples (2x MIC CGlcN) at a retention time of 18.7 min. (B) In the presence of CGlcN besides UDP-MurNAc-5P a compound with a 2 Da lower mass was detected. MS scans at a retention time of 25.1 min reveal a mixture of UDP-MurNAc-5P and UDP-CMurNAc-5P. Structures and theoretical exact masses are shown. Created with BioRender.com.

We next tested whether the inhibition of certain steps during the peptidoglycan biosynthesis pathway influences the accumulation of specific cell wall precursor molecules, both in the absence and presence of CGlcN. In this regard, we investigated the known antibiotics fosfomycin and vancomycin without or in combination with CGlcN. Fosfomycin inhibits the conversion of UDP-GlcNAc to UDP-MurNAc. Thus, in the presence of fosfomycin UDP-GlcNAc accumulates (**Fig. 3**). When CGlcN was administered together with fosfomycin a strong accumulation of UDP-CGlcNAc was measurable whereas UDP-GlcNAc was no longer detectable. The treatment of *B. subtilis* with vancomycin leads to the accumulation of UDP-MurNAc-5P. In the presence of CGlcN, a considerable amount of UDP-CMurNAc-5P accumulates in addition to UDP-MurNAc-5P (**Fig. 3**). Thus, in contrast to the treatment with fosfomycin, both precursors were detected during vancomycin treatment.

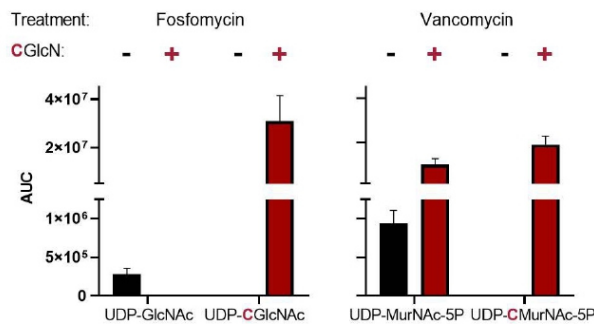


Figure 3. Accumulation of peptidoglycan nucleotide precursors and carbocyclic variants thereof by inhibition with antibiotics. Shown are the relative metabolite concentrations expressed as area under the curve (AUC) values from three independent experiments determined by extracted ion chromatograms with 0.02 Da mass tolerance and baseline adjusted to 500 cps. CGlcN was added at a concentration of 64 μ g/ml (2x MIC) and the antibiotic concentrations were 512 μ g/ml for fosfomycin and 1 μ g/ml vancomycin (2x MIC in all cases as determined for *B. subtilis* grown in CDM medium). Black bars: w/o CGlcN; red bars: w/ CGlcN.

In conclusion, we describe the *in vivo* generation of antimetabolites, generated from CGlcN along the cell wall precursor biosynthetic pathway (Fig. 4). These antimetabolites add a second effect to the antibiotic profile of CGlcN, besides its previously shown induction of the *glmS* riboswitch.³ Compounds acting on riboswitches are promising antibiotics.^{18,19} However, metabolite analogues of riboswitch activators bear the advantage of secondary effects that add to the initial mode of action.²⁰ Further analysis towards carba-variants of lipid I/II are of interest, however this requires developing elaborated protocols to address hydrophobic membrane associated compounds. According to our current model, we believe that the main antibiotic activity relates to the antimetabolite effect of CGlcN and only secondary to *glmS* riboswitch activation. This perception is supported by the characteristics observed from F-CGlcN, which are similar to CGlcN regarding bacterial growth inhibition, PTS dependency and induction of cell envelope stress.^{3,10} In terms of riboswitch activation *in vitro* they are substantially different though. F-CGlcN also inhibits bacterial growth by metabolization, but whether the extend of antimetabolite generation is identical or differs from the one observed by CGlcN remains elusive. Due to limited availability of the fluoro-compound we could not test it in this study. Taken together, these data demonstrate that CGlcN is not only a putative antibiotic molecule with previously unknown antimetabolite properties, but also a promising tool compound broadly applicable to unravel bacterial cell wall metabolism, e.g., in synergy with other antibiotics or by using carba-variants for targeted labelling of cell wall precursors.

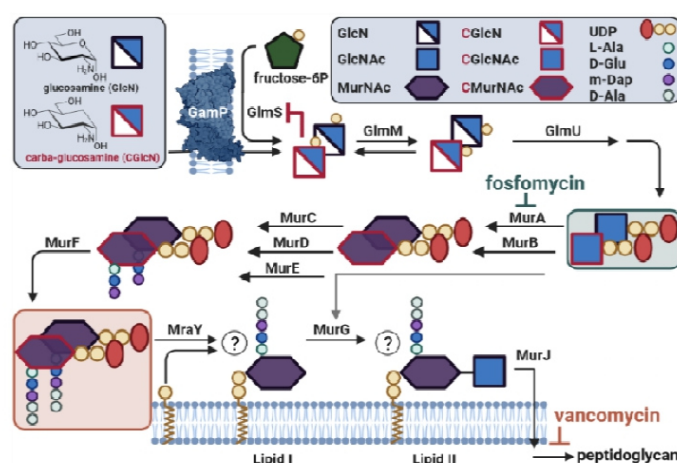


Figure 4. Schematic of the metabolism of glucosamine and CGlcN in *Bacillus subtilis* along the peptidoglycan biosynthetic pathway. The carbocyclic analog is taken up from the growth medium and phosphorylated via the phosphotransferase GamP. The phosphorylated product can interfere with GlmS regulation^{2,10}, however also serves as fortuitous substrate of the glucosamine isomerase GlmM and further on in the pathway as substrate of the bifunctional acetyl-uridylyltransferase GlmU, yielding UDP-CGlcNAc, which subsequently is metabolized via the Mur enzymes up to UDP-CMurNAc-5P. The latter apparently serves as the deadend product. Whether MraY and MurG also accept the carbon derivatives as substrates is currently unclear. Shown are the target steps of the antibiotics fosfomycin and vancomycin used to accumulate the carba-analogs of the peptidoglycan nucleotide precursors. Created with BioRender.com.

Supporting Information available

Supporting Information contain experimental procedures, materials and methods description and supporting tables.

Author Contributions

This study was conceived by GM and CM. GM and CM supervised the experiments, MM and SF conducted the metabolomics experiments, evaluated and interpreted results. DaM and AE synthesized the F-CGlcN and examined its biological activity. AB and DiM synthesized CGlcN. HBO supervised the promoter-dependent assays of F-CGlcN. The manuscript was written by GM, CM, MM and SF. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

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4.5 Manuscript 5

Friz S, Stahl Y, Stincione P, Franz-Wachtel M, Macek B, Petras D, Mayer C. Native mass spectrometry reveals that MurA carboxyvinyltransferases are primarily phosphoenolpyruvate-bound and therefore fosfomycin insensitive.

Ready for submission. Manuscript and appendix are attached.

Native mass spectrometry reveals that MurA carboxyvinyltransferases are primarily phosphoenolpyruvate-bound and therefore fosfomycin insensitive.

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Key words: MurA, AroA, peptidoglycan, *Bacillus subtilis*, enzyme kinetics, inhibition, fosfomycin, UDP-GlcNAc, PEP, native mass spectrometry, proteomics

ABSTRACT

Peptidoglycan (PGN) is an essential component of the bacterial cell wall and its *de novo* synthesis is stringently regulated and adapted to growth rate. A key enzyme in the PGN biosynthetic pathway is the UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase MurA. MurA catalyses the conversion of phosphoenolpyruvate (PEP) and UDP-*N*-acetylglucosamine (UDP-GlcNAc) to UDP-GlcNAc-enolpyruvate with the concomitant release of inorganic phosphate, the first committed step of PGN *de novo* biosynthesis. Surprisingly, *Bacillus subtilis* and many other Firmicutes, have two genetically very similar versions of MurA: MurAA (MurA) is essential but MurAB (MurZ) is not, although both catalyse identical reactions. The reason for having two versions of MurA is still unclear. Both enzymes, heterologously expressed as recombinant N-terminal His₆ fusions, show different sensitivities and properties with regard to fosfomycin (FOS) sensitivity. Intriguingly, native mass spectrometry analyses revealed that they are both isolated as posttranslational covalently modified PEP-bound enzymes. This modification occurs at a cysteine (Cys) located in the active site as shown by bottom up proteomics after trypsin digestion. The modification is located at the same Cys residue that plays a crucial role in sensitivity to the antibiotic FOS, a well-known broad-spectrum antibiotic active against Gram-positive and Gram-negative bacteria. MurAA and MurAB of *B. subtilis* are both sensitive to and thus primary molecular targets for FOS. However, the native MS analyses showed that the PEP-modified native enzymes are not a target for FOS because the binding site is already covered. Only in the presence of the second substrate UDP-GlcNAc is FOS able to bind to the now accessible Cys and achieve the permanent inactivation of the enzyme. Enzyme kinetic analyses showed that the relative concentration of UDP-GlcNAc and PEP determines the sensitivity of MurA to FOS: higher levels of UDP-GlcNAc increase the sensitivity to FOS, while higher levels of PEP make the enzyme more resistant to FOS. The FOS effect depends exclusively on the Cys modification. This is supported by the fact that the related carboxyvinyltransferase AroA, the 5-enolpyruvylshikimate-3-phosphate synthase of the shikimate pathway, albeit catalysing a similar reaction mechanism as MurA (i.e. a carboxyvinyl transfer from PEP), is not inhibited by FOS. AroA has a glutamic acid instead of a Cys in the active site. FOS cannot bind and AroA becomes naturally resistant to FOS, which was confirmed in this study using a colourimetric phosphate assay. Cys to aspartic acid substitutions in MurA, found in natural variants, retain enzymatic activity but render intrinsically resistant to FOS, highlighting the critical importance of the active site Cys for FOS inhibition. If the Cys is replaced by an amino acid with the same properties, the enzymatic reaction is still possible because the binding to Cys is not critical for catalysis.

INTRODUCTION

The classification of bacteria on the basis of their cell wall structure dates back to the Gram staining method developed by Hans Christian Gram almost 140 years ago [1, 2].

The basis of Gram staining resides in the structure of the bacterial cell wall, which differs considerably between Gram-positive and Gram-negative bacteria. The cell wall of Gram-positive bacteria is comparatively simple, consisting of up to 40 layers of peptidoglycan (PGN). In addition, polyol phosphates, firstly wall teichoic acids (covalently linked) and secondly lipoteichoic acids (non-covalently linked), interact with the PGN [3]. The cell wall of Gram-negative bacteria has a more complex structure. It contains an additional outer membrane consisting of phospholipids, proteins (e.g. porins) and polysaccharides. This membrane layer encloses one to two layers of PGN. Further towards the protoplasm, in Gram-negative bacteria, is the periplasmic space between the PGN and the cytoplasmic membrane [4, 5].

PGN is essential for stability, shaping of the cell, and resistance to turgor pressure [6]. The importance and the central role of PGN makes it a predestined target for antibiotics, but the cell itself also controls its metabolism via and with the enzymes of PGN *de novo* synthesis [7].

A substantial amount of the bacterial cell resources goes into the *de novo* synthesis of the bacterial cell wall, which involves a large number of enzymes catalysing about 20 reactions. The synthesis of nucleotide PGN precursors takes place in the cytoplasm, followed by the production of lipid-bound cell wall precursors on the inside of the cytoplasmic membrane, while the final polymerisation and assembly of the *de novo* PGN takes place on the outside of the cytoplasmic membrane. Amino acids, supplied by primary metabolism, partly non-codogenic D-isomers, and the amino sugars *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) are required for PGN synthesis. GlmS catalyses the reaction of fructose-6-phosphate to glucosamine-6-phosphate, which is isomerised by GlmM and activated by GlmU to uridine diphosphate-GlcNAc (UDP-GlcNAc). Fructose-6-phosphate is permanently present in the cell as a result of glycolysis or gluconeogenesis. The addition of phosphoenolpyruvate (PEP) to UDP-GlcNAc is catalysed by MurA, followed by the reduction of the UDP-GlcNAc-enolpyruvate product (MurB) to generate UDP-MurNAc. The MurNAc carbohydrate is characteristic of bacteria and its synthesis is an important step in PGN synthesis. The enzymes MurC, MurD, MurE and MurF subsequently attach the bacteria-specific amino acids to the lactyl group of UDP-MurNAc, before the sugar derivative can be transferred by *MraY* to the membrane carrier undecaprenyl phosphate (C55-P) on the inner membrane, yielding lipid I, the first lipid-bound PGN precursor. MurG links GlcNAc from UDP-GlcNAc to lipid I to form lipid II, which is then transported to the outer leaflet of the inner membrane with the help of a flippase (MurJ). The transported lipid II molecules are polymerised via a glycosyl transfer reaction to build up the extracellular expanding glycan chains.

The polymerisation goes along with the cleavage of the undecaprenyl-pyrophosphate (C55-PP), which is subsequently converted to C55-P and recycled for a new precursor transfer across the membrane. Transpeptidase enzymes, known as penicillin-binding proteins (PBPs), cross-link the peptide chains. Some PBPs (class A PBPs) carry a glycosyltransferase and a second transpeptidase domain.

This results in glycan chains of alternating GlcNAc and MurNAc residues connected by β -1-4 bonds, which are cross-linked via peptide bridges. Normally, the peptide chain consists of the pentapeptide stem L-Ala- γ -D-Glu-*meso*-A2pm (or L-Lys)-D-Ala-D-Ala (A2pm, 2,6-diaminopimelic acid), the last D-Ala residue being either hydrolysed by a transpeptidase at the end of synthesis in exchange for a peptide cross-link, or removed by carboxypeptidases and missing in the PGN network. The cross-linking of the glycan chains generally takes place between the carboxyl group of the D-Ala at position 4 and the amino group of the diamino acid at position 3, either directly or via a short peptide bridge. [4, 8, 9, 10]

A key enzyme in the *de novo* synthesis of PGN is MurA a UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase (EC 2.5.1.7 (IUBMB)). MurA catalyses the conversion of PEP and UDP-GlcNAc to UDP-*N*-acetyl-3-O-(1-carboxyvinyl)-D-glucosamine (UDP-GlcNAc-enolpyruvate) with the release of inorganic phosphate, the first committed step of PGN *de novo* biosynthesis. This highly unusual enzyme reaction, which involves the cleavage of a very stable C-O bond and a special reaction mechanism, is barely known. Only one other enolpyruvyltransferase is known which can catalyse such a reaction, the 5-enolpyruvylshikimate-3-phosphate synthase (EPSP) AroA (EC 2.5.1.19), a central enzyme in the shikimic acid pathway. [11, 12, 13, 14, 15, 16]

While the enzyme reaction is almost identical, the AroA and MurA enzymes differ fundamentally in their sensitivity to the antibiotic fosfomycin (FOS). While AroA is not affected by FOS, MurA shows a strong effect on FOS. [17, 18]

The inhibition of MurA by FOS is based on an unusual covalent binding of FOS to a sulfhydryl group of a cysteine (Cys, *Escherichia coli* MurA:115, *Bacillus subtilis* MurAA: 117, *B. subtilis* MurAB: 116), located in the region of the active site crucial for the catalysed reaction. This position is also the initial binding site for the first substrate, PEP. During the reaction, PEP is transferred to UDP-GlcNAc and the binding site is regenerated. When FOS binds, the enzyme is permanently and irreversibly inactivated. The Cys is crucial and therefore the main difference to AroA. In the active centre of AroA, a glutamic acid (position 341) takes over the function. This is confirmed by mutation experiments in *E. coli* in which the cysteine was replaced by an aspartic acid, resulting in a FOS-resistant but catalytically active MurA. Some species such as *Chlamydia*, *Exiguobacterium* or *Terribacillus* are naturally resistant to FOS due to this amino acid substitution. [19, 20, 21]

The active site Cys has a dual function. Firstly, as a binding site for PEP and secondly as a general acid for catalysis. During the reaction mechanism, Cys-PEP is formed as a temporary by-product of a general acid-base mechanism. This is necessary because the tetrahedral intermediate must not be bound to the enzyme. Therefore, no covalent binding of PEP to the corresponding Cys is required for catalytic conversion. [20, 22]

MurA consists of two globular domains, I and II, each with six subdomains. Both consist of six helices and three four-stranded β -sheets and are connected by a double-stranded hinge region. The N- and C-termini and most of the C-terminal part of the sequence are located in domain I, while domain II contains mainly the N-terminal part. The fixed hinge region connecting the two domains consists of two strands of equal length connected by hydrogen bonds and a salt bridge.

Each of the six subdomains consists of the same secondary structures in exactly the same order ($\beta 1$, $\alpha 1$, $\beta 2$, $\alpha 2$, $\beta 3$, $\beta 4$), so that the structure resembles a sixfold repeat of a folding subunit. A highly flexible loop connecting $\beta 2$ and $\alpha 2$ in subdomain IIc is the active site, which also contains the PEP-binding amino acid Cys. The catalytic centre is located in the cavity between domains I and II. When no substrate binds to MurA, the flexible loop forms an open conformation, which in turn changes to a closed conformation upon substrate binding, in which two hydrophobic surfaces interact with the uridyl ring of UDP-GlcNAc. MurA undergoes a conformational change from the open to the closed conformation following an induced fit mechanism. This has a significant impact on sensitivity to FOS. Only UDP-GlcNAc-bound MurA is susceptible to FOS binding due to this induced fit mechanism. [23, 24, 25]

The function of MurA is essential for bacteria with active PGN. In many organisms, such as *E. coli*, there is only one copy of MurA, so there is no need to explain its essentiality. *B. subtilis*, in contrast, has two genetically very similar versions: MurAA and MurAB. In this case, MurAA is essential but MurAB is not, although both catalyse the identical reaction and both are inhibited by FOS. The enzymes seem to be able to substitute each other only partially, at certain stages of growth or under certain conditions. [26, 27, 28]

In this study, we set out to gain detailed insights into the key enzyme of PGN *de novo* synthesis, MurA. Central to this is the investigation of the cysteine-PEP binding the MurA reaction intermediate, which has never been shown in this form by native mass spectrometry, especially in combination with Cys-FOS binding. The aim of the study is to identify differences between the MurA enzymes in *B. subtilis*, but also in relation to variants that are naturally resistant to FOS. The combination of techniques used, from simple enzyme assays to inhibition assays and mass spectrometry analysis, in combination with recombinant enzymes, will allow a detailed insight and investigation of MurA under FOS treatments.

MATERIAL AND METHODS

Bacterial strains

Bacillus subtilis subsp. *subtilis* str. 168 (trpC2). (BGSC, 1A1, [29])

Escherichia coli DH5 α B F⁻ *endA1* *glnV44* *thi-1* *recA1* *relA1* *gyrA96* *deoR* *nupG* *purB20* $\phi 80$ *lacZ* Δ M15 Δ (*lacZYA-argF*)U169, *hsdR17*(r_K⁻m_K⁺), λ ⁻

Escherichia coli BL21(DE3) B F⁻ *ompT* *gal* *dcm* *lon* *hsdS_B*(r_B⁻m_B⁻) λ (DE3 [*lacI* *lacUV5*-T7p07 *ind1* *sam7* *nin5*]) [*malB*⁺]_{K-12}(λ ^S)

Escherichia coli K-12 BW25113 *lacI*⁺*rrnB*_{T14} Δ *lacZ*_{WJ16} *hsdR514* Δ *araBAD*_{AH33} Δ *rhaBAD*_{LD78} *rph-1* Δ (*araB-D*)567 Δ (*rhaD-B*)568 Δ *lacZ*4787(::*rrnB-3*) *hsdR514* *rph-1*

Plasmids

pET-28a(+) (MilliporeSigma (Novagen), [30])

Cloning of recombinant proteins (*E. coli*: *aroA*, *B. subtilis*: *murAA*, *murAB*)

The corresponding genomic DNA (*Escherichia coli* K-12 BW25113 and *Bacillus subtilis subsp. subtilis str. 168*) for cloning was isolated according to the manufacturer's instructions (GeneJET Aufreinigungskits für genomische DNA (K0721, Thermo Scientific). The plasmid pET-28a(+) was linearised using the restriction enzymes *NcoI* and *XhoI* to generate a C-terminal his₆-tag (AroA) or *NdeI* and *XhoI* to generate a N-terminal his₆-tag (MurAA, MurAB) (R0193S, R0146S, R0111S: New England Biolabs) and subsequently purified using a 1 % agarose gel (GeneJET Gel-Extraktionskit (K0691, Thermo Scientific™). The genes were amplified by PCR (Q5, 50 µL according to New England Biolabs (M0491), primers see table1, GeneJET PCR-Aufreinigungskits (K0702, Thermo Scientific™)) and cloned into the prepared plasmid using Gibson assembly (Gibson Assembly Protocol (E5510) and Cloning Kit (E5510S), New England Biolabs). Subsequently, the cloned plasmid was transformed into chemically competent *E. coli* DH5α cells [31] using heat shock (30 min thawing, 30 min incubation with 10 µL Gibson clone, 1 min 42 °C, addition of 900 µL lysogenic broth (LB) medium and regeneration for 1 h at 37 °C followed by plating on LB_{Kan50} agar plates.). The grown colonies were checked by colony PCR (Red MasterMix (2x) (M3029.0100, GENAXXON bioscience), primer: T7, T7term), and if positive, they were multiplied overnight, plasmid was isolated (GeneJET Plasmid-Miniprep-Kit (K0502, Thermo Scientific™)) and then sequenced (TubeSeq, Eurofins Genomics). Verification was achieved using 1 % agarose gel stained with MIDORI Green (MG04, NIPPON Genetics) and the GeneRuler 1 kb DNA Ladder (SM0311, Thermo Scientific) for size evaluation. Upon confirmation, the plasmids were transformed into chemical competent *E. coli* BL21 (DE3).

Table 1: Primer used for cloning (DNA Oligos, MERCK)

AroA_fwd	TGTTTAACTTTAAGAAGGAGATATACCATGgaatccctgacgttacaacc
AroA_rev	AGTGGTG GTGTGTGTGTGTGCTCGAGggctgcctggctaatacgcg
MurAA_fwd	AGTGGTG GTGTGTGTGTGTGCTCGAGttatgcatttaagtcagaaacgacttc
MurAA_rev	CTGGTGCCGCGCGGCAGCCATATGaaaaaatcatcgctccgcg
MurAB_fwd	TGGTG GTGTGTGTGTGCTCGAGttatgaattttgaagctgttctattcttcg
MurAB_rev	GCCGCGCGGCAGCCATATGaaaagtgaatatgccg
T7	TAATACGACTCACTATAGGG
T7term	CTAGTTATTGCTCAGCGGT

Expression and cell disruption of recombinant proteins MurAA, MurAB and AroA

A pre-culture of expression tested recombinant *E. coli* BL21 (DE3) constructs with antibiotic selection pressure (LB_{Kan5}) was inoculated into 1 L LB to OD_{600nm} of 0.05 and incubated at 37 °C and 120 rpm until an OD_{600nm} of 0.4-0.8 is reached. Expression is induced with Isopropyl β -D-1-thiogalactopyranoside (IPTG, 1 mM) and carried out either at 37 °C for 3 h or at 20 °C for 24 h. Cells were harvested (5000 g, 10 min, 4 °C) and frozen until further use. Before induction and after expression, 1 mL samples were taken to evaluate expression by SDS-PAGE. Cell disruption was performed by sonication. 1 L of centrifuged culture was dissolved in 20 mL of cell disruption buffer (500 mM NaCl, 20 mM Tris-HCl, 1 tablet Pierce™ Protease Inhibitor (Thermo Scientific), pH 7.5) and sonicated for 5 x 2 min (Output Control: 5, Duty Cycle 60 %, 5 min pause per run, Branson Ultrasonics Sonifier Modell 250 with macro tip). The cell lysate was centrifuged (18000 g, 1 h, 4 °C) and the supernatant was purified with all soluble proteins inside.

Protein purification using IMAC and SEC

The protein lysate was filtered (0.45 μm) and then purified using an ÄKTApurifier-Systems (GE Healthcare/Cytiva) system. The cloned his₆-tag was used to separate the recombinant protein from the other proteins by Immobilised Metal Chelate Affinity Chromatography (IMAC) and appropriate HisTrap HP 1 mL (29051021, Cytiva). The system starts with 96 % buffer A (500 mM NaCl and 20 mM Tris-HCl for MurAA and MurAB and 20 mM Na₂HPO₄ for AroA, both pH 7.5) and 4 % buffer B (500 mM imidazole, 500 mM NaCl and 20 mM Tris-HCl for MurAA and MurAB and 20 mM Na₂HPO₄ for AroA, both pH 7.5). Over the period of 30 min, buffer A is continuously reduced to 0 %, whereas buffer B is increased to 100 %. The flow rate was 1 mL min⁻¹. UV-active fractions (280 nm) are analysed by SDS-PAGE to determine the fractions with the desired recombinant protein. The confirmed fractions were further purified by size exclusion chromatography (SEC). For this purpose, amounts and volumes of protein solution corresponding to the manufacturer were separated with the same ÄKTApurifier-System using a HiLoad 16/600 Superdex (MurAA and MurAB: PN28989333, 75 pg; AroA: PN28989335, 200 pg, both from Cytiva) at a flow rate of 1 mL min⁻¹ through 1.5 column volumes of buffer A. For analysis, SDS-PAGE was used. Protein concentration was determined using a Nano Photometer (N60/N50, Implen).

SDS-PAGE analysis

To separate the proteins according to their charge, depending on their molecular weight, an SDS-PAGE was performed. 10 μL sample was added to 1x SDS sample buffer (Laemmli (6x): 10 % glycerol, 5 % β -mercaptoethanol, 2 % 20 % SDS solution, 0.01 % bromophenol blue, 60 mM Tris-HCl (pH 6.8), all calculated w/v.) and heated at 95 °C for 10 min. The Mini-PROTEAN TGX™ precast Gels (4561046, BioRad) were run at 50 mA for 35 min in SDS running buffer (14.0 g L⁻¹ glycine, 3.0 g L⁻¹ Tris base, 2 mL L⁻¹ 20 % SDS solution, pH 8.5). PageRuler Prestained Protein Ladder, 10 to 180 kDa. (26616X4, Thermo Scientific) was used as a marker. The gel was washed in water and stained with CBB staining solution.

LC-MS activity test of enzymes

Enzyme activity was measured by liquid chromatography-mass spectrometry (LC-MS). For this purpose, the 1 mM substrates shikimate-3-phosphate (S3P) (S0702-1MG, Merck) and phosphoenolpyruvate (PEP) (10108294001, Roche) were added to AroA with 5 μM enzyme in 50 mM HEPES buffer (pH 7). MurAA and MurAB were combined with 5 μM enzyme, 1 mM PEP (10108294001, Roche) and 1 mM uridine-5'-diphospho-N-acetylglucosamine (UDP-GlcNAc) (U4375, Merck) in Tris buffer (50 mM NaCl, 20 mM Tris-HCl, adjusted to pH 7.5). The reactions were incubated at 37 °C for 1-3 h and heat-inactivated at 95 °C. The samples were centrifuged, and the supernatant was analysed. LC-MS measurement was performed using an electrospray ionisation time-of-flight (ESI-TOF) MS (MicroTOF II; Bruker Daltonics) operated in negative ion mode, covering the range of 85-900 m z⁻¹, and connected to an UltiMate 3000 high-performance liquid chromatography (HPLC) system (Dionex).

For the HPLC separation, a SeQuant ZIC-pHILIC column (150460, PEEK 150 × 2.1 mm, 5 μm , 110 Å, Merck) was used at 30 °C with a buffer system of CH₃CN (buffer A) and 100 mM (NH₄)₂CO₃, pH 9 (buffer B).

A single run (injection volume: 5 μL) was performed with a flow rate of 0.2 mL min^{-1} and a linear gradient of 25 min, reducing the concentration of buffer A from 82 % to 42 %. Before (5 min) and after (10 min) the gradient, the column was equilibrated with 82 % buffer A. Alternatively, an LC-MS measurement was carried out with the identical instruments in positive ion mode. For this purpose, a Gemini column (00F-4435-E0, 5 μm C18 110 $\text{\AA}$, Phenomenex) was used at a flow rate of 0.2 mL min^{-1} with the following elution programme: a 5 min wash step with 100% CH_3CN , followed by a linear gradient over 30 min to 40 % elution buffer (0.05 % ammonium formate, 0.1 % formic acid), a 5 min time delay of the gradient and a 5 min re-equilibration. 5 μL of each sample was injected. The MS analysis range was 120-900 m z^{-1} . All analyses were performed with a noise threshold of 1000 and a measurement tolerance of 0.02 Da using the OpenMS based tool for metabolomics data analysis UmetaFlow [32] with its graphical user interface (<https://github.com/axelwalter/umetaflow-gui>).

Phosphate assay activity test of enzymes

For the further analysis of the enzymes, a quantitative phosphate assay was carried out. The quantitative extension compared to the purely qualitative LC-MS analysis makes it possible to generate inhibitions and kinetics. Tests with AroA (5 μM) were performed in 50 μL final volume, with final concentrations of 1 mM PEP and S3P. Incubation was for 4 min at RT, followed by 800 μL CS-solution (98 % AM/MG solution (1:3: 4,2 % ammonium molybdate in 4 M HCl (AM), (277908, Merck): 0,045 % malachite green (oxalate salt) (MG), (M9015, Merck), 2 % Triton (X-100, Merck)), after 1 min 100 μL NC-solution (35 % sodium citrate x 2 H_2O (6448, Merck)) and after another 30 min incubation at RT (in darkness) the OD was determined at 660 nm (SPARK multimode plate reader, Tecan). Inhibition experiments were carried out with 0.1-10 mM FOS (P5396, Merck) and 0.1-1 mM glyphosate (45521, Merck). [33, 34]

For MurAA and MurAB, 1 μM enzyme, 0.1-10 mM UDP-GlcNAc and 0.1-10 mM PEP in Tris buffer (50 mM NaCl, 20 mM Tris-HCl, adjusted to pH 7.5) were used, varying depending on the experiment. 30 μL per reaction are added to a well of a 96-well plate and incubated at 37 $^\circ\text{C}$ and 120 rpm for 20 (kinetic) or 60 min. For visualisation, the 30 μL reaction volume was added to 800 μL CS-solution. Absorbance at 650 nm was measured with plate reader (SPARK multimode plate reader, Tecan). Inhibition experiments were carried out with up to 200 $\mu\text{g mL}^{-1}$ FOS (P5396, Merck). [33, 34]

Native MS Analysis

To detect MurAA and MurAB interactions with UDP-GlcNAc and FOS, native MS assays were performed on a Q Exactive HF Orbitrap instrument (Thermo Scientific). Prior to native MS analysis purified enzymes (10 μM) were incubated (45 min, 37 $^\circ\text{C}$, no heat inactivation) in different substrate concentrations: enzyme only, enzyme with UDP-GlcNAc, enzyme with UDP-GlcNAc and PEP, and each of the combinations with 128 $\mu\text{g mL}^{-1}$ FOS. The reaction, performed in Tris buffer, was buffered with centrifugal filters (Amicon Ultra- 0.5 mL, 10 K, UFC5003BK, Merck) in 10 mM ammonium acetate (pH 8) according to the manufacturer's instructions in 4 steps (12000 g, 12 min, 4 $^\circ\text{C}$). Native proteins were injected via the integrated syringe pump set at a flow rate of 5 $\mu\text{L min}^{-1}$ (10 $\mu\text{L min}^{-1}$ to run in). Proteins were infused together with a buffer solution of ammonium acetate (10-100 mM mixed on the fly with water) (Quaternary Pump) and 5 % ACN in water with 0.1 % FA (Binary Pump), the conditions ensured the protein ionisation in the source.

The ESI settings used for the analysis included a sheath gas flow of 20 arb. units, the auxiliary gas flow was set to 7 arb. units and the sweep gas flow was set to 0 arb. units. The auxiliary gas temperature was set to 250 °C. The spray voltage was set to 3 kV and the inlet capillary was heated to 253 °C. S-lens level was set to 70 V applied. MS scan range was set to 1500-6000 m/z with a resolution $R_{m/z\ 200}$ 15,000 with positive polarity and 5 microscans. AGC target was set at 5e6 and the maximum ion injection time set 2000/3000. MS acquisition was performed in all-ion fragmentation (AIF) mode with $R_{m/z\ 200}$ with 20 % HCD collision energy and an isolation window of 2500-4000 m/z . Online data acquisition was realised, and data files generated were analysed by using the Thermo Xcalibur Qual Browser. Subsequently, the data were deconvoluted and analysed via the UniDec software. [35, 36]

Proteomic MS Analysis

Proteomics MS analysis with a trypsin digest was performed to accurately determine specific protein modification. The identical substrate combinations as already analysed by native MS were used. The samples were incubated at 37 °C for 1 h and then heat-inactivated at 95 °C for 10 min. 15-25 mg of protein was used per sample. Samples were digested in solution with trypsin with and without disulfide bond reduction, followed by carbamidomethylation, and peptides from protein extracts were further fractionated by high pH reversed phase chromatography [37].

Desalted peptides [38] were analysed on an Easy-nLC 1200 UHPLC system coupled to an Orbitrap Exploris 480 quadrupole mass spectrometer (both Thermo Scientific) as previously described [39], using 35 min (in vitro assays), 87 min fraction-specific (fractions) or 127 min segmented gradients for peptide separation.

MS data were analysed using the MaxQuant software suite (vs 1.5.2.8 and 2.2.0.0) [40, 41]. Spectra from *in vitro* assays were searched against an *B. subtilis* database from UniProt, the sequences of His₆-MurAA and His₆-MurAB, and a database of 286 commonly observed contaminants. In addition, spectra from fractions were searched against the UniProt *B. subtilis* database (retrieved 16 December 2022; 4,271 entries).

Full tryptic specificity was required and up to two missed cleavages were allowed. In addition to acetylation of the protein N-terminus and oxidation of methionine, cysteine carbamidomethylation was set as a variable modification in cases where digestion of proteins was performed after treatment of samples with dithiothreitol (DTT) and iodoacetamide (IAA). Additional variable modifications were defined as PEP, FOS and, as a product after loss of phosphonic acid under acidic conditions, fosfomycin-aldehyde (FOS-aldehyde).

The target decoy strategy [42] was used to calculate the false discovery rate (FDR) at the protein, peptide and modification site level, and the threshold was set at 0.01 % FDR. Precursor and product ion mass tolerances were set to 4.5 ppm and 20 ppm, respectively.

RESULTS AND DISCUSSION

Cloning and purification of the recombinant carboxyvinyltransferases AroA of *E. coli* and MurAA and MurAB of *B. subtilis*

Gibson assembly was used for gene cloning to obtain access to the following recombinant proteins with a His₆-tag, AroA from *E. coli* (C-terminal His₆-tag), MurAA and MurAB from *B. subtilis* (N-terminal His₆-tag). To confirm correct cloning, the recombinant constructs were analysed by colony PCR (see appendix A2) and finally by DNA sequencing (A3). There were no mutations in the sequence of the coding gene. However, the sequence of the cloned gene encoding AroA is missing a single histidine codon, so that AroA has a His₅-tag instead of an His₆-tag at the C-terminal end. Fortunately, this change did not affect the purification and activity of AroA. A single base change was identified in the MurAA coding sequence, resulting in an amino acid exchange at position 20 (histidine (H) to arginine (R)), the linker sequence before the MurAA start codon, which should not affect enzyme performance (A3). The sequence of the cloned MurAB was as theoretically predicted (A3). After IPTG-induced expression, all proteins were purified by IMAC and SEC (A4). Based on the amino acid sequence, the size of the recombinant proteins was calculated to be 47.0 kDa for AroA, 48.9 kDa for MurAA and 48.2 kDa for MurAB (A4). Analyses performed by SDS-PAGE after IMAC and after SEC showed that the proteins were successfully purified (partly depending on the temperature of protein expression) and appeared with the expected sizes. The amount of protein was sufficient in all cases. The least pure protein was MurAB, which still contained some impurities on SDS-PAGE, whereas all other proteins were basically free of any additional bands.

Biological activity validation of the recombinant proteins: *E. coli* AroA, *B. subtilis* MurAA, *B. subtilis* MurAB

As a first step, the activity of all three recombinant enzymes was analysed. For this purpose, the appropriate substrates were added and the reaction mixtures, as well as enzyme-free negative controls, were analysed by LC-MS. For all enzymes, the specific product was identified in the corresponding sample. AroA produces 5-enolpyruvylshikimate-3-phosphate (EPSP) and both MurA enzymes produce UDP-*N*-acetylglucosamine-enolpyruvate (UDP-GlcNAc-ep) (A6, A7, A8). In addition, the activity was also independently determined using the phosphate assay, as shown in the results in the following sections. Thus, all recombinant enzymes were active after purification and catalysed the expected reaction.

Development and evaluation of phosphate assay to determine enzyme activity and characteristics

The catalytic MurA reaction yields UDP-GlcNAc-ep and inorganic phosphate from PEP and UDP-GlcNAc. Detection of released phosphate can be measured with a colourimetric assay at an OD of 650 nm and is based on quantification of a green complex formed between malachite green, molybdate and free inorganic phosphate. To verify the phosphate assay used as described, which is constructed from different sources, a commercial phosphate assay containing recombinant *E. coli* MurA (MURA100KE, ProFoldin) was performed for comparison.

When comparing the staining solutions between the commercial product and the staining solution used in this work, there were relatively few differences after subtraction of the measurement background (A9). The proportionality and the detection limits of the developed assay were additionally tested and confirmed with a phosphate standard (A10).

Activity and fosfomycin sensitivity of recombinant *E. coli* AroA

The similarity of the reaction mechanism of AroA and MurA, which both catalyse the carboxyvinyl transfer from PEP-like substrates (shikimate-3-phosphate (S3P) and UDP-GlcNAc, respectively), suggested that they could both be inhibited by fosfomycin (FOS). The AroA enzyme activity could be proven without any doubt by the described phosphate assay. An addition of $128 \mu\text{g mL}^{-1}$ glyphosate (GLP; a known AroA inhibitor), resulted in a 50 % reduction in enzyme activity, whereas an approximately 10-fold higher amount of FOS had no influence on the activity (Fig.1). How the potent inhibitor GLP, which is competitive with PEP and uncompetitive with S3P, binds to the active site of AroA enzymes has been described in the literature [43, 44]. Therefore, GLP is used as a positive control for inhibition. GLP occupies the binding of the second substrate (PEP), resulting in an mimick of the intermediate state of the ternary enzyme-substrate complex [45]. Due to the lack of cysteine in the active site, FOS is unable to covalently block AroA and does not appear to be potent as a competitive PEP mimic (Fig.1).

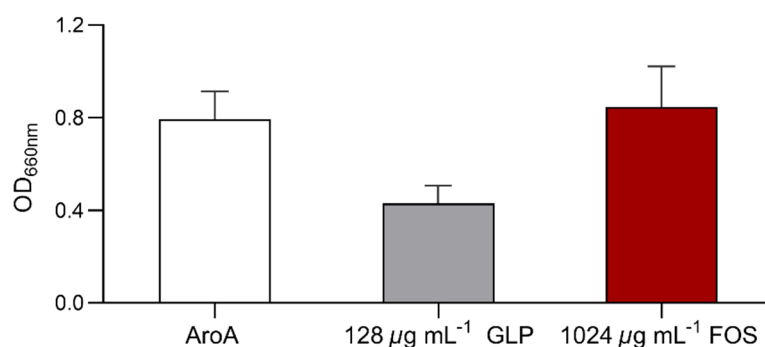


Figure 1: *E. coli* AroA activity and inhibition by GLP and FOS. The enzyme activity of *E. coli* AroA was determined by phosphate assay. The reaction mixture consisted of $5 \mu\text{M}$ AroA in $50 \mu\text{L}$ final volume, with final substrate concentrations of 1 mM PEP and S3P. Inhibition of glyphosate (GLP, $128 \mu\text{g mL}^{-1}$) and fosfomycin (FOS; $1024 \mu\text{g mL}^{-1}$) was determined identically. Phosphate was determined by a colour reaction with malachite green and ammonium molybdate. The OD was determined at 660 nm , plotted is the mean with SD from triplicates.

Reaction and protein storage parameters of the recombinant *B. subtilis* enzymes MurAA and MurAB

The reaction parameters of the phosphate assay of MurAA and MurAB were optimised to give higher value to subsequent further analysis of inhibition and kinetic parameters. The optimum pH and temperature were found to be 7.5 and 37°C , respectively. This is known from the literature but was additionally verified (result not shown). The influence of the buffer properties on enzyme activity was also evaluated. After purification, the enzymes were solved in 20 mM Tris-HCl containing 500 mM NaCl (required for SEC purification) at pH 7.5 .

The use of 20 mM HEPES and the reduction to 50 mM NaCl had no significant effect on the actual enzyme activity (A11). However, the NaCl, or general salt concentration, becomes crucial in inhibition experiments with FOS.

Under high salt conditions, the enzyme was more resistant to FOS (A11). To increase the sensitivity and to approximate physiologically relevant conditions, all experiments were performed in the buffer system 20 mM Tris-HCl with 50 mM NaCl at pH 7.5. The enzyme and substrate concentrations were also optimised (A12). An enzyme concentration of 1 μ M and substrate concentrations of PEP and UDP-GlcNAc of 1 mM each were found to be optimal. Reaction time was studied over a period of 20-120 min. A linear correlation was found between 20 and 60 min. Full conversion could be assumed after 60 min, depending on experimental feasibility (A13). All tests were performed with *B. subtilis* MurAA to establish comparability between the MurAA and MurAB paralogs, and the identical conditions were used for MurAB. Storage, and therefore stability, of the proteins was demonstrated in this work by means of an activity test and SDS-PAGE. To ensure a broad comparison, the proteins were stored at room temperature (RT), at 4 °C and at -10°C. After one week the enzymes were active regardless of the storage conditions, even after 6 weeks the enzymes still showed (A14, A15 not all tests shown) comparable activity at 4 °C and -20 °C. 10 % glycerol and 20 mM dithiothreitol (DTT) did not affect the activity or lead to a higher activity after prolonged storage.

Influence of fosfomycin and differing substrate concentrations on the activity of the recombinant *B. subtilis* enzymes MurAA and MurAB

In contrast to the investigated AroA, FOS inhibits MurAA and MurAB. However, the sensitivity varied considerably. Fig.2 (top) shows that already at 10 μ g mL⁻¹ FOS and equimolar substrate concentrations (1 mM), only 60 % of the MurAA enzyme activity is retained, whereas MurAB was unaffected by this FOS concentration. The situation was the similar at higher FOS concentrations. At 100 μ g mL⁻¹ FOS, a maximum of 10 % MurAA residual activity remained, whereas MurAB still had 50 % activity. When substrate concentrations were varied, it was found that increasing the initial PEP concentration from 1 mM to 5 mM resulted in higher enzyme activity and, conversely, lower sensitivity to FOS. The explanation is obvious because, as already described, FOS and PEP compete for the identical site in the enzyme (Cys). The PEP-bound enzyme is protected from FOS inactivation by a covalent bond. When the concentration of UDP-GlcNAc was increased by the same amount, both enzymes showed a slight decrease in enzyme activity and thus an increased sensitivity to FOS. When UDP-GlcNAc consumes the available PEP, the enzyme becomes available for FOS. When the FOS inhibition of the two MurA paralogs is considered, it becomes even clearer that FOS has no effect on the activity of AroA. The importance of Cys in the active site is essential for FOS inhibition.

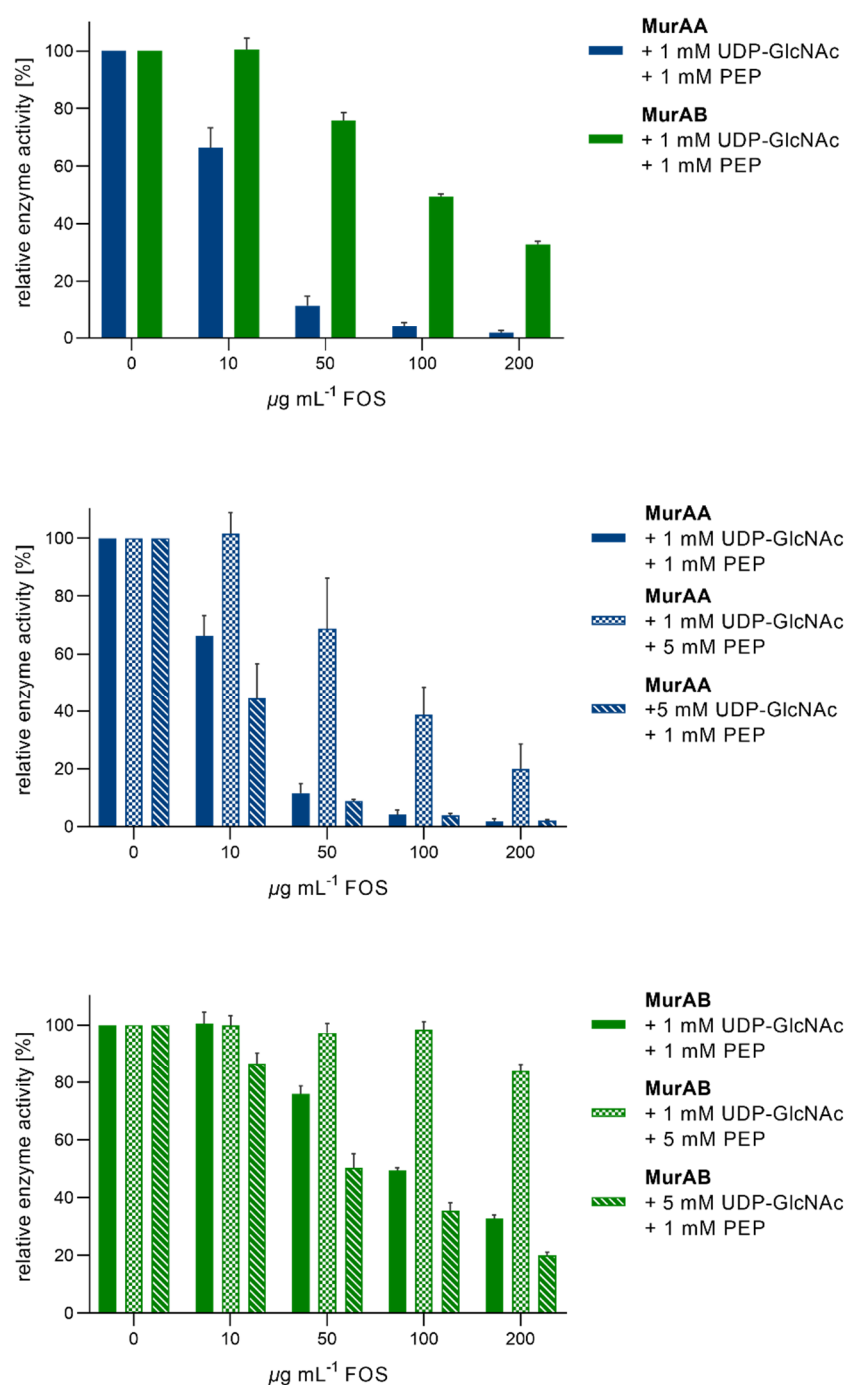


Figure 2: FOS inhibition of *B. subtilis* MurAA and MurAB at different substrate concentrations. The reaction is carried out at 37 °C for 1 h. The inhibition of 1 µM *B. subtilis* MurAA and MurAB is tested with different FOS concentrations (0-200 µg mL⁻¹) and different substrate concentrations 1 mM or 5 mM for PEP and UDP-GlcNAc. Absorbance is measured at 650 nm, mean values with SD are shown. Buffer: 20 mM Tris-HCl, 50 mM NaCl pH 7.5. The enzyme activities are shown in relation to a reaction without FOS. Absolute values of the uninhibited reactions are shown in A16.

Kinetic characterization of recombinant *B. subtilis* MurAA and MurAB

A final comparison of the two recombinant *B. subtilis* MurA paralogs is made on the basis of the kinetic parameters (Table2). MurAB had a lower affinity for both substrates, as indicated by a lower K_m value, but a higher maximum velocity V_{max} for both substrates. MurAB has a higher turnover number for both substrates, while MurAA has a higher efficiency for both substrates, indicated by a higher turnover number ($k_{cat} K_m^{-1}$) value. The two paralogs have distinctly different properties. MurAA had a higher substrate affinity (smaller K_m , higher number of changes), whereas MurAB showed a higher total activity (higher K_{cat} and V_{max}). In conclusion, MurAA worked better at lower substrate concentrations, whereas MurAB had an advantage at substrate saturated conditions. To the best of our knowledge, there are no kinetic data from the *B. subtilis* MurA paralogs in the literature.

Table 2: Kinetic parameters of *B. subtilis* MurAA and MurAB

	MurAA		MurAB	
	UDP-GlcNAc	PEP	UDP-GlcNAc	PEP
K_m (mM)	0.664	0.500	2.477	2.672
V_{max} ($\mu M \text{ min}^{-1}$)	35.32	29.76	67.63	53.75
k_{cat} (1 s^{-1})	0.589	0.496	1.127	0.896
k_{cat}/K_m ($1 \text{ s}^{-1} \text{ mM}$)	0.887	0.992	0.455	0.335

Kinetic parameters were determined with different amounts of UDP-GlcNAc or PEP. One of the two substrates was kept at 1 mM while the concentration of the other substrate was varied. 1 μM enzyme was used and the reaction was incubated at 37 °C for 20 min. Absorbance is measured at 650 nm, mean values with SD are shown with a nonlin fit of kinetic by GraphPad Prism (A17). The calculation of the kinetic parameters presented in the table were calculated with by GraphPad Prism.

Native mass spectrometry and proteomics analyses of paralogous MurAA and MurAB from *B. subtilis*

Native MS provides the outstanding possibility to determine the exact mass of proteins with an intact fold. This provides the opportunity to identify binding and interactions with the protein by appropriate mass shifts. Fig.3 and Fig.4 show native mass spectrometry (native MS) spectra deconvoluted using UniDec. To investigate the shown influence of substrates on enzyme activity and FOS sensitivity, as well as possible post-translational modifications (ptm), in vitro approaches of MurAA and MurAB with and without UDP-GlcNAc and each with and without FOS were performed. Due to the nature of the method, masses may differ by approximately 1 Da and are therefore given without decimals. The enzymes were each identified with a loss of the N-terminal methionine (M). Surprisingly, MurAA and MurAB showed PEP ptm without the addition of substrates. The addition of FOS did not change the fact that the enzymes exclusively occurred with a PEP ptm. No PEP was added. This means that PEP binds extremely strong to MurAA and MurAB and this binding withstands the purification (IMAC and SEC), the enzymatic reaction and the rebuffering. The PEP ptm thereby protects the enzyme from reacting with FOS and makes PEP-bound MurA not an FOS target. Skarzynski *et. al* also demonstrated the co-purification of PEP and MurA [46, 47, 48]. When the enzymes are incubated together with UDP-GlcNAc, the theoretical apo-mass of the enzyme can be identified without ptm.

The reaction with the bound PEP proceeds. According to the hypothesis, the respective Cys in the active site is no longer protected by PEP and is bound by FOS. This can be determined without any ambiguity. In the case of MurAA, as shown in Fig.3. the result was not different when PEP was added to the reaction with UDP-GlcNAc. In this particular case, the paralog MurAB behaved differently. When MurAB was analysed together with UDP-GlcNAc, PEP and with and without FOS, several main masses were obtained. Without FOS, in addition to the apo enzyme and the PEP-bound enzyme, a mass of 48880 Da was identified as the largest percentage contribution. It is known from the literature that a complex of MurA the product UDP-GlcNAc-ep and PEP has sufficient stability for crystallisation [25]. The calculated mass differs by 3 Da from the theory, so it is quite possible that the corresponding complex can be measured by native MS. This hypothesis is supported by the fact that the same effect was observed when FOS was added. The mass changes from PEP to FOS and also the ratios shifted in favour of the exclusively FOS-bound MurAB. Detailed proteomic analysis and prior trypsin digestion of the identical experimental setups allowed unambiguous analysis of the MurAA and MurAB ptm. The specific method identified the Cys (MurAA: 137, MurAB: 136) with both PEP and FOS in the presence of UDP-GlcNAc, as expected from the described native MS analyses (A19, A20). Furthermore, the fundamental similarity of the MurAB and MurAA paralogs was also demonstrated by the native MS experiments. In addition, the differences were also revealed by this method. Differences are shown in a reaction with all substrates, MurAB is less specific, which was already predicted by the previously determined different K_m values.

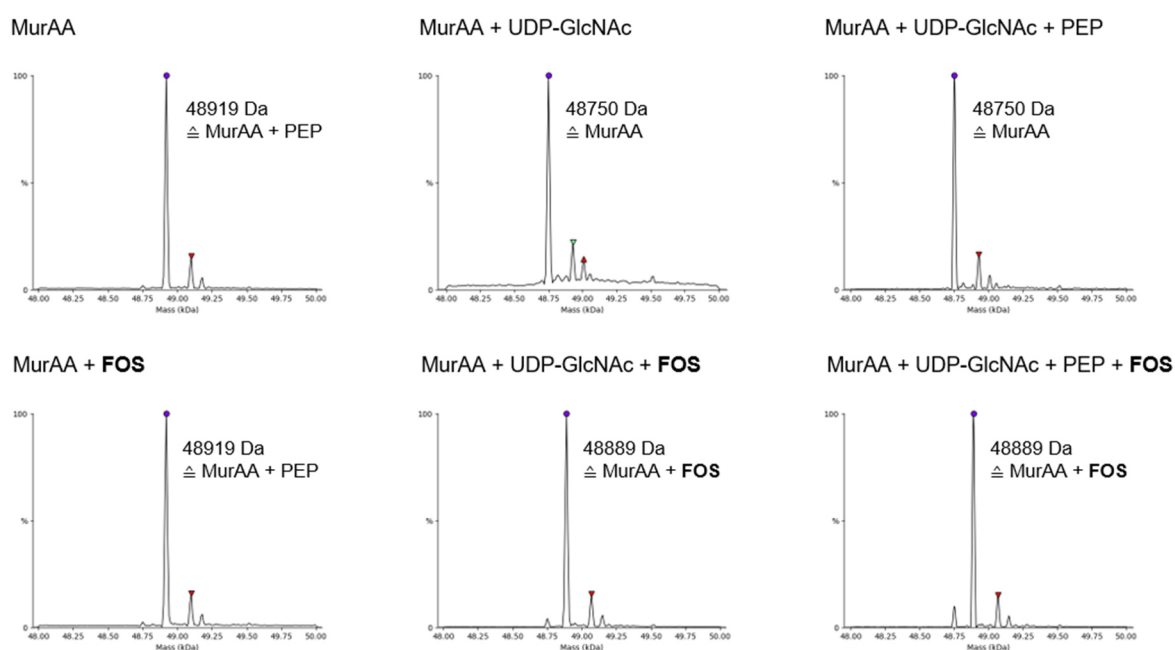


Figure 3: Native MS spectra of *B. subtilis* MurAA under FOS influence in different substrate combinations. Enzymatic reaction was performed with final concentrations of 10 μ M MurAA, 1 mM UDP-GlcNAc, 1 mM PEP and 128 μ g mL⁻¹ FOS for 45 min at 37 °C in 20 mM Tris-HCl, 500 mM NaCl pH 7.5. After the reaction, for native MS analysis, the reaction was rebuffed in 10 mM ammonium acetate pH 8. Headers explain the reaction approaches; the detected masses are noted in the spectrum. Due to the native method, the mass accuracy is around 1 Da. Relevant theoretical masses (Da): his₆-MurAA (-M): 48752, UDP-GlcNAc: 607, PEP: 168, FOS: 138, detailed information: A3, A18. Evaluation was performed with UniDec.

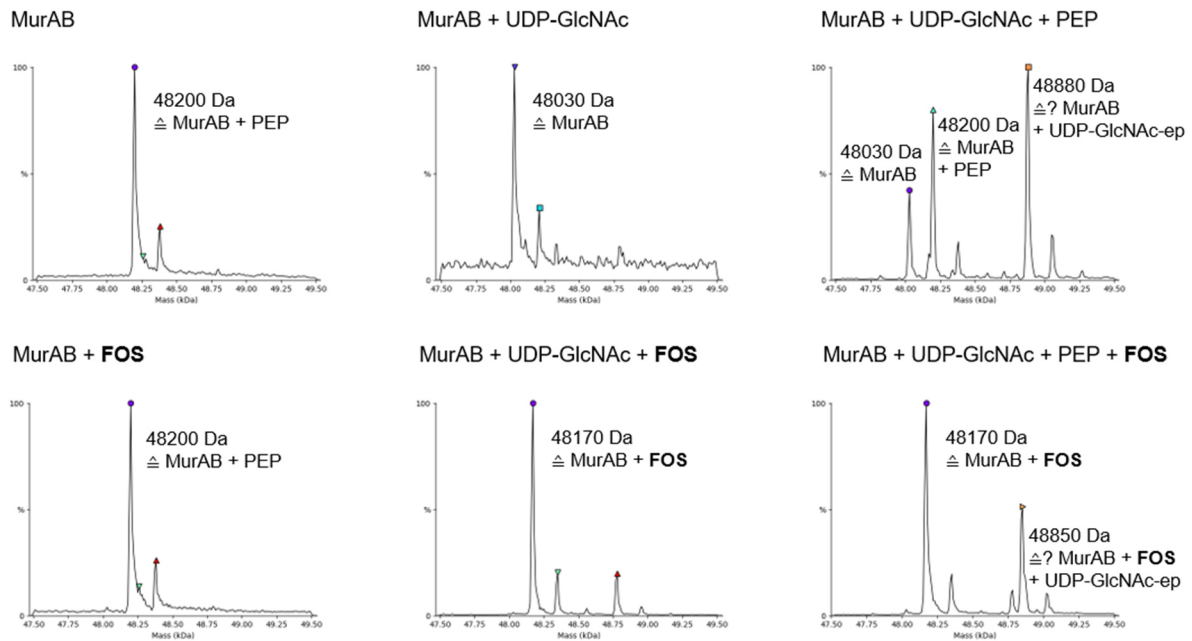


Figure 4: Native MS spectra of *B. subtilis* MurAB under FOS influence in different substrate combinations. Enzymatic reaction was performed with final concentrations of 10 μ M MurAA, 1 mM UDP-GlcNAc, 1 mM PEP and 128 μ g mL⁻¹ FOS for 45 min at 37 °C in 20 mM Tris-HCl, 500 mM NaCl pH 7.5. After the reaction, for native MS analysis, the reaction was rebuffered in 10 mM ammonium acetate pH 8. Headers explain the reaction approaches; the detected masses are noted in the spectrum. Due to the native method, the mass accuracy is around 1 Da. Relevant theoretical masses (Da): his₆-MurAB (-M): 48032, UDP-GlcNAc-ep: 677, UDP-GlcNAc: 607, PEP: 168, FOS: 138, detailed information: A3, A18. Evaluation was performed with UniDec.

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AUTHOR CONTRIBUTIONS

SF developed the experiments, evaluated them, visualised the results and wrote the MS. SF carried out the cloning. SF and YS purified the protein and performed all enzyme assays. PS and DP performed the native mass spectrometry. MFW and BM performed the bottom-up proteomics analyses. CM provided scientific assistance and reviewed the MS.

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APPENDIX

A1: chemically competent *E. coli* DH5 α

A2. Colony PCR analyses of the cloned constructs.

A3. Information about the recombinant proteins AroA, MurAA and MurAB

A4. UV chromatograms of IMAC and SEC purification of the recombinant proteins AroA, MurAA and MurAB

A5. SDS-PAGE analysis of the IMAC and SEC purifications of the recombinant proteins AroA, MurAA and MurAB

A6. Activity verification of *E. coli* AroA by LC-MS

A7. Activity verification of *B. subtilis* MurAA by LC-MS.

A8. Activity verification of *B. subtilis* MurAB by LC-MS

A9. Evaluation of the in-house developed MurA phosphate assay.

A10. Proportionality of the in-house developed MurA phosphate assay.

A11. Evaluation of buffer system used for the MurA phosphate assay.

A12. Evaluation of enzyme and substrate concentrations for the MurA phosphate assay.

A13. Time dependence of the in-house developed MurA phosphate assay.

A14. Storage stability of the purified the recombinant proteins MurAA and MurAB

A15. SDS-PAGE analysis to validate the stability of the recombinant proteins MurAA and MurAB

A16. FOS inhibition of *B. subtilis* MurAA and MurAB at different substrate concentrations.

A17. Kinetic characterization of recombinant *B. subtilis* MurAA and MurAB.

A18: Mass list with the exact masses for native mass spectrometric analysis

A19: Proteomic analysis of the in vitro enzyme reaction of recombinant purified MurAA from *B. subtilis* in different combinations.

APPENDIX:

Native mass spectrometry reveals that MurA carboxyvinyltransferases are primarily phosphoenolpyruvate-bound and therefore fosfomycin insensitive.

Simon Friz¹, Yannis Stahl¹, Paolo Stincone², Mirita Franz-Wachtel³, Boris Macek³, Daniel Petras², Christoph Mayer¹

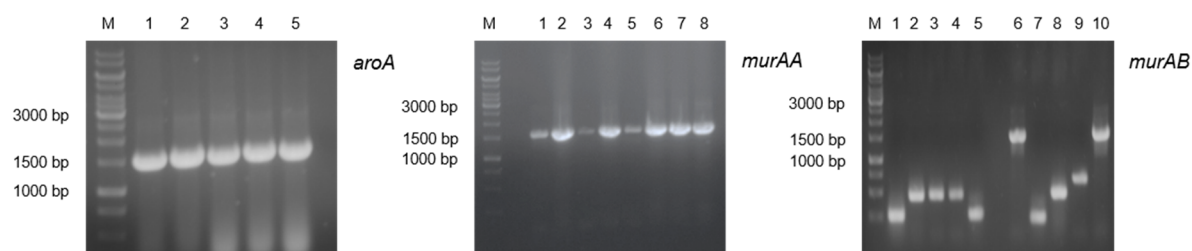
¹Interfaculty Institute of Microbiology and Infection Medicine, Organismic Interactions/ Microbial Glycobiology, Eberhard-Karls-Universität Tübingen, Tuebingen 72076, Auf der Morgenstelle 28, Germany, ²Cluster of Excellence-Controlling Microbes to Fight Infection, Eberhard-Karls-Universität Tübingen, Tuebingen 72076, Auf der Morgenstelle 28, Germany, ³Proteome Center Tuebingen, Eberhard-Karls-Universität Tübingen, Tuebingen 72076, Auf der Morgenstelle 15, Germany.

A1: Chemically competent *E. coli* DH5α *

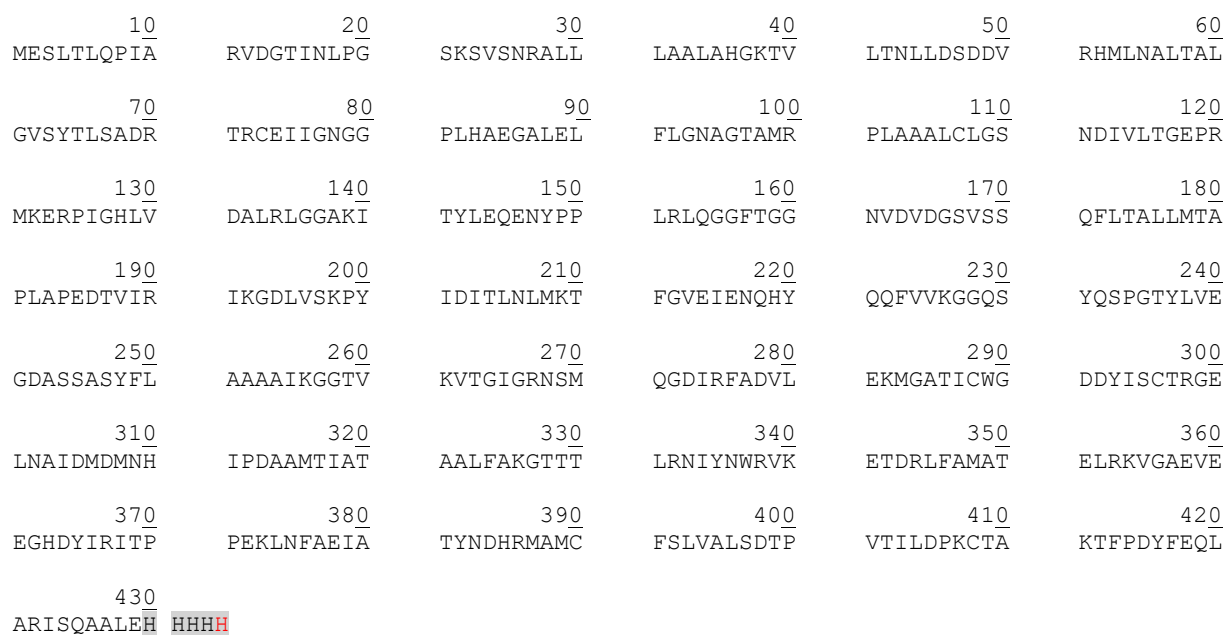
To produce chemically competent cells, an overnight culture of the desired *E. coli* strain was inoculated in 50 mL trypton-yeast medium (TYM, recipe below) with the antibiotics required for selection in a ratio of 1:200. The cells were incubated at a temperature of 37 °C and 180 rpm to an OD_{600nm} of 0.5-0.7. The culture was cooled down to ice and harvested by centrifugation (4700 rpm, 20 min, 4 °C). The cell pellet was resuspended in 5 mL of 4 °C cold TFB I medium and incubated for 1.5 h. The cell pellet was then removed by centrifugation. After another centrifugation step (4700 rpm, 20 min, 4 °C), the cells were resuspended in 2.5 mL cold TFB II medium and then aliquoted to 50 µL each. For storage, the aliquots were snap-frozen in liquid nitrogen and stored at -80 °C.

TYM:	TFBI:	TFBII:
20 g tryptone	0.3 mL 5 M potassium acetate	2.5 mL 2 M MOPS
5 g yeast extract	495 mg manganese(II) chloride	3.7 mL 1 M calcium chloride
5.84 g sodium chloride	2.5 mL 2 M potassium chloride	250 µL 2 M potassium chloride
2.46 g magnesium sulphate	0.5 mL 1 M calcium chloride	7.5 mL glycerol
ad 1 L H ₂ O _{dd}	7.5 mL glycerol	H ₂ O _{dd} ad 50 mL
	H ₂ O _{dd} ad 50 mL	

*Green R, Rogers EJ. Methods in Enzymology - 28: Transformation of Chemically Competent *E. coli* 2013.



A2. Colony PCR analyses of the cloned constructs. Verification of colony PCR using 1 % agarose gel and a GeneRuler 1 kb DNA Ladder (SM0311, Thermo Scientific) as size marker. The expected sizes of. *aroA*: 1462, *murAA*: 1552, *murAB*: 1531, can be identified in at least one clone each. This was subsequently transformed into *E. coli* BL21 (DE3).



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Expression plasmid for recombinant *B. subtilis* his₆-tag-MurAA

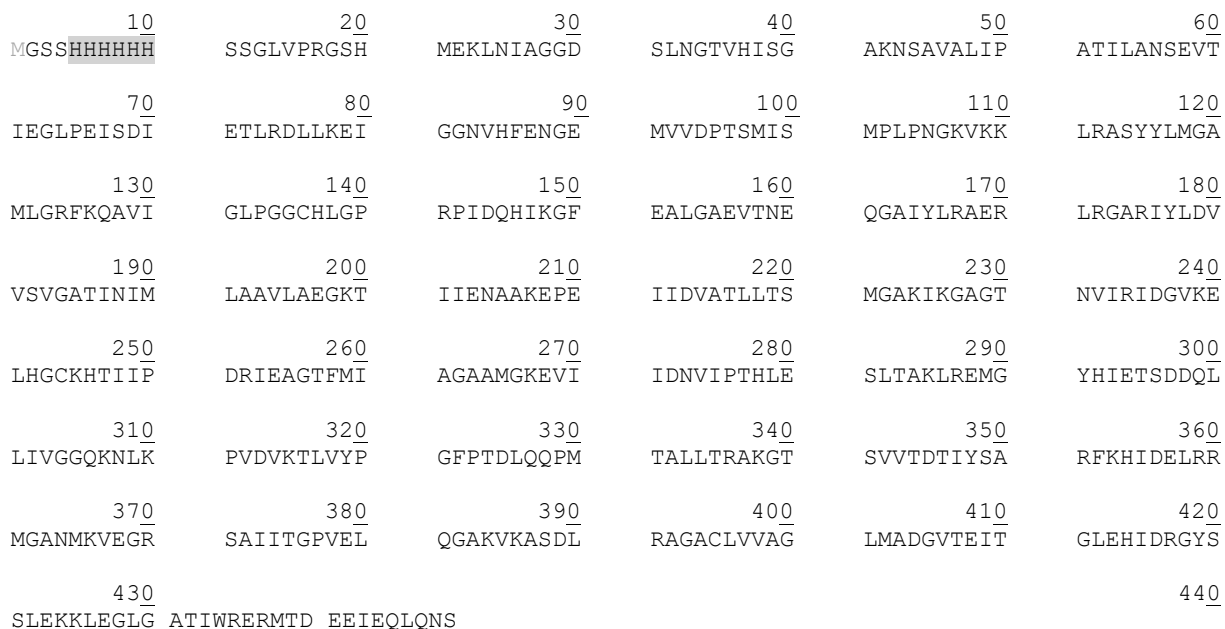


10	20	30	40	50	60
MGSSHHHHHH	SSGLVPRGSR	MEKIIVRGGQ	KLNGTVKVEG	AKNAVLPIVIA	ASLLASEEKS
70	80	90	100	110	120
VICDVPTLSD	VYTINEVLRH	LGADVHFENN	EVTVNASYAL	QTEAPFEYVR	KMRASVLVMG
130	140	150	160	170	180
PLLARTGHAR	VALPGGCAIG	SRPIDQHLKG	FEAMGAEIKV	GNGFIEAEVK	GRLQGAKIYL
190	200	210	220	230	240
DFPSVGATEN	LIMAAALAEQ	TTTLENVAKE	PEIVDLANYI	NGMGGKIRGA	GTGTIKIEGV
250	260	270	280	290	300
EKLHGVKHHI	IPDRIEAGTF	MVAAAITEGN	VLVKGAVPEH	LTSLIAKMEE	MGVTIKDEGE
310	320	330	340	350	360
GLRVIGPKEL	KPIDIKTMPH	PGFPTDMQSQ	MMALLLRASG	TSMITETVFE	NRFMHAEFR
370	380	390	400	410	420
RMNGDIKIEG	RSVIINGPVQ	LQGAEVAATD	LRAGAALILA	GLVAEGHTRV	TELKHLDRGY
430			440		450
VDFHQKLAAL	GADIERVNDE	SASEQENKEV	VSDLNA		

number of amino acids: 456, molecular weight: 48883.13, theoretical pI: 5.96

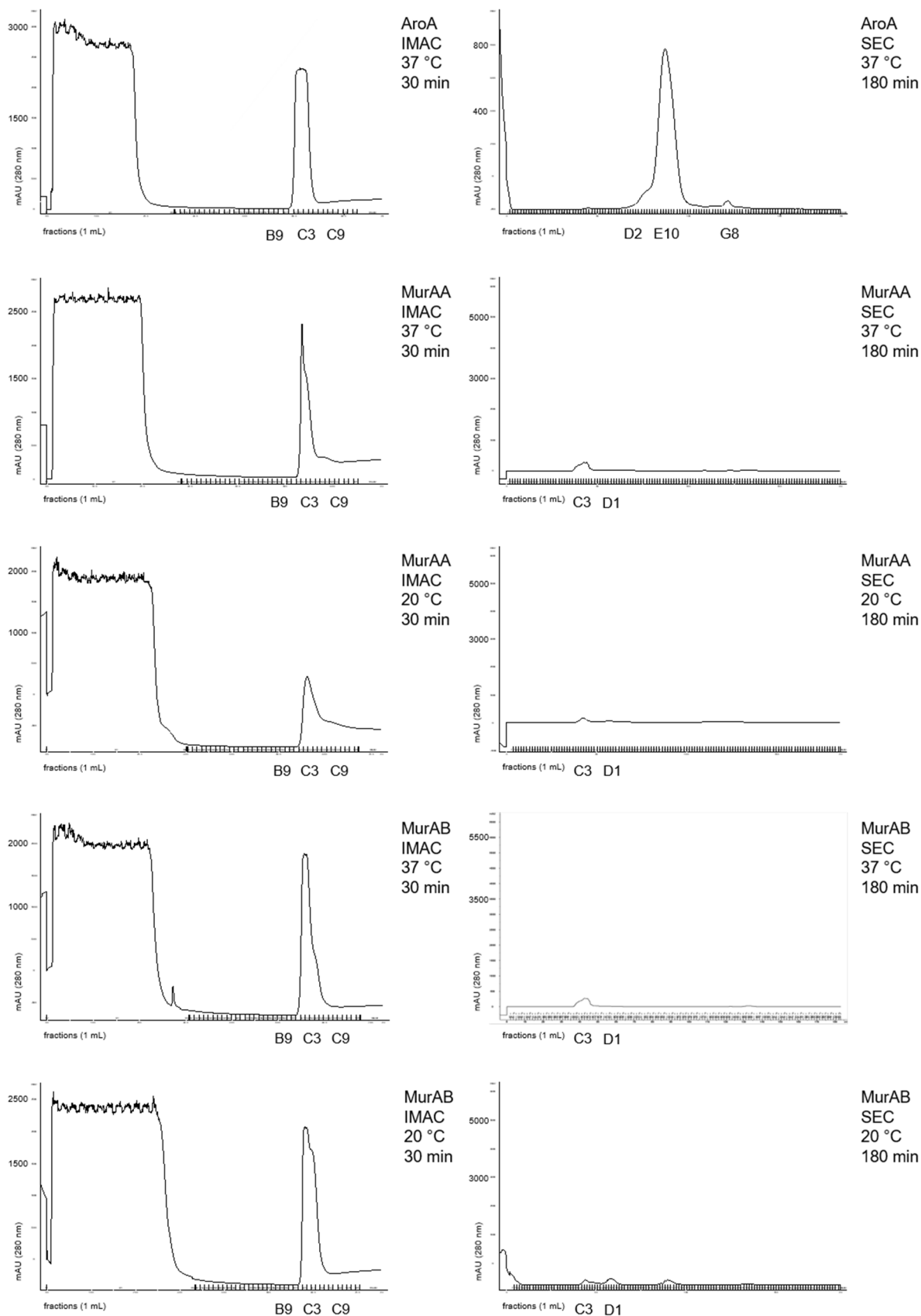
without methionine (M) at the beginning:

number of amino acids: 455, molecular weight: 48751.94, theoretical pI: 5.97

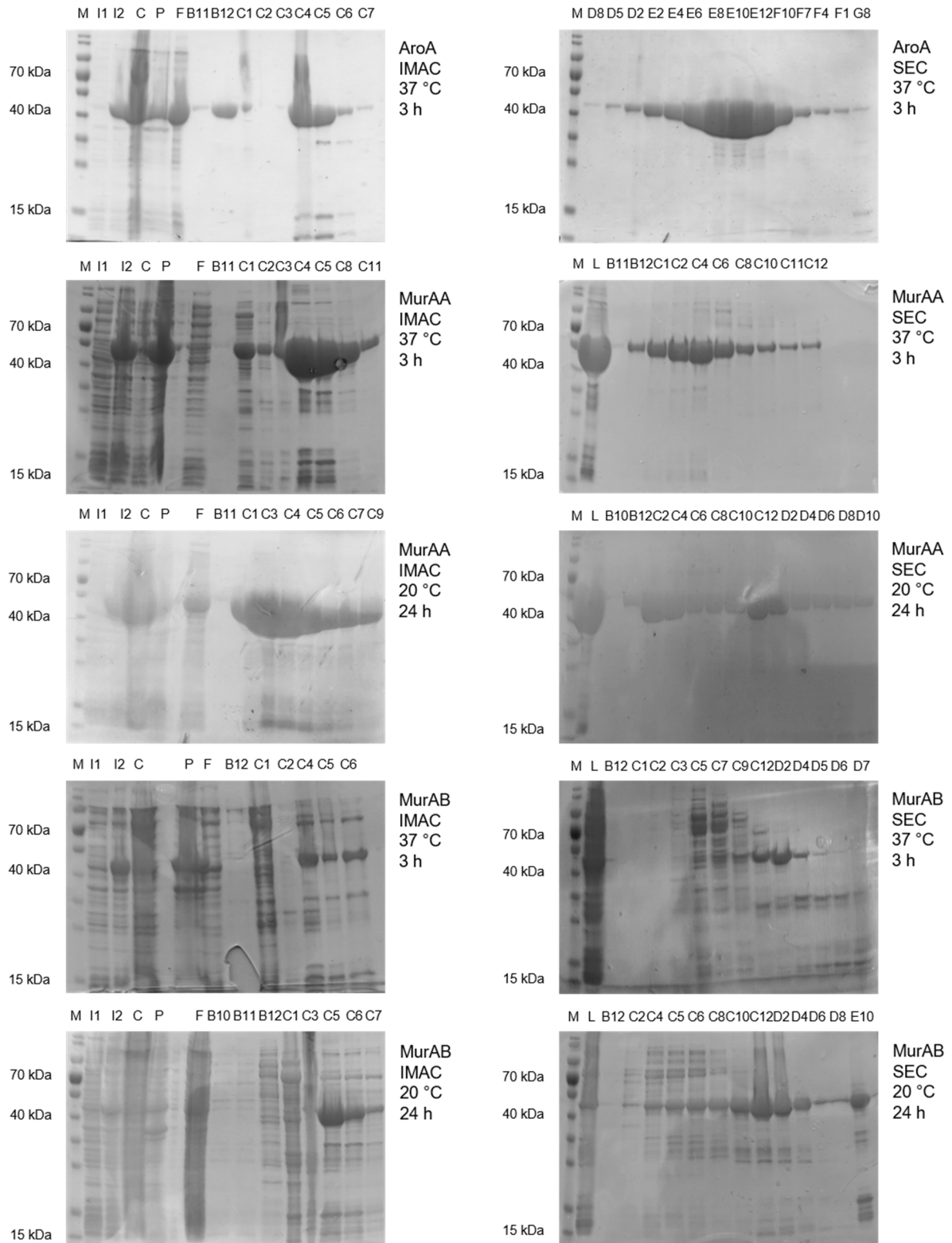


without methionine (M) at the beginning:

A3. Information about the recombinant proteins AroA, MurAA and MurAB. The respective plasmid map (created with SnapGene), the amino acid code and the information about the protein size and the isoelectric point are shown.

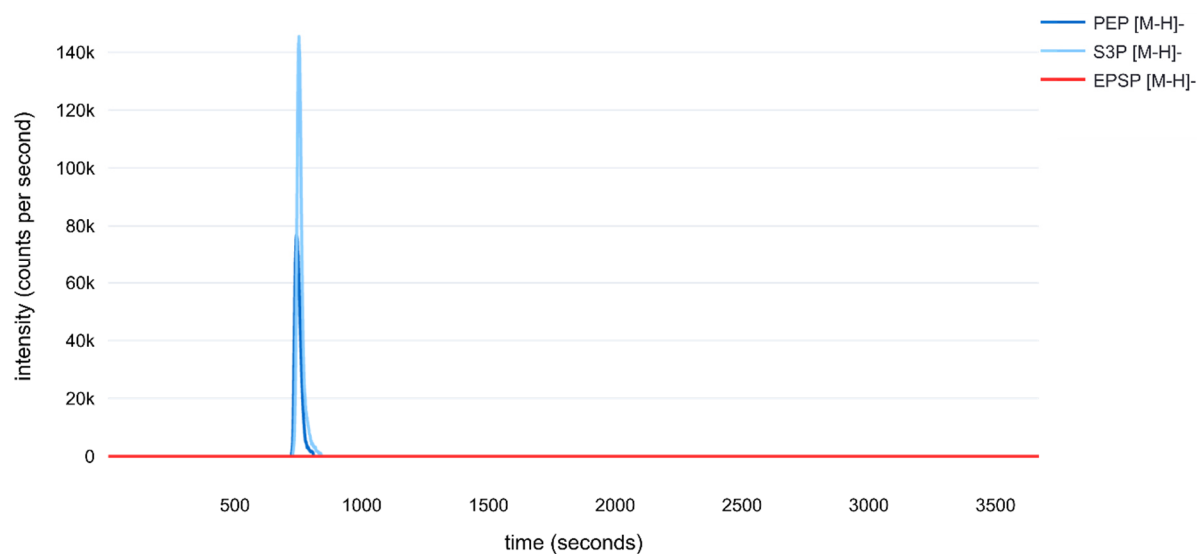


A4. UV chromatograms of IMAC and SEC purification of the recombinant proteins AroA, MurAA and MurAB. Shown are the chromatograms of the UV detector (280 nm) of the ÄKTApurifier system plotted in mAU versus fractions in mL. The IMAC and SEC chromatograms at different temperatures are shown for each recombinant protein.

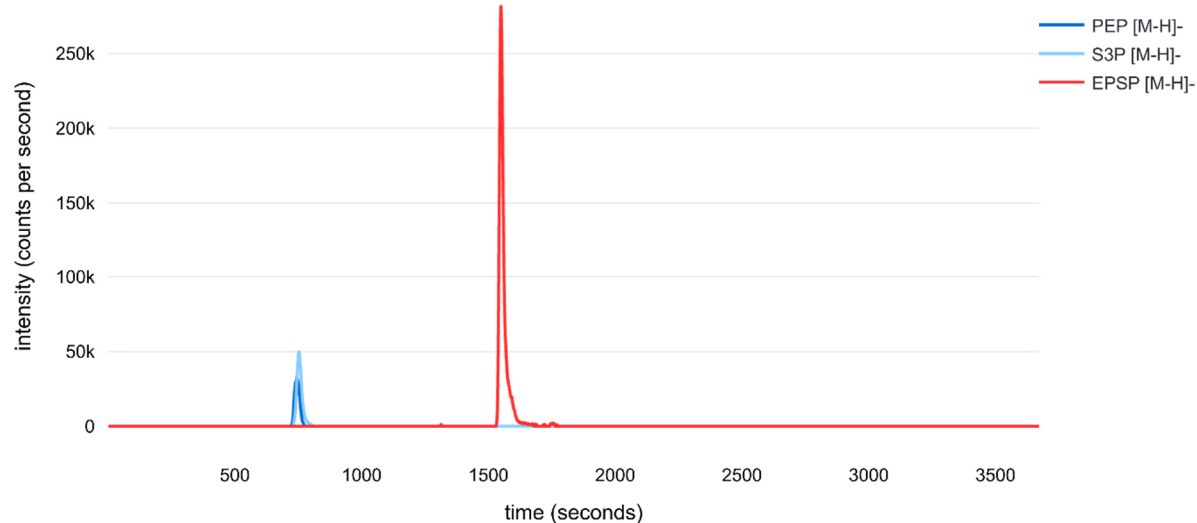


A5. SDS-PAGE analysis of the IMAC and SEC purifications of the recombinant proteins AroA, MurAA and MurAB. Shown are the stained (CBB staining) SDS-PAGE gels for analysis of IMAC and SEC purifications. To determine the sizes, a PageRuler Prestained Protein Ladder, 10 to 180 kDa. (26616X4, Thermo Scientific) was used. C=crude extract (supernatant after cell disruption), P= pelet after cell disruption, F = flow through during IMAC, L = loop (applied protein solution after IMAC purification).

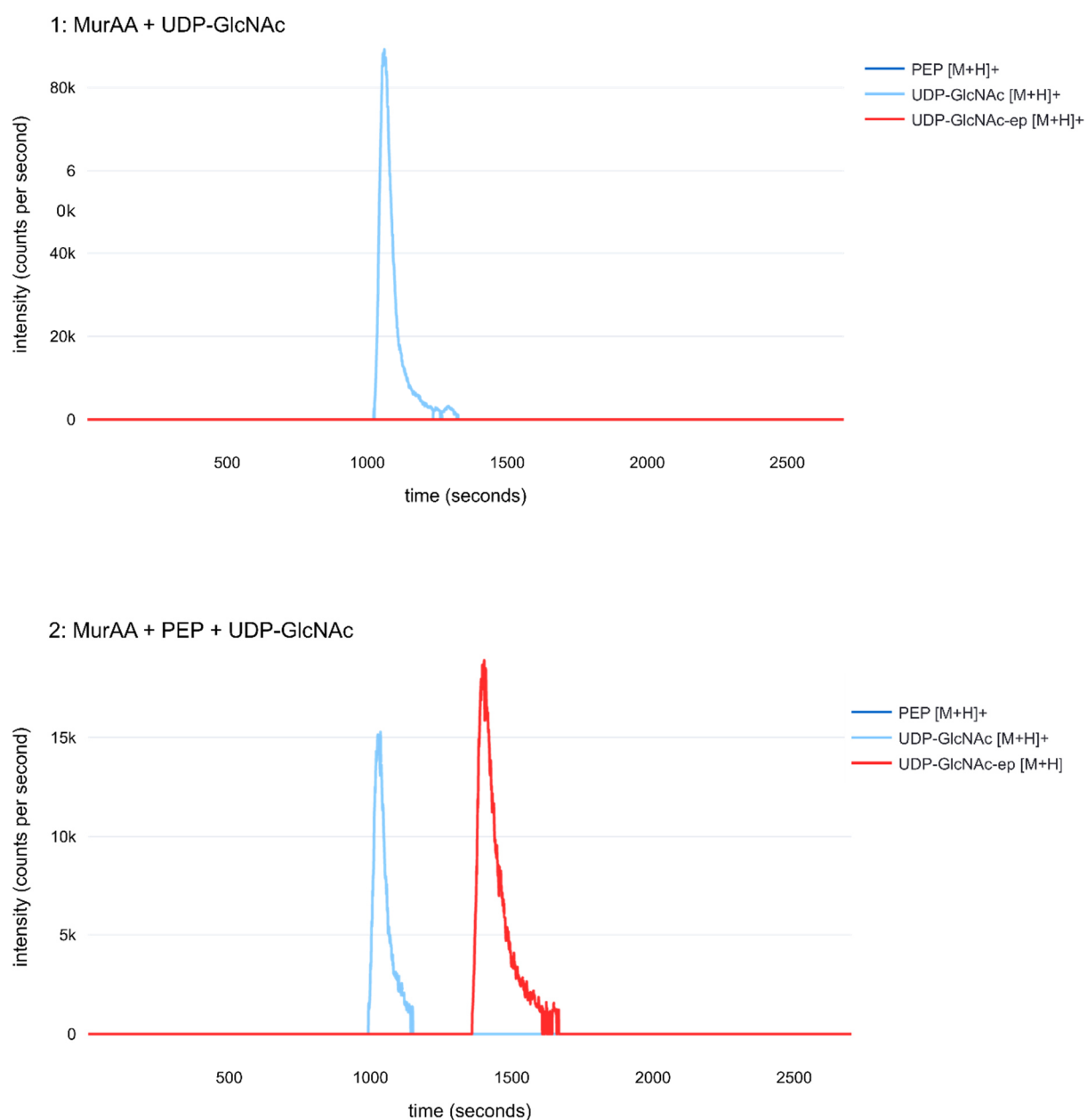
1: PEP + S3P



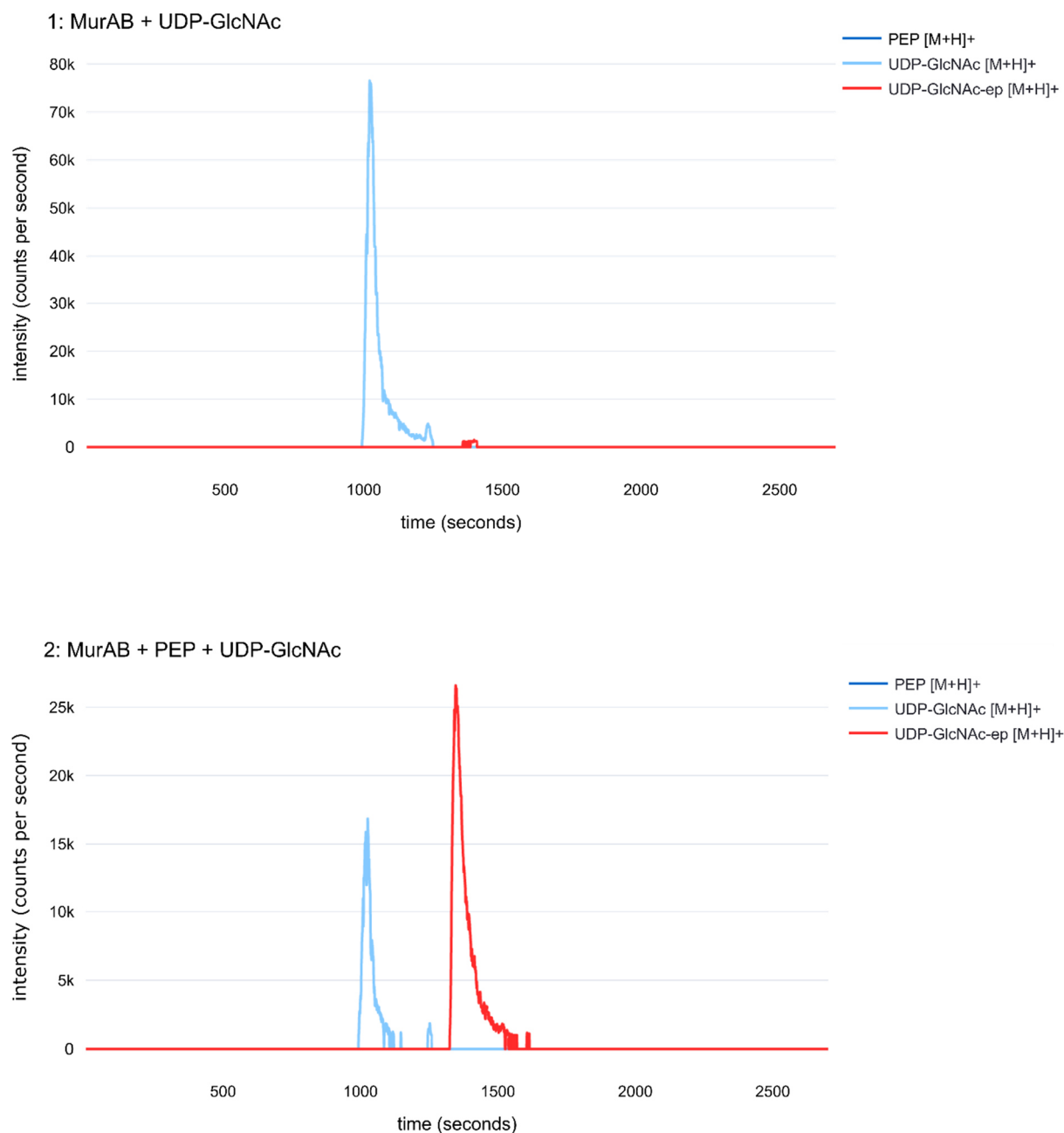
2: AroA + PEP + S3P



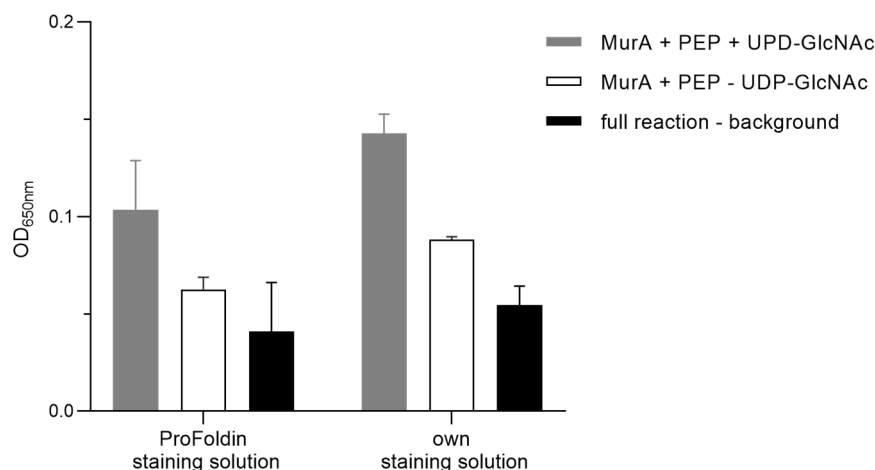
A6. Activity verification of *E. coli* AroA by LC-MS. Extracted ion chromatograms (EIC) with the exact masses in negative ion mode: phosphoenolpyruvate (PEP) [M-H]⁻: 166.6751 m z⁻¹, shikimate-3-phosphate (S3P) [M-H]⁻: 253.0119 m z⁻¹, 5-enolpyruvylshikimate-3-phosphat (EPSP) [M-H]⁻: 323.0174 m z⁻¹ are displayed. All analyses were performed with a noise threshold of 1000 and a measurement tolerance of 0.02 Da using the OpenMS based tool for metabolomics data analysis UmetaFlow [32] with its graphical user interface (<https://github.com/axelwalter/umetaflow-gui>). 1 shows a reaction without AroA (negative control), 2 corresponds to a complete enzymatic reaction. All substrates: 1 mM, buffer: 20 mM Tris-HCl, 500 mM NaCl, pH 7.5.



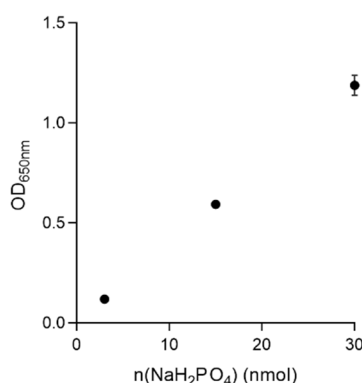
A7. Activity verification of *B. subtilis* MurAA by LC-MS. Extracted ion chromatograms (EIC) with the exact masses in positive ion mode of: phosphoenolpyruvate (PEP) $[M+H]^+$: $168.9897 \text{ m z}^{-1}$, UDP-N-acetylglucosamine (UDP-GlcNAc) $[M+H]^+$: $608.0889 \text{ m z}^{-1}$, UDP-N-acetylglucosamine-enolpyruvate (UDP-GlcNAc-ep) $[M+H]^+$: $678.0943 \text{ m z}^{-1}$, are displayed. All analyses were performed with a noise threshold of 1000 and a measurement tolerance of 0.02 Da using the OpenMS based tool for metabolomics data analysis UmetaFlow [32] with its graphical user interface (<https://github.com/axelwalter/umetaflow-gui>). 1 shows a reaction without PEP (negative control), 2 corresponds to a complete enzymatic reaction. All substrates: 1 mM, buffer: 20 mM Tris-HCl, 500 mM NaCl, pH 7.5.



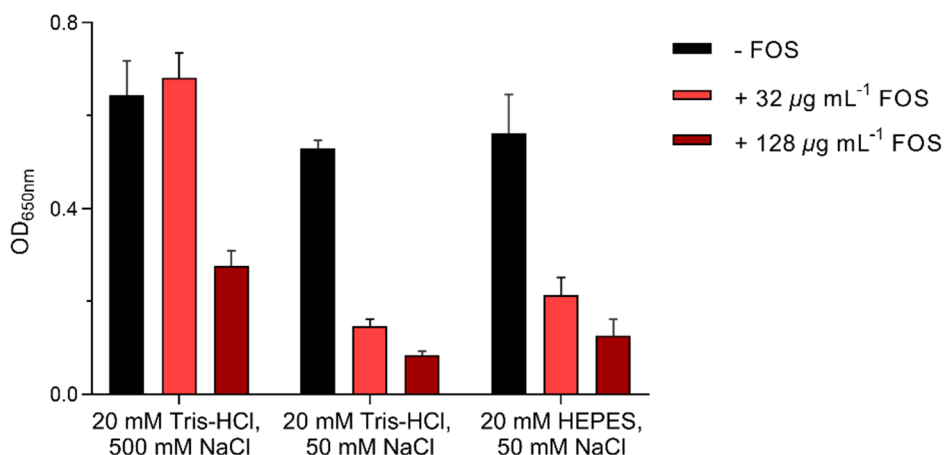
A8. Activity verification of *B. subtilis* MurAB by LC-MS. Extracted ion chromatograms (EIC) with the exact masses in positive ion mode of: phosphoenolpyruvate (PEP) $[M+H]^+$: 168.9897 m z^{-1} , UDP-N-acetylglucosamine (UDP-GlcNAc) $[M+H]^+$: 608.0889 m z^{-1} , UDP-N-acetylglucosamine-enolpyruvate (UDP-GlcNAc-ep) $[M+H]^+$: 678.0943 m z^{-1} , are displayed. All analyses were performed with a noise threshold of 1000 and a measurement tolerance of 0.02 Da using the OpenMS based tool for metabolomics data analysis UmetaFlow [32] with its graphical user interface (<https://github.com/axelwalter/umetaflow-gui>). 1 shows a reaction without PEP (negative control), 2 corresponds to a complete enzymatic reaction. All substrates: 1 mM, buffer: 20 mM Tris-HCl, 500 mM NaCl, pH 7.5.



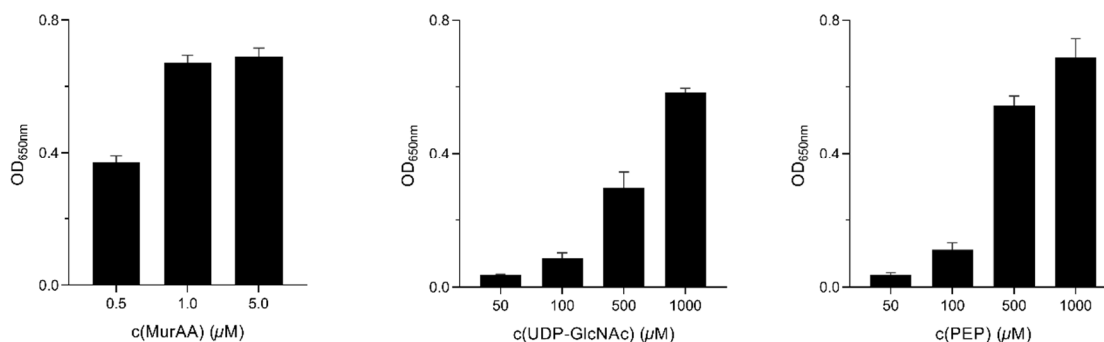
A9. Evaluation of the in-house developed MurA phosphate assay. Two reactions were performed with 20 μ M *E. coli* MurA (ProFoldin) in triplicate: one reaction with all components and one reaction without UDP-GlcNAc, reflecting the non-specific background signal of each dye. Absorbance was measured at 650 nm, mean values with SD are shown. The dye of the ProFoldin kit and the dye described in the methods section were used for the staining reaction. The reaction was performed at 37 °C for 1 h according to the manufacturer's instructions and concentrations. All substrates: 1 mM, buffer: 20 mM Tris-HCl, 500 mM NaCl, pH 7.5.



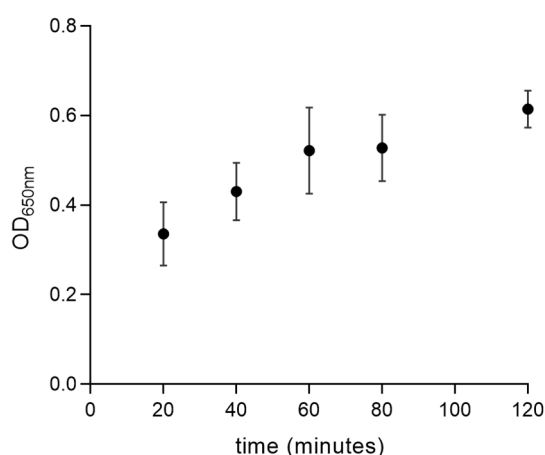
A10. Proportionality of the in-house developed MurA phosphate assay. Test for proportionality and detection limits of the developed phosphate assay with NaH₂PO₄ as standard for inorganic phosphate. Absorbance was measured at 650 nm, mean values with SD are shown.



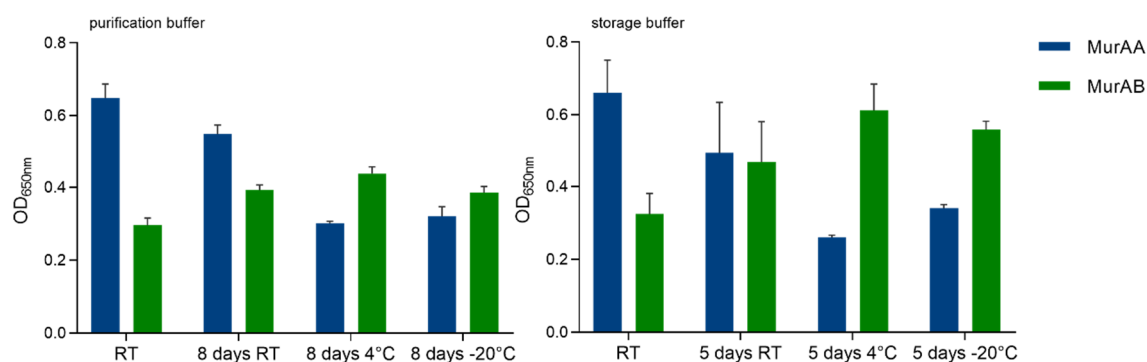
A11. Evaluation of buffer system used for the MurA phosphate assay. The reaction was carried out with 5 μ M MurAA, 1 mM PEP, and 1 mM UDP-GlcNAc for 1 h at 37 °C in the three buffers (all pH 7.5) indicated. Absorbance is measured at 650 nm, mean values with SD are shown.



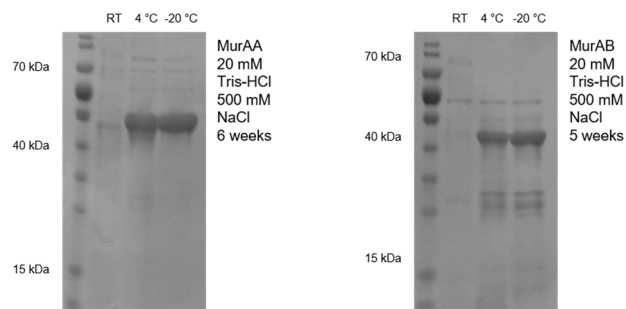
A12. Evaluation of enzyme and substrate concentrations for the MurA phosphate assay. The reaction was carried out with 0.5-5.0 μM MurAA, 50-1000 μM PEP, and 50-1000 μM UDP-GlcNAc for 1 h at 37 °C in 20 mM Tris-HCl, 50 mM NaCl pH 7.5. Absorbance was measured at 650 nm, mean values with SD are shown.



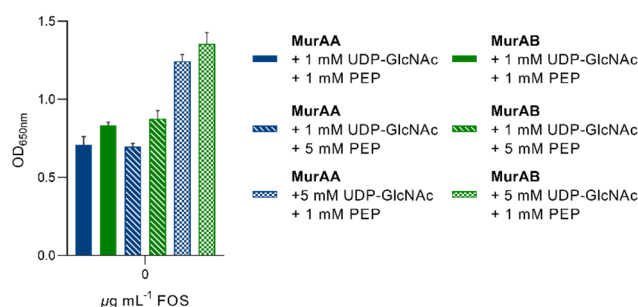
A13. Time dependence of the in-house developed MurA phosphate assay. Test for proportionality and detection limits of the developed phosphate assay with NaH₂PO₄ as standard for inorganic phosphate. Absorbance was measured at 650 nm, mean values with SD are shown. Buffer: 20 mM Tris-HCl, 50 mM NaCl pH 7.5.



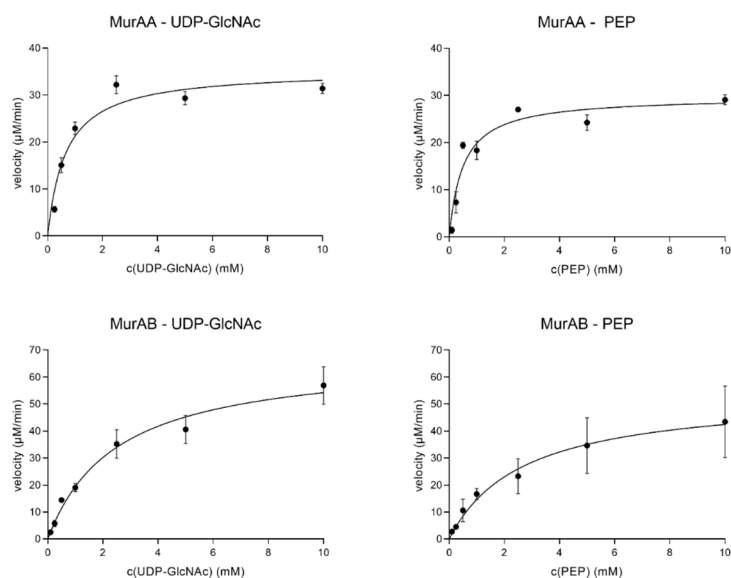
A14. Storage stability of the purified the recombinant proteins MurAA and MurAB. The reaction was carried out with 1 μM enzyme, 1 μM PEP, and 1 μM UDP-GlcNAc for 1 h at 37 °C in 20 mM Tris-HCl, 50 mM NaCl pH 7.5 (purification buffer) or additionally with + 10 % glycerol + 20 mM Dithiothreitol (DTT). Absorbance was measured at 650 nm, mean values with SD are shown. RT=room temperature. The temperature specifications refer to storage. If a date is specified, it corresponds to the time after purification.



A15. SDS-PAGE analysis to validate the stability of the recombinant proteins MurAA and MurAB. Shown are the stained (CBB staining) SDS-PAGE gels for analysis of the enzymatic stocks. To determine the sizes, a PageRuler™ Prestained Protein Ladder, 10 to 180 kDa. (26616X4, Thermo Scientific™) was used. RT=room temperature. The temperature specifications refer to storage. If a date is specified, it corresponds to the time after purification.



A16. FOS inhibition of *B. subtilis* MurAA and MurAB at different substrate concentrations. The reaction was carried out at 37 °C for 1 h. The inhibition of 1 μM *B. subtilis* MurAA and MurAB was tested with different FOS concentrations (0-200 μg mL⁻¹) and different substrate concentrations 1 mM or 5 mM for PEP and UDP-GlcNAc. Absorbance was measured at 650 nm, mean values with SD are shown. Buffer: 20 mM Tris-HCl, 50 mM NaCl pH 7.5. The absolute absorptions of the uninhibited experiments are shown (in addition to relative activities in Fig.2).



A17. Kinetic characterization of recombinant *B. subtilis* MurAA and MurAB. Kinetic parameters were determined with different amounts of UDP-GlcNAc or PEP. 1 mM of the other substrate and 1 μM enzyme was used. The reaction was incubated at 37 °C for 20 min. Absorbance is measured at 650 nm, mean values with SD are shown with a nonlin fit of kinetic by GraphPad Prism. Buffer: 20 mM Tris-HCl, 50 mM NaCl pH 7.5.

A18: Mass list with the exact masses for native mass spectrometric analysis

	sum formula	neutral monoisotopic mass (Da)
UDP-GlcNAc-ep	C ₂₀ H ₂₉ N ₃ O ₁₉ P ₂	677.0871
UDP-GlcNAc	C ₁₇ H ₂₇ N ₃ O ₁₇ P ₂	607.0816
PEP	C ₃ H ₅ O ₆ P	167.9824
FOS	C ₃ H ₇ O ₄ P	138.0082

A19: Proteomic analysis of the in vitro enzyme reaction of recombinant purified MurAA from *B. subtilis* in different combinations.

	MurAA: PEP	MurAA: FOS
Positions within proteins	137	137
Score diff	73.4977	77.8708
PEP	2.01E-11	3.31E-17
Score	123.4	154.48
Delta score	122.3	146.93
Score for localization	73.498	77.871
Localization prob R52	1	
Score diff R52	94.5693	
PEP R52	2.27E-07	
Score R52	94.569	
Localization prob R53	1	
Score diff R53	51.0887	
PEP R53	2.01E-11	
Score R53	123.4	
Localization prob R54	1	
Score diff R54	83.4044	
PEP R54	2.31E-11	
Score R54	120.9	
Localization prob R55	1	1
Score diff R55	73.4977	77.8708
PEP R55	2.63E-11	3.31E-17
Score R55	118.31	154.48
Number of PEP (C)	1	1
Amino acid	C	C
Sequence window	LLARTGHARVALPGGCAIGSRPIDQHLK GFE	LLARTGHARVALPGGCAIGSRPIDQHLK GFE
Modification window	X;X;X;X;X;X;X;X;X;X;X;X; Carbamidomethyl (C);PEP (C);X;X;X;X;X;X;X;X;X;X;X	X;X;X;X;X;X;X;X;X;X;X;X; Carbamidomethyl (C);FOS (C);X;X;X;X;X;X;X;X;X;X;X
Peptide window coverage	XXXXXXXXXPPPPPPPPPPPPPPPPPP XXX	XXXXXXXXXPPPPPPPPPPPPPPPPPP XXX
PEP (C) Probabilities	VALPGGC(1)AIGSRPIDQHLK	VALPGGC(1)AIGSRPIDQHLK
PEP (C) Score diffs	VALPGGC(73.5)AIGSRPIDQHLK	VALPGGC(77.87)AIGSRPIDQHLK
Position in peptide	7	7
Charge	4	3
Mass error [ppm]	-0.080315	-0.346
Intensity MurAA	7893700	0
Intensity MurAA + FOS	37621000	0
Intensity MurAA + UDP-GlcNAc	31491000	0
Intensity MurAA + UDP-GlcNAc + FOS	24265000	44823000

MurAA and MurAA + UDP-GlcNAc reactions were analysed with and without FOS. 5 µM (25 µg in total) MurAA was used and incubated at 37 °C for 1 h in 20 mM Tris-HCl, 50 mM NaCl pH 7.5. Digestion with trypsin in solution with DTT/IAA.

A20: Proteomic analysis of the in vitro enzyme reaction of recombinant purified MurAB from *B. subtilis* in different combinations.

	MurAB: PEP	MurAB: FOS
Positions within proteins	136	136
Score diff	48.1464	53.6247
PEP	8.48E-32	2.90E-77
Score	159.14	246.66
Delta score	139.89	244.97
Score for localization	48.146	53.625
Localization prob R61_A	1	
Score diff R61_A	50.784	
PEP R61_A	3.06E-09	
Score R61_A	92.563	
Localization prob R62_A	1	
Score diff R62_A	72.5852	
PEP R62_A	2.94E-09	
Score R62_A	89.011	
Localization prob R63_A	1	
Score diff R63_A	46.3496	
PEP R63_A	2.54E-11	
Score R63_A	118.44	
Localization prob R64_A	1	1
Score diff R64_A	50.2553	145.767
PEP R64_A	2.03E-21	2.90E-77
Score R64_A	150.91	246.66
Number of PEP (C)	1	1
Amino acid	C	C
Sequence window	MLGRFKQAVIGLPGGCHLGPRPIDQHIKGF E	MLGRFKQAVIGLPGGCHLGPRPIDQHIKGF E
Modification window	Oxidation (M);X;X;X;X;X;X;X;X;X;X;X; Carbamidomethyl (C);PEP (C);X;X;X;X;X;X;X;X;X;X;X;X;X;X;X;X	Oxidation (M);X;X;X;X;X;X;X;X;X;X;X; Carbamidomethyl (C);FOS (C);X;X;X;X;X;X;X;X;X;X;X;X;X;X;X;X
Peptide window coverage	XXXXXXXXXXXXXXXXXXXXXXXXX X	XXXXXXXXXXXXXXXXXXXXXXXXX X
PEP (C) Probabilities	QAVIGLPGGC(1)HLGPRPIDQHIK	QAVIGLPGGC(1)HLGPRPIDQHIK
PEP (C) Score diffs	QAVIGLPGGC(48.15)HLGPRPIDQHIK	QAVIGLPGGC(53.62)HLGPRPIDQHIK
Position in peptide	10	10
Charge	4	3
Mass error [ppm]	0.54561	-0.24738
Intensity MurAB	37287000	0
Intensity MurAB + FOS	25729000	0
Intensity MurAB + UDP-GlcNAc	39552000	0
Intensity MurAB + UDP-GlcNAc + FOS	51247000	2.0502E+10

MurAB and MurAB + UDP-GlcNAc reactions were analysed with and without FOS. 5 µM (15 µg in total) MurAB was used and incubated at 37 °C for 1 h in 20 mM Tris-HCl, 50 mM NaCl pH 7.5. Digestion with trypsin in solution with DTT/IAA.

4.6 Manuscript 6

Friz S, Stahl Y, Mayer C. Investigating the inhibition of *Bacillus subtilis* growth by fosfomycin: The role of the targets MurAA and MurAB, drug transport, intracellular accumulation and tolerance development.

Ready for submission. Manuscript and appendix are attached.

Investigating the inhibition of *Bacillus subtilis* growth by fosfomycin: The role of the targets MurAA and MurAB, drug transport, intracellular accumulation and tolerance development.

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Key words: fosfomycin, peptidoglycan, MHC, *Bacillus subtilis*, growth curves, resistance LC-MS

ABSTRACT

The epoxide-phosphonate antibiotic fosfomycin (FOS) inhibits the carboxyvinyltransferase MurA, a key enzyme in peptidoglycan synthesis, by covalently binding to a cysteine residue in the active site. When *Bacillus subtilis* is treated with FOS above the minimum inhibitory concentration (MIC), a clear lytic effect is seen, caused by previously unknown side effects. We aimed to study these secondary cellular effects in response to FOS and to investigate factors that influence FOS sensitivity, effective intracellular concentrations of target and antibiotic, transport, adaptation and resistance development and growth behaviour of *B. subtilis*. FOS sensitivity of *B. subtilis* is significantly influenced by four genetically manifested factors: *glpT* (transport: glycerol-3-phosphate permease), *fosB* (FOS detoxification mechanism: bacillithiol S-transferase), *murAA* and *murAB* (main targets of FOS, only MurAA is essential in *B. subtilis*, unclear interaction and relationship) and *reoM* (proteolysis of MurAA and possibly MurAB: adaptor protein to initiate proteolytic degradation). To investigate these factors in relation to the influence of FOS on *B. subtilis*, the commonly used MIC test strips, broth microdilution and growth curves in perforated plates were used to ensure continuous long-term measurement. Different media and the development of resistance in both liquid and solid cultures were analysed. The intracellular and extracellular concentration of FOS was measured by LC-MS over a period of 20 hours during FOS treatment and subsequent culture regeneration to provide a direct insight into the availability and physiologically relevant levels of FOS. It was clearly shown that FOS is actively transported via GlpT, not only because the MIC increased by a factor of 10 to $117.3 \mu\text{g mL}^{-1}$, but also because the growth behaviour in liquid cultures of the GlpT mutant was significantly different from the wt. When $128 \mu\text{g mL}^{-1}$ FOS was added during the exponential growth phase, the wt showed a lytic effect, whereas the GlpT mutant and the ReoM mutant were unaffected. Although the MIC of $42.7 \mu\text{g mL}^{-1}$ without ReoM was only twice as high as the wt MIC ($18.7 \mu\text{g mL}^{-1}$), the importance of degradation and the resulting intracellular MurA levels has been demonstrated. Apart from a small growth deficit in stationary phase, the MurAB mutant behaves identically to the wt. MurAA can indeed override the function of MurAB (the reverse is not possible, MurAA is essential), but the relationship and effects of the interaction between the two paralogs are unclear. In contrast, deletion of FosB significantly increases the sensitivity, the MIC is $1.8 \mu\text{g mL}^{-1}$ and there is a clear growth effect in liquid culture over $1 \mu\text{g mL}^{-1}$. Excitingly, the cells recovered from FOS treatment (and lysis) after about 10-15 hours and began to grow again. Apparently, chromosomal mutations are not responsible for this phenotype, but rather the resistance of *B. subtilis* is caused by metabolic adaptations to the presence of FOS. The resistance tests that were carried out showed that the effect of FOS on the growth of *B. subtilis* is very complex and depends to a large extent on GlpT and FosB.

INTRODUCTION

The epoxide-phosphonate Fosfomycin (FOS: L-cis-1,2-epoxypropyl phosphoric acid) was first isolated in 1969 from cultures of *Streptomyces* sp. species. It is produced by *Streptomyces* sp. and *Pseudomonas* sp. using distinct biosynthetic pathways [1, 2, 3, 4]. FOS was later shown to target the carboxyvinyltransferase MurA, a key enzyme in peptidoglycan (PGN) synthesis, by covalently binding to a cysteine residue in the active site [5].

MurA catalyses the first committed step of PGN synthesis: the carboxyvinyl transfer from phosphoenolpyruvate (PEP) to uridine diphosphate *N*-acetyl-glucosamine (UDP-GlcNAc) [1, 2, 3, 4, 5]. Inhibition by FOS is caused by covalent binding to a sulfhydryl group of an active site cysteine in MurA, which is originally the binding site for PEP, thereby permanently inactivating the enzyme [5, 6]. Gram-negative bacteria generally have only a single, essential MurA. Instead, most Gram-positive bacteria have two paralogs, both of which are targeted by FOS; in *Bacillus subtilis*, these paralogs are MurAA (=MurA) and MurAB [7]. The reason for this redundancy is not clear.

The transport of FOS in *B. subtilis* is dependent on the glycerol-3-phosphate permease GlpT. This transporter is not essential and its knockout results in a significant increase in FOS resistance. [8, 9, 10, 11]. Moreover *B. subtilis* has a FOS resistance protein, the thiol-S-transferase FosB, which degrades FOS with the aid of the redox regulator compound bacillithiol [11, 12]. The secondary cellular effects of FOS detoxification by FosB in *B. subtilis* have not been adequately investigated. In addition, the recently identified MurA adaptor protein ReoM has been shown to control the proteolytic degradation of MurA. Although ReoM is not directly involved in resistance, it is important for understanding the influence of FOS on growth [13]. ReoM is an activator of ClpCP-dependent proteolytic degradation of MurA. PrkA senses muropeptides and phosphorylates and thereby inactivates ReoM, which in turn prevents MurA binding and the proteolytic degradation of MurA. MurAB further activates to the proteolytic decay of MurAA. The effect of non-reversible inactivation of MurAA by FOS in this regulatory cascade is currently unknown. [13, 14]

Our study aimed to unravel the intricate and multi-layered influence of FOS on *B. subtilis* resistance, exploring the controllable transport via GlpT, the effective degradation facilitated by FosB, and the regulated degradation of MurAA and possibly MurAB via ReoM. Additionally, we sought insight into the unclear physiological role and unexplained relationship between MurAA and MurAB, as well as the potential ability of MurAB to complement MurAA, thus highlighting the complexity of FOS impact on *B. subtilis*.

Evaluating the efficacy of antimicrobial agents, like FOS, against specific pathogens poses significant challenges. However, the widely used method of determining the minimum inhibitory concentration (MIC) of an antimicrobial agent against relevant bacteria provides valuable insight into the efficacy of an antimicrobial agent. The phenotype-based method provides a fundamentally simple approach to efficacy against different bacteria. Combined with mutants, different growth conditions, media and different methods of MIC determination, the simple methodology provides a deep insight into the effect of antimicrobial agents.

The combination of meaningful results and effort makes MIC determinations a powerful tool. Depending on the antibiotic, certain methods for determining the MIC work differently. [15, 16, 17]

To get a general overview of the impact of FOS, we compared the MIC that kills *B. subtilis* wt (i.e. *Bacillus subtilis* subsp. *subtilis* str. 168 (trpC2)) and mutants of the *glpT*, *fosB*, *murAB* and *yrzL* (= *reoM*) genes. In the case of FOS, there are considerable differences depending on the MIC determination. If only the common method of MIC determination is considered, e.g. using test strips on agar plates, a completely different picture emerges from the monitoring of liquid cultures. Based on these different MIC analyses and the observation of the development of resistance (genetically manifested resistance is caused by rare point mutations in MurA (e.g. cysteine > aspartic acid), which make inactivation of FOS impossible [1, 5]), a detailed picture of the phenotypic response of *B. subtilis* to FOS was obtained by monitoring the growth of the different mutants. In addition, the intracellular and extracellular concentration of FOS was measured by LC-MS during treatment and subsequent regeneration of the cultures.

MATERIAL AND METHODS

Bacterial strains

Bacillus subtilis subsp. *subtilis* str. 168 (trpC2). (BGSC, 1A1, [18])

Bacillus subtilis subsp. *subtilis*, trpC2 $\Delta fosB::erm$. (BGSC, BKE17840, [18])

Bacillus subtilis subsp. *subtilis*, trpC2 $\Delta fosB$. (this work)

Bacillus subtilis subsp. *subtilis*, trpC2 $\Delta murAB::kan$. (BGSC, BKK37100, [18])

Bacillus subtilis subsp. *subtilis*, trpC2 $\Delta yrzL::kan$. (*yrzL*=*reoM*, BGSC, BKK27400, [18])

Bacillus subtilis subsp. *subtilis*, trpC2 $\Delta glpT::erm$. (BGSC, BKE02140, [18])

Generation of a marker less $\Delta fosB$ mutant

Bacillus subtilis subsp. *subtilis*, trpC2 $\Delta fosB::erm$ were made naturally competent for transformation by the following procedure and were used to transform plasmid pDR244 [18].

20 mL MGE medium was inoculated with 250 μ L o/n culture and incubated at 130 rpm at 37 °C. Cells were harvested (4 min, 4°C, 2500 g) 120 min after the transition to stationary phase, monitored by OD_{600nm}. The pellet was suspended in 1/10 of the supernatant volume spiked with 17 % glycerol (v/v) and stored at -80 °C. For transformation, 300 μ L of the regeneration medium (RM) was mixed with 700 ng DNA (pDR244) and incubated at 37 °C for 30 min. Subsequently, 100 μ L of expression medium (EM) was added. After 90 min at 37 °C, the cells were plated onto lysogeny broth (LB) plates containing 100 μ g mL⁻¹ spectinomycin for selection. Plates were incubated at 30 °C o/n, and the growing colonies were then heat-shifted to 42 °C o/n to remove the plasmid pDR244 again, which harbours a temperature-sensitive origin of replication and allows temperature-induced expression of Cre recombinase. Successful removal of the *erm*^R cassette was confirmed by sequencing.

Media:

10x MM: 440 mM KH_2PO_4 , 600 mM K_2HPO_4 , 29 mM sodium citrate, 150 mM $(\text{NH}_4)_2\text{SO}_4$

MGE: 2000 μL 10xMM, 800 μL glucose (50%), 144 μL L-glutamate (40%), 100 μL ammonium iron(II) citrate (2 mg mL^{-1}), 100 μL yeast extract (5 %), 200 μL tryptophan (5 mg mL^{-1}), 100 μL CAA (10%), 40 μL MgSO_4 (1 M), 40 μL CaCl_2 (0.5 M), filled up to 20 mL. RM: 170 μL 10xMM, 17 μL glucose (50%), 34 μL MgSO_4 (1 M), 1700 μL $\text{H}_2\text{O}_{\text{dd}}$.

RM: 170 μL 10xMM, 17 μL glucose (50 %), 34 μL MgSO_4 (1 M), 1700 μL $\text{H}_2\text{O}_{\text{dd}}$.

EM: 300 μL yeast extract (5 %), 150 μL CAA (10 %), 15 μL tryptophan (5 mg mL^{-1}), 150 μL $\text{H}_2\text{O}_{\text{dd}}$.

Determination of the minimum inhibitory concentration (MIC)

The MIC was determined both on agar plates (solid medium) and in liquid cultures. The investigations on different media were carried out using lysogeny broth (LB), Mueller-Hinton-Bouillon 2 (cation-adjusted) (MHII), modified M9 [19] and MC [20].

liquid medium

For the determination of MIC by broth microdilution according to CLSI [16, 17], 5.5×10^5 CFU mL^{-1} were incubated in a 96 well plate with a serial dilution of FOS (0.5-1024 $\mu\text{g mL}^{-1}$). The effect of FOS on the cells was also determined by measuring $\text{OD}_{600\text{nm}}$ over 24 h. The following values were measured. For this purpose, FOS (1-8192 $\mu\text{g mL}^{-1}$, depending on the strain used) was added during the exponential growth phase. The experiment was performed in 24 well plates covered with a semi-permeable sealing membrane (Breathe-Easy, Z380059, Sigma-Aldrich) in a plate reader (SPARK multimode plate reader, Tecan or EPOCH2, BioTek Absorbance Microplate Reader, Agilent Technologies). All incubations were performed overnight at 37 °C with shaking.

solid medium

The MIC determination was performed according to CLSI standard [16, 17] with LB, MHII, modified M9 [19] and MC [20] medium.

O/n single colonies were adjusted to MC-Farland 0.5 according to the manufacturer's instructions (MIC Test Strip Technical Sheet-MTS45-Rev.1/09.11.2017), spread out and provided with FOS MIC test strips (Liofilchem MIC Test Strip Fosfomycin (includes glucose-6-phosphate) 0.064-1024 $\mu\text{g mL}^{-1}$, 92079). Alternatively, 6 mm DISCS containing 10 μL FOS (250-500 μg) were added to the identically prepared plates. Incubation was carried out overnight at 37 °C.

LC-MS monitoring of intra- and extracellular fosfomycin concentrations

Cultures were inoculated to an $\text{OD}_{600\text{nm}}$ of 0.05 in a total volume of 30 mL. The 200 mL flasks were incubated at 37 °C and 120 rpm until reaching the exponential growth phase (0.5-0.8 $\text{OD}_{600\text{nm}}$) and treated with FOS at a concentration of 128 $\mu\text{g mL}^{-1}$. Samples for measurement of extracellular FOS concentration were prepared by transferring 1 mL of culture supernatant to a new reaction tube and centrifuging (12044 g, 5 min, RT).

The supernatant was filtered through a 0.22 μm sterile filter (ROTILABO PVDF, Carl Roth) and stored at $-80\text{ }^{\circ}\text{C}$. All samples for measurement of intracellular FOS concentration were adjusted to a sample size of 20 mL at an $\text{OD}_{600\text{nm}}$ of 0.8. If the reference amount could not be achieved, 25 mL with the appropriate $\text{OD} < 0.8 \text{ OD}_{600\text{nm}}$ was taken and included in the analysis. The corresponding amount was cooled with liquid nitrogen (three immersions for 3-5 s each time, without freezing). The sample then has a temperature of approximately $5\text{ }^{\circ}\text{C}$. The cooled sample was passed in less than 5 s through a cooled filter system (XX1014700, Millipore Classic Glass Filter Holder-Kit 47 mm, glass frit membrane holder, 300 mL funnel, S-Pak filter, $0.45\text{ }\mu\text{m}$, 47 mm, all chilled at $4\text{ }^{\circ}\text{C}$ for at least 30 min). The chilled sample was then passed through the filter. The filters were washed with 5 mL of chilled sterile isotonic saline. They were then placed directly into 5 mL of 60 % ethanol (50 mL Falcon), frozen in liquid nitrogen, and stored at -80°C at least overnight. Cells were disrupted by thawing (room temperature), shaking, and vortexing, 10-15 times each. After centrifugation at $4\text{ }^{\circ}\text{C}$ for 5 min at 10000 g, 4 mL of the supernatant was removed, and the extraction was repeated with 2 mL of chilled water. This yielded a 6 mL sample which was dried in a SpeedVac system at $30\text{ }^{\circ}\text{C}$ and then dissolved in $\text{H}_2\text{O}_{\text{dd}}$.

Liquid chromatography-mass spectrometry (LC-MS) analysis was conducted using an ESI-TOF mass spectrometer (MicroTOF II; Bruker Daltonics) operated in negative ion mode coupled to an UltiMate 3000 high-performance liquid chromatography (HPLC) system (Dionex, Thermo Fisher Scientific). Separation in the HPLC was obtained using a SeQuant ZIC-pHILIC column (PEEK $150 \times 2.1\text{ mm}$, $5\text{ }\mu\text{m}$, $110\text{ }\text{\AA}$, Merck) at $30\text{ }^{\circ}\text{C}$ with a buffer system of CH_3CN and 100 mM $(\text{NH}_4)_2\text{CO}_3$, pH 9. A single run (injection volume of $5\text{ }\mu\text{L}$) was performed with a flow rate of 0.2 mL min^{-1} and a linear gradient of 25 min, reducing the concentration of CH_3CN from 82 % to 42 %. The column was equilibrated with 82 % CH_3CN for 5 min before and 10 min after the gradient. The evaluation was performed using extracted ion chromatograms (accuracy 0.02 Da), the AUC (baseline 1000 cps) was determined using the OpenMS based tool for metabolomics data analysis UmetaFlow [21] with its graphical user interface (<https://github.com/axelwalter/umetaflow-gui>). The AUCs obtained from UmetaFlow were presented in Prism 8 (GraphPad). The AUC was determined using the $[\text{M}-\text{H}]^-$ mass of FOS: 137.0009 m z^{-1} (FOS: $\text{C}_3\text{H}_7\text{O}_4\text{P}$, neutral: 138.0082 m z^{-1}) in the retention time range between 10 and 16 min.

RESULTS AND DISCUSSION

FOS susceptibility of *B. subtilis* mutants on agar plates

The most convenient method of assessing the susceptibility of bacteria to antibiotics is to use MIC test strips. The advantage of this method is that a predefined, stable concentration range of an antibiotic is provided on a strip and the MIC can be determined directly from a plate (Fig.1, A1). Using FOS test strips, the MIC of *B. subtilis* 168 wt was determined to be $18.7\text{ }\mu\text{g mL}^{-1}$ (Fig.1, Table1, A1). In the absence of the known FOS resistance and detoxification protein FosB (*B. subtilis* ΔfosB (ΔfosB)), colonies became 10-fold more sensitive ($1.8\text{ }\mu\text{g mL}^{-1}$). Deletion of the glycerol-3P transporter GlpT (*B. subtilis* $\Delta\text{glpT}::\text{erm}$ (ΔglpT)) resulted in a 10-fold increase to $117.3\text{ }\mu\text{g mL}^{-1}$ compared to the wt MIC. Deletion of *B. subtilis* MurAA is lethal, however deletion of the paralogous *murAB* gene (*B. subtilis* $\Delta\text{murAB}::\text{kan}$ (ΔmurAB)) grows normally and this mutation did not have a strong effect on the MIC, as only a slight increase in resistance to $24.0\text{ }\mu\text{g mL}^{-1}$ was seen.

The absence of the MurA adaptor protein ReoM (*B. subtilis* Δ yrzL::kan. (yrzL=reoM), (Δ reoM)) showed a two-fold increase in MIC ($42.7 \mu\text{g mL}^{-1}$). If the cell is deficient in the adaptor protein ReoM, the induction of ClpC-ClpP dependent proteolytic degradation of MurAA is no longer possible [13]. This results in a higher level of MurAA in the cell, which is likely to be the reason for the increased FOS resistance of Δ reoM compared to the wt. A more detailed look at the plates (Fig.1, A1) showed that in addition to the clear growth-free zone, a second zone was visible in all strains (although less so in Δ fosB), which will be termed “halo” from now on. The halo represents an area of intermediate growth. It is possible that cells in this area are exposed to concentrations of FOS around the MIC, resulting in a bacteriostatic behaviour that is also seen in liquid cultures exposed to concentrations of FOS in the MIC range (see below). However, the MIC changes of the mutants relative to wt do not change when the halo areas are taken into account.

The Δ fosB mutant behaved differently because the non-growing zone can be divided into two areas. When the plates were incubated for two or more days instead of only one, the colonies in the non-growing intermediate zone regrown rapidly, while the inner zone remained without growth, the halo became denser after a few days.

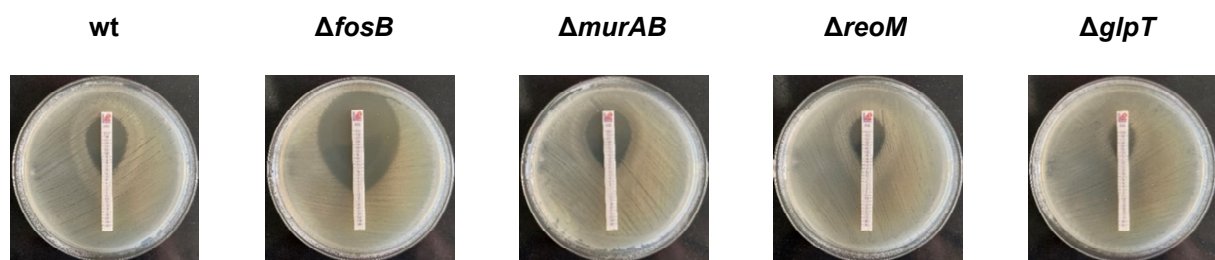


Figure 2: Visualisation of the MIC determination of *B. subtilis* mutants against FOS on LB agar plates. Pictures of the MIC test (LB) analyses. MIC test strips (Liofilchem MIC test strip fosfomycin (includes $50 \mu\text{g mL}^{-1}$ glucose-6-phosphate) with $0.064\text{--}1024 \mu\text{g mL}^{-1}$ FOS) were used. The following mutants were tested for MIC: *B. subtilis* 168 (trpC2) = wt, *B. subtilis* (trpC2) Δ fosB = Δ fosB, *B. subtilis* (trpC2) Δ murAB::kan = Δ murAB, *B. subtilis* (trpC2) Δ yrzL::kan (yrzL=reoM) = Δ reoM, *B. subtilis* (trpC2) Δ glpT::erm = Δ glpT. The cultures were spread according to the CLSI regulations, adjusted to MC-Farland 0.5 [16, 17]. A differentiation is made between the non-growing area (=MIC) and the growing area, which is different from the rest of the colonies (=MIC HALO). All replicates can be found in the appendix (A1, the calculations are presented in table 1).

A surprisingly different result was obtained when the MIC tests were carried out on Mueller Hinton (MHII) agar plates. In the gluconeogenic MHII, the overall resistance to FOS was higher (A2). The wt showed two halo zones at ≈ 128 and $6 \mu\text{g mL}^{-1}$ FOS, whereas the Δ fosB mutant showed no halo and a MIC of $\approx 12 \mu\text{g mL}^{-1}$. The reasons for this effect and the difference to LB are unclear. The use of minimal media with *B. subtilis* was experimentally difficult. Both modified M9 (carbon source was glucose) and MC medium (minimal C: the carbon source was glucose) were tested (results not shown) [19, 20]. Due to the slow growth (2 days), diffusion and therefore FOS concentration differences occur. Therefore, the slow growing colonies appeared to be less affected by FOS. For this reason, no further studies were carried out with minimal media. Overall, and in all preliminary experiments, the use of LB gave the most detailed and differentiated picture of the mutants, and LB is also the most relevant medium for *B. subtilis*. A more meaningful comparison of the different media is shown below with liquid culture media.

Table 2: Summary of the MIC determination of *B. subtilis* mutants against FOS on LB agar plates.

	MIC $\mu\text{g mL}^{-1}$ FOS			mean	std. dev.
	1	2	3		
wt	16	24	16	18.7	3.8
ΔfosB	2.0	1.5	2.0	1.8	0.2
ΔmurAB	24	24	24	24.0	0.0
ΔreoM	48	48	32	42.7	7.5
ΔglpT	128	96	128	117.3	15.1

	MIC HALO $\mu\text{g mL}^{-1}$ FOS			mean	std. dev.
	1	2	3		
wt (out)	1	1.5	1.5	1.3	0.2
ΔfosB (in, no growth)	16	16	16	16	0.0
ΔmurAB (out)	3	4	4	3.7	0.5
ΔreoM (out)	6	6	4	5.3	0.9
ΔglpT (out)	16	16	32	21.3	7.5

MIC test strips (Liofilchem MIC Test Strip Fosfomycin (includes $50 \mu\text{g mL}^{-1}$ glucose-6-phosphate) with 0.064-1024 $\mu\text{g mL}^{-1}$ FOS were used. The following mutants were tested for MIC: *B. subtilis* 168 (*trpC2*) = wt, *B. subtilis* (*trpC2*) ΔfosB = ΔfosB , *B. subtilis* (*trpC2*) $\Delta\text{murAB}::\text{kan}$ = ΔmurAB , *B. subtilis* (*trpC2*) $\Delta\text{yrzL}::\text{kan}$ (*yrzL*=*reoM*) = ΔreoM , *B. subtilis* (*trpC2*) $\Delta\text{glpT}::\text{erm}$ = ΔglpT . The cultures were spread according to the CLSI regulations, adjusted to MC-Farland 0.5 [16, 17]. A differentiation is made between the non-growing area (=MIC) and the growing area, which is different from the rest of the colonies (=MIC HALO). Pictures of all replicates can be found in the appendix (A1).

MIC determination by LB broth microdilution (BM) (carried out for wt and ΔfosB according to CLSI [16], results not shown) gave a similar picture, although the difference is much smaller with this method in liquid medium. The MIC of FOS determined by broth microdilution was $\approx 32 \mu\text{g mL}^{-1}$ for wt and $\approx 8 \mu\text{g mL}^{-1}$ for ΔfosB . This resulted in a MIC fold change of 4, which is significantly lower than the 10-fold difference with MIC test strips. This comparison is unavoidable as BM and antibiotic test strips are the main methods for MIC determination. It is difficult to compare MIC determinations for FOS due to of other effects in liquid cultures (see below).

FOS susceptibility and growth behaviour of *B. subtilis* in liquid media

To study the influence of FOS on the growth of *B. subtilis*, it is advantageous to have a continuous observation rather than an end-point measurement as in MIC determinations using MIC test strips or broth microdilution. A simple but very insightful method is the continuous measurement of the optical density ($\text{OD}_{600\text{nm}}$), which also allows the determination of the MIC. Many factors relevant to understanding the effect of FOS on *B. subtilis* have been discovered in this way and are described below. Media differences are also important in liquid cultures, similar to what has been shown and described above for agar plate cultures. The observation of liquid cultures in different media again illustrates the problem of minimal media use. In the appendix (A3) a comparison of growth of wt in four different media, two rich and two minimal media, was made. As for the solid media studies (see above), LB and MHII were used as rich media and MC and modified M9 as minimal media (carbon source in both minimal media was glucose).

LB and MHII showed no significant differences. In the minimal media, the initial lag phase was markedly longer, and the maximum cell density of the cultures was considerably lower than in the rich media. After the lag phase, cultures in minimal media continued to show atypical growth patterns. In modified M9 medium, cultures died after about 8 h and then reached only half the cell density of rich medium cultures. Cultures in MC medium died at approximately 12 h in the stationary phase compared to the rich media cultures. A method development for treatment with FOS was therefore also rejected for liquid minimal media. As LB and MHII showed no differences, LB was used for all further experiments as described. The experiments with the different media were also carried out with the $\Delta fosB$ mutant and the results were identical (results not shown). An effect of strain can therefore be excluded. All four mutants and the wt were treated with different amounts of FOS. The time of FOS addition was in the middle of the exponential growth phase after 3.5 h. As shown in Fig.2, all strains behave very similarly when not treated with FOS (FOS0). Very little difference in growth behaviour relative to wt was observed with the $\Delta murAB$ mutant, but the culture clearly begins to lyse earlier in the stationary phase, possibly because the lack of MurAB activity becomes crucial in stationary phase [14]. In all experiments with different concentrations of FOS, $\Delta fosB$ showed an increase in OD_{600nm} in stationary phase, whereas the OD_{600nm} of $\Delta glpT$ decreased more rapidly towards the end of the stationary growth phase. Extreme differences in the growth behaviour of *B. subtilis* wt and the mutants can be observed under FOS treatment. While $\Delta fosB$ showed a clear lytic effect at 4 $\mu\text{g mL}^{-1}$ FOS, $\Delta glpT$ was hardly affected by 1024 $\mu\text{g mL}^{-1}$ FOS (Fig.2, A4). This result already indicates the high level of GlpT induction during FOS treatment in *B. subtilis* (see below). As the mutant strains showed very different FOS sensitivities, it was already clear that not all mutants could be compared at identical FOS concentrations. Therefore, a selection of representative concentrations is shown in Fig.1 to illustrate the differences between the mutants. $\Delta fosB$ (FOS 1024) and $\Delta glpT$ (FOS 4) were not included in all cases because the effect was already maximum or minimum at the corresponding lower or higher concentrations. An overview of all relevant concentrations for each mutant is shown in A4. When cultures were treated with FOS 4, only $\Delta fosB$ showed a lytic effect. $\Delta glpT$ was not affected at all and $\Delta reoM$ also showed no effect. Wt and $\Delta murAB$ behave very similarly, both showed no lytic effect but did not reach the identically high OD_{600nm} in the stationary phase compared to the untreated cultures. $\Delta reoM$ and $\Delta glpT$ were also unaffected by the 32-fold amount (FOS 128) and showed no lytic effect, only a slightly lower OD_{600nm} in the stationary phase (A4). $\Delta murAB$ and the wt showed a lytic effect at this concentration. Interestingly, $\Delta fosB$ and $\Delta murAB$ (also at other concentrations, A4) seemed to behave differently from wt with respect to regrowth after the lytic effect. $\Delta murAB$ regenerated significantly faster than the wt at all concentrations (up to 2048, A4), comparable to the ratio of $\Delta fosB$ to wt at lower concentrations. The effect of regrowth mentioned above is crucial. The difference in OD_{600nm} after 24 h was often very small, but the cultures had been lytic for the previous 20 h and then adapted to FOS conditions and regrown. The basis for this regrowth behaviour is not a genetic mutation that has manifested resistance, but an adaptation to FOS through conversion and adaptation of metabolism (see below). When the concentration was further increased (FOS 1024), $\Delta reoM$ also showed a lytic effect. However, this phase did not lead to a decrease in OD_{600nm} comparable to the wt, but only to a decrease in OD_{600nm} comparable to the middle of the exponential phase.

As mentioned above, the lack of proteolytic degradation of MurAA can lead to increased resistance which, in combination with the MurAB present, can cause the culture to grow much faster, even in stationary phase [7, 13]. The significantly earlier regeneration of OD_{600nm} by MurAB cannot be explained trivially. MurAA can compensate for the absence of MurAB (as it is non-essential) [14], but activation of MurAA expression is required by a still unclear regulatory mechanism, which may involve MurAB controlling ReoM-induced MurAA degradation as in *Listeria monocytogenes* [22]. This seems to result in an easier and faster adaptation to FOS. The wt apparently had problems adapting to FOS (at FOS 512 and higher, A4) and started to regrow about 20 h after treatment, whereas $\Delta fosB$ and $\Delta murAB$ showed regrowth already after 12 h, considering the reasonable concentrations that give an identical course in relation to the initial lytic effect. $\Delta glpT$ was much more resistant and showed an initial effect at FOS 2048, but this was not apparent until FOS 4096 (A4). However, even at these high FOS concentrations, no initial lysis event was visible, but lysis was initiated earlier in the stationary phase. From an experimental and cell physiological point of view, it does not make sense to use concentrations higher than 8192 $\mu\text{g mL}^{-1}$, as this did not show the intended initial lytic effect for $\Delta glpT$, limiting the comparison with the other mutants. FOS is known to be actively transported via nutrient transport systems. These are the glycerol-3-phosphate permease (GlpT) and the glucose-6-phosphate ABC transporter (UhpT) [5]. UhpT is not present in *B. subtilis*. The effect of significantly increasing resistance by deleting the transporter has been shown in other organisms [5, 8, 9, 11]. These experiments showed that FOS is transported in a very specific way and does not enter the cell by chance or in very small amounts. The influence of GlpT on FOS sensitivity is therefore considerable. Interestingly, although the cultures showed a high tolerance to FOS (up to 4 $\mu\text{g mL}^{-1}$, except $\Delta fosB$ (up to 4 $\mu\text{g mL}^{-1}$), without growth deficit, A4) in the exponential phase, they took up only a small amount of FOS in the lag phase. In such experiments where the FOS treatment starts with the inoculation of the culture, only concentrations of 0.5 $\mu\text{g mL}^{-1}$ for wt and 0.1 $\mu\text{g mL}^{-1}$ for $\Delta fosB$ can be used (results of previous experiments not shown), higher concentrations result in an extremely prolonged lag phase. For further experiments and also for the determination of FOS concentrations in the context of this work, FOS concentrations were chosen to be able to observe two different effects. The first effect was an adaptation to FOS in which the treated cultures (start of FOS treatment with inoculation) showed no growth deficiency compared to the untreated cultures. The chosen FOS concentration was 0.5 $\mu\text{g mL}^{-1}$ for wt, $\Delta murAB$ and $\Delta reoM$ and 0.1 $\mu\text{g mL}^{-1}$ for $\Delta fosB$. The second effect was a FOS stress response with a clear lytic effect on the cultures (start of FOS treatment in the exponential phase, as it was not possible to add the required high concentrations of FOS from the start as described above). The FOS concentration chosen was 128 $\mu\text{g mL}^{-1}$ for the wt, $\Delta murAB$ and $\Delta reoM$ and 16 $\mu\text{g mL}^{-1}$ for $\Delta fosB$. Both effects were reproduced in 30mL cultures. GlpT was not scaled up due to the non-physiological concentrations and costly amounts of FOS.

Development of resistance against FOS in *B. subtilis*

In the literature, the development of resistance is mainly associated with dysfunctional import caused by mutations affecting transporter expression. Less commonly, a point mutation in MurA leads to complete resistance to FOS. In addition, resistance is caused by degradation of FOS in Firmicutes, mainly by the thiol S-transferase FosB, which produces the GlcNAc-containing thiol compound bacillithiol. [1, 8, 9, 10].

In none of the many FOS treatment experiments carried out in the laboratory has a genetically manifested resistance to FOS been observed in *B. subtilis* wt. An example for liquid medium and an example for solid medium are shown in A5, A6 and table A1. The culture treated with FOS in the liquid medium was again subjected to an identical treatment.

No change or increase in FOS tolerance was observed for $\Delta glpT$ and wt. Identical results were obtained when colonies growing in the non-growing zone of the DISC diffusion assay (1-2 days) were grown overnight and the identical DISC diffusion assay was performed with the potentially resistant colonies (RI-RIII). Unaltered inhibition zones are evident. Differences in FOS tolerance are found in both experiments with $\Delta fosB$. In liquid cultures, the $\Delta fosB$ mutant seemed to adapt to the significantly lower FOS concentrations of $16 \mu\text{g mL}^{-1}$ compared to the wt ($128 \mu\text{g mL}^{-1}$) (A5). On agar plates (A6), the inner growth-free area did not change. The outer HALO area became smaller in some cases, making the colonies thus more resistant to FOS (TableA1). Considering that $\Delta fosB$ has not yet reached a wt tolerance level (lysis: 1.5 cm and HALO lysis: 3.0 cm diameter, for wt $250 \mu\text{g}$ FOS (plate not shown)), despite the halving of the DISC concentration, the change suggests an adaptation rather than a genetic change. The same is true for the liquid cultures where $\Delta fosB$ did not reach the wt tolerance level. Because of the markerless deletion, it was not possible to reverse the $\Delta fosB$ activity. If this had been the case, the mutant would have had the same FOS tolerance as the wt. A transporter deletion could also be excluded as the effect of the transporter was higher (A1, A4).

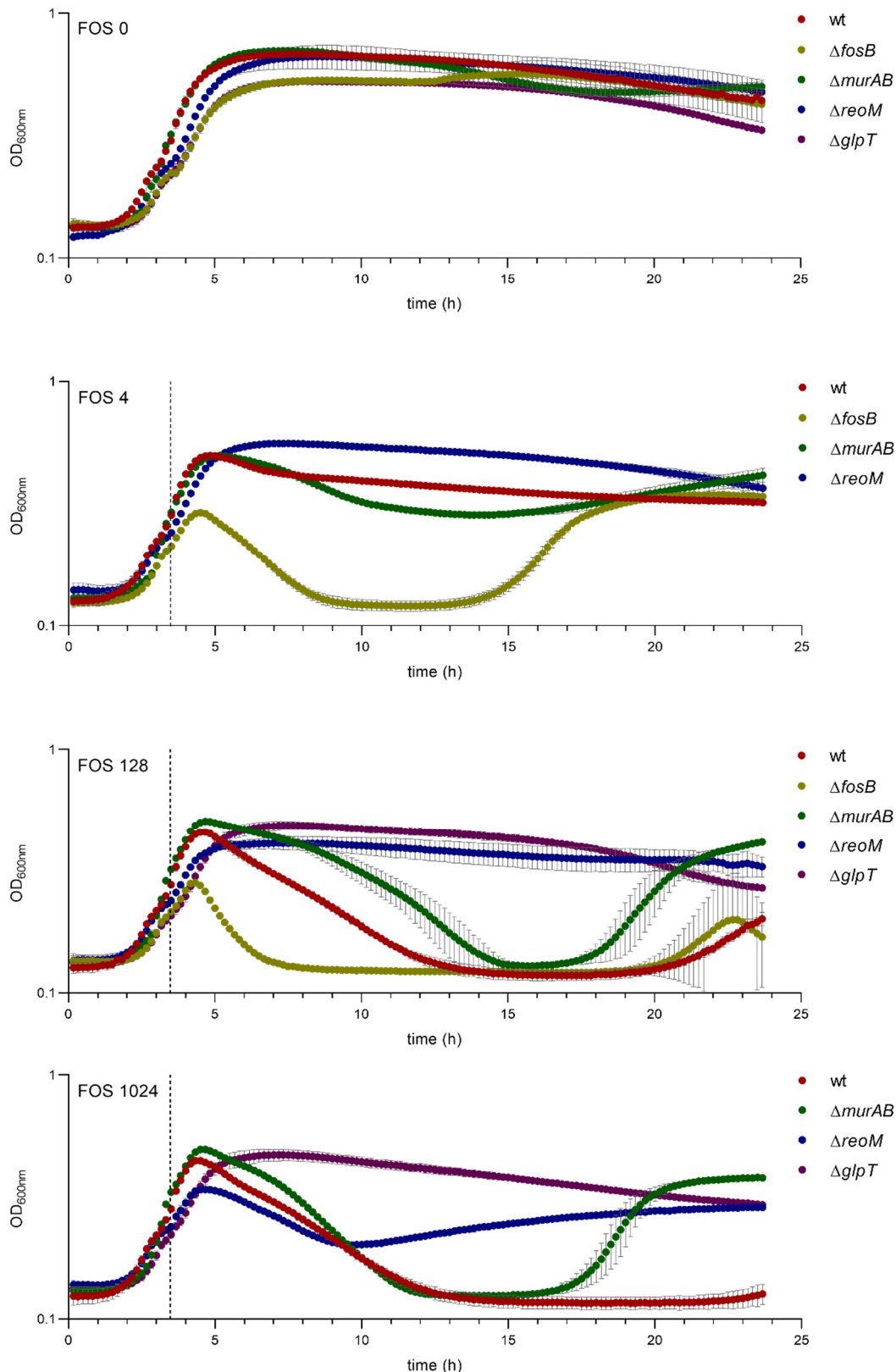


Figure 2: Monitoring of growth behaviour and FOS sensitivity of *B. subtilis*. LB growth curves of the strains: *B. subtilis* 168 (*trpC2*) = wt, *B. subtilis* (*trpC2*) $\Delta fosB$ = $\Delta fosB$, *B. subtilis* (*trpC2*) $\Delta murAB::kan$ = $\Delta murAB$, *B. subtilis* (*trpC2*) $\Delta yrzL::kan$ (*yrzL*=*reoM*) = $\Delta reoM$, *B. subtilis* (*trpC2*) $\Delta glpT::erm$ = $\Delta glpT$. FOS treatment with concentrations from 0-1024 $\mu\text{g mL}^{-1}$ after 3.5 h of incubation. Experiments were conducted in triplicate or duplicate in 24-well plates covered with a semi-permeable sealing membrane (Breathe-Easy, Z380059, Sigma-Aldrich) shaken at 37 °C using the EPOCH2, BioTek Absorbance Microplate Reader, Agilent Technologies, by measuring OD_{600nm} every 10 min.

Determination of intracellular and extracellular FOS concentration in treated *B. subtilis* cultures

To substantiate the statements and observations not only based on OD_{600nm} measurements, the FOS concentration in the culture medium and in the cells was determined by LC-MS. The experiment with *ΔglpT* was not performed because of the non-physiological FOS concentrations mentioned and the absolute overabundance of FOS before any effect was visible. The FOS concentrations used were 128 $\mu\text{g mL}^{-1}$ for wt, *ΔmurAB*, and *ΔreoM*, *ΔfosB* was treated at 16 $\mu\text{g mL}^{-1}$. The behaviour of the cultures in a larger volume (30 mL) and in 100 mL flasks was identical to the experiments performed in the 24-well plates (Fig.2). Figure A7 shows that wt, *ΔmurAB* and *ΔfosB* showed a lytic effect, while *ΔreoM* was hardly affected by FOS at the concentrations used.

In Fig.3, all FOS concentrations are shown intracellularly and extracellularly. In the wt, there was little decrease in extracellular concentrations. The area under the curve of FOS corresponds to the level of a FOS standard of 128 $\mu\text{g mL}^{-1}$ in LB. The extracellular concentration was therefore in a considerable excess and was not considerably reduced by cell uptake. Cell uptake was not only ensured by the visible lytic effect, but also by the fact that FOS could be measured intracellularly. Immediately after the addition of FOS, the intracellular concentration increased abruptly, decreased just as quickly and stabilised at a clearly measurable level.

The intracellular concentration is at a level between 10-20 $\mu\text{g mL}^{-1}$ (approximation based on a 2-point calibration). As the extracellular levels of FOS remained unchanged, the cells had to adjust their metabolism and adapt to the FOS, otherwise the cultures would not be able to grow again. The *ΔfosB* mutant showed an identical picture at lower FOS concentrations. As a result, there was a change in the extracellular environment. About a tenth of the amount could be measured in the interior, which was in a similar ratio to the concentration used.

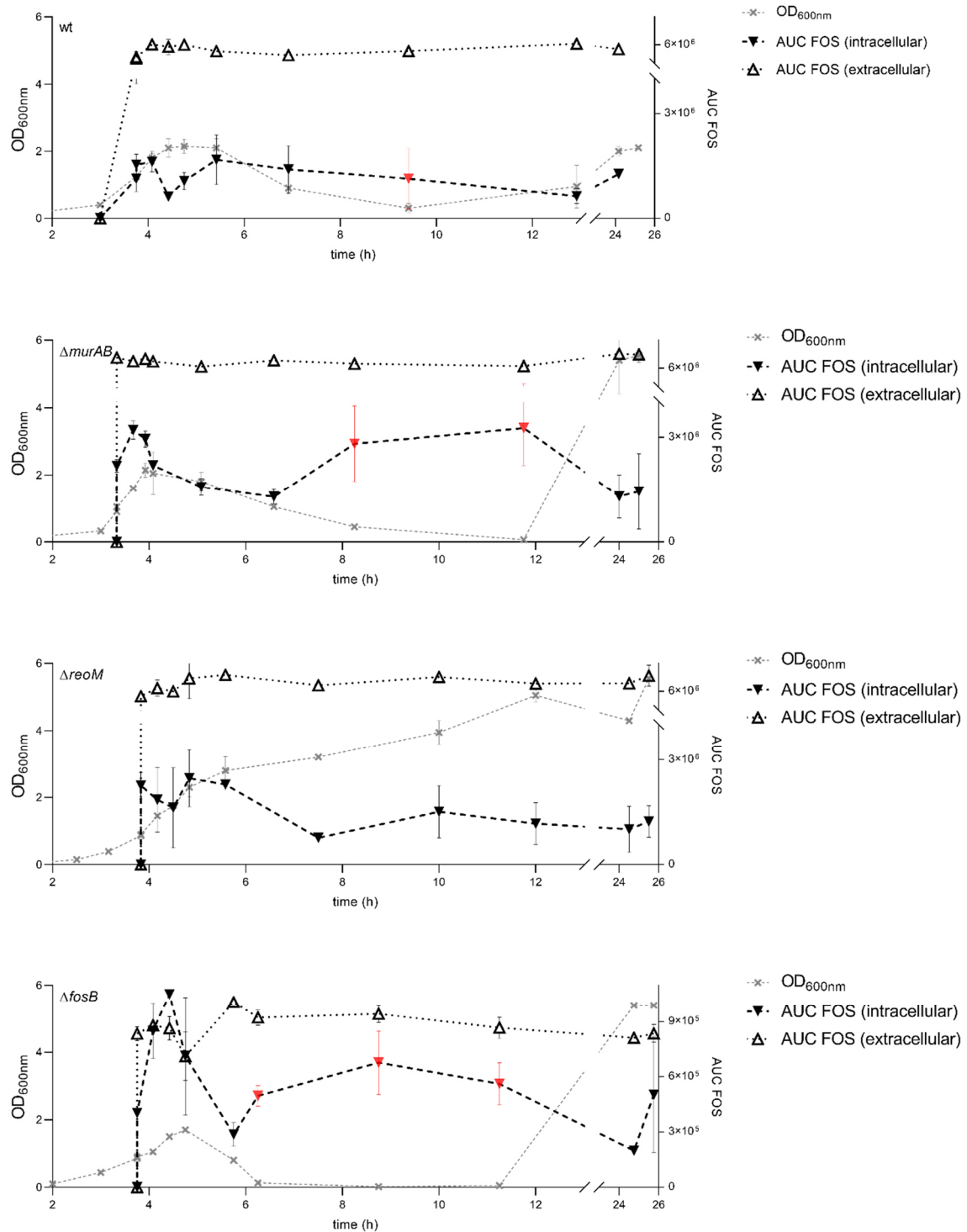


Figure 3: Determination of intracellular and extracellular FOS concentration in treated *B. subtilis*. Experiments were conducted with the following strains: *B. subtilis* 168 (*trpC2*) = wt, *B. subtilis* (*trpC2*) Δ *fosB* = Δ *fosB*, *B. subtilis* (*trpC2*) Δ *murAB::kan* = Δ *murAB*, *B. subtilis* (*trpC2*) Δ *yrzL::kan* (*yrzL*=*reoM*) = Δ *reoM*. Cultures were treated with FOS at concentrations of 16 μ g mL⁻¹ (Δ *fosB*) or 128 μ g mL⁻¹ (wt, Δ *murAB*, Δ *reoM*) after 3-4 h of incubation, as specified by the first measurement of the extracellular concentration of FOS (=0) immediately before the FOS treatment. Experiments were performed in duplicate in 100 mL flasks shaken at 37 °C. For samples marked in red, the reference volume of 20 mL OD_{600nm} 0.8 could not be taken, because the OD_{600nm} was lower, here 25 mL was taken (see method section).

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AUTHOR CONTRIBUTIONS

SF developed, performed, evaluated the experiments visualised the results and wrote the MS. YS performed part of the FOS concentration determination experiments. CM provided scientific assistance and reviewed the MS.

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APPENDIX

A1. Table 1: LB MIC tests on agar plates.

A2. MHII MIC tests on agar plates.

A3. *B. subtilis* growth curves in different media

Table A1: Summary of the resistance determination of *B. subtilis* mutants against FOS on LB agar plates.

A4. Monitoring of growth behaviour and FOS sensitivity of *B. subtilis*.

A5. Monitoring the growth behaviour of *B. subtilis* to study the development of resistance to FOS

A6. Resistance determination of *B. subtilis* mutants against FOS on LB agar plates

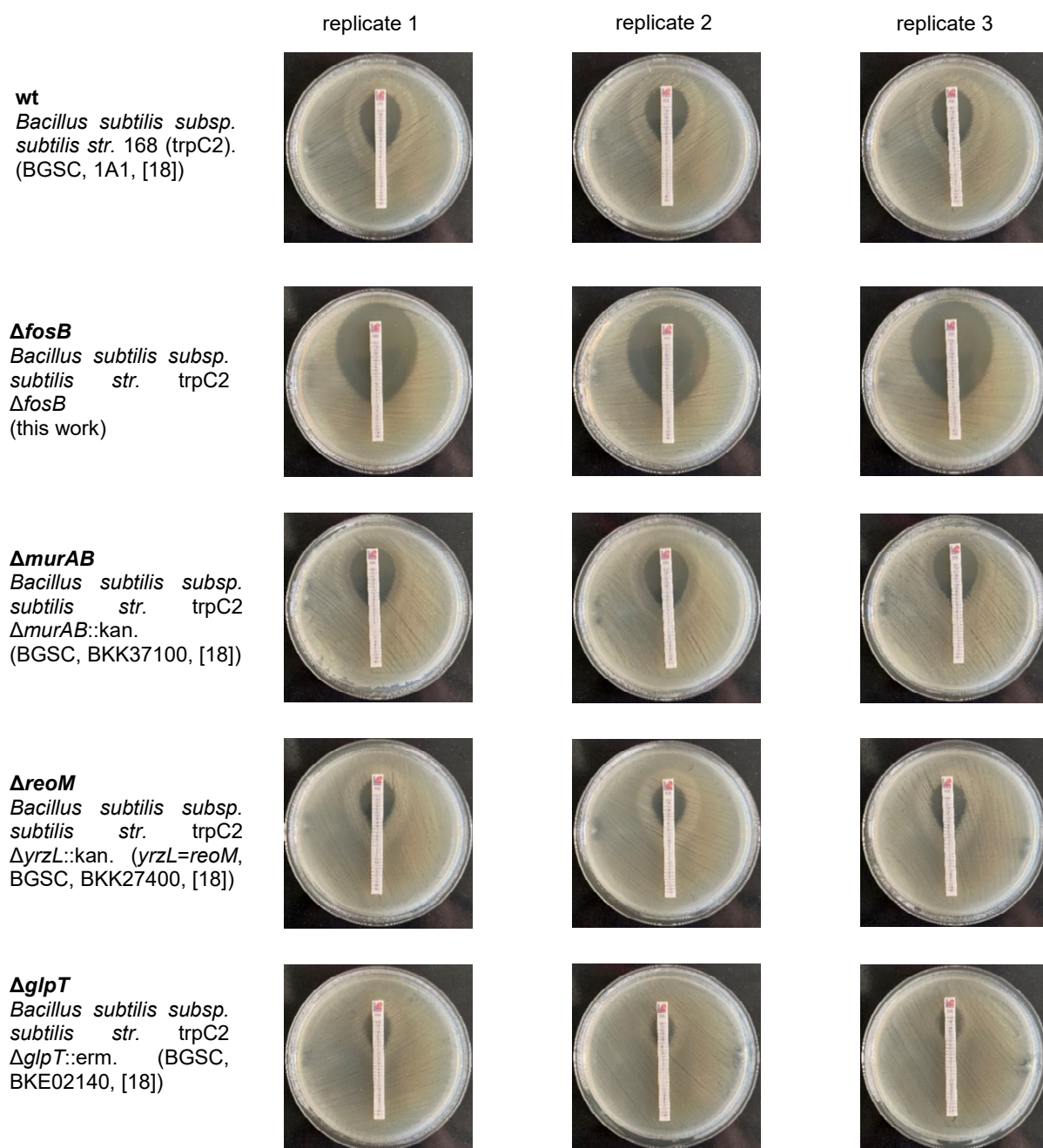
A7. Culture flask (30 mL) growth curves to determine intracellular and extracellular FOS concentration.

APPENDIX:

Investigating the inhibition of *Bacillus subtilis* growth by fosfomycin: The role of the targets MurAA and MurAB, drug transport, intracellular accumulation and tolerance development.

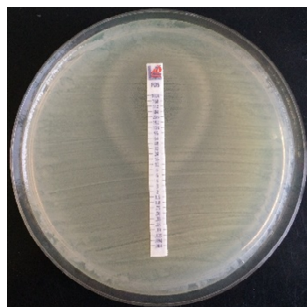
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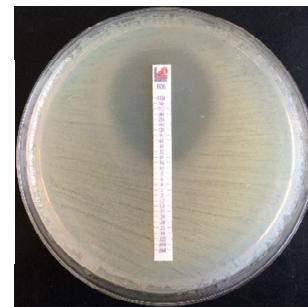


A1. Table 1: LB MIC tests on agar plates. Pictures of the MIC test (LB) analyses. MIC test strips (Liofilchem MIC test strip fosfomycin (includes $50 \mu\text{g mL}^{-1}$ glucose-6-phosphate) with $0.064\text{-}1024 \mu\text{g mL}^{-1}$ FOS were used. The cultures were spread according to the CLSI regulations, adjusted to MC-Farland 0.5 [16, 17].

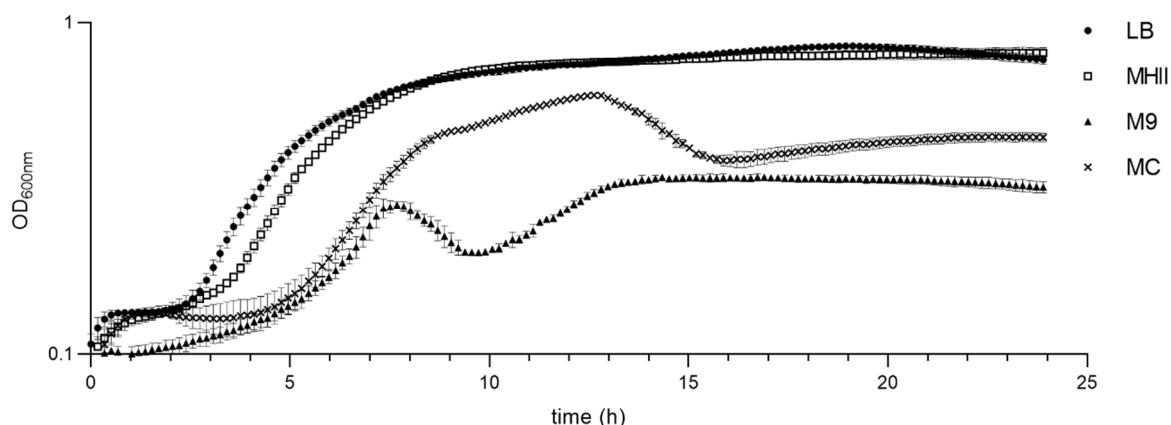
wt
Bacillus subtilis
 subsp. *subtilis* str.
 168 (trpC2). (BGSC,
 1A1, [18])



$\Delta fosB$
Bacillus subtilis
 subsp. *subtilis* str.
 trpC2
 $\Delta fosB$
 (this work)



A2. MHII MIC tests on agar plates. Pictures of the MIC test (MHII) analyses. MIC test strips (Liofilchem MIC test strip fosfomycin (includes 50 $\mu\text{g mL}^{-1}$ glucose-6-phosphate) with 0.064-1024 $\mu\text{g mL}^{-1}$ FOS were used. The cultures were spread according to the CLSI regulations, adjusted to MC-Farland 0.5 [16, 17].

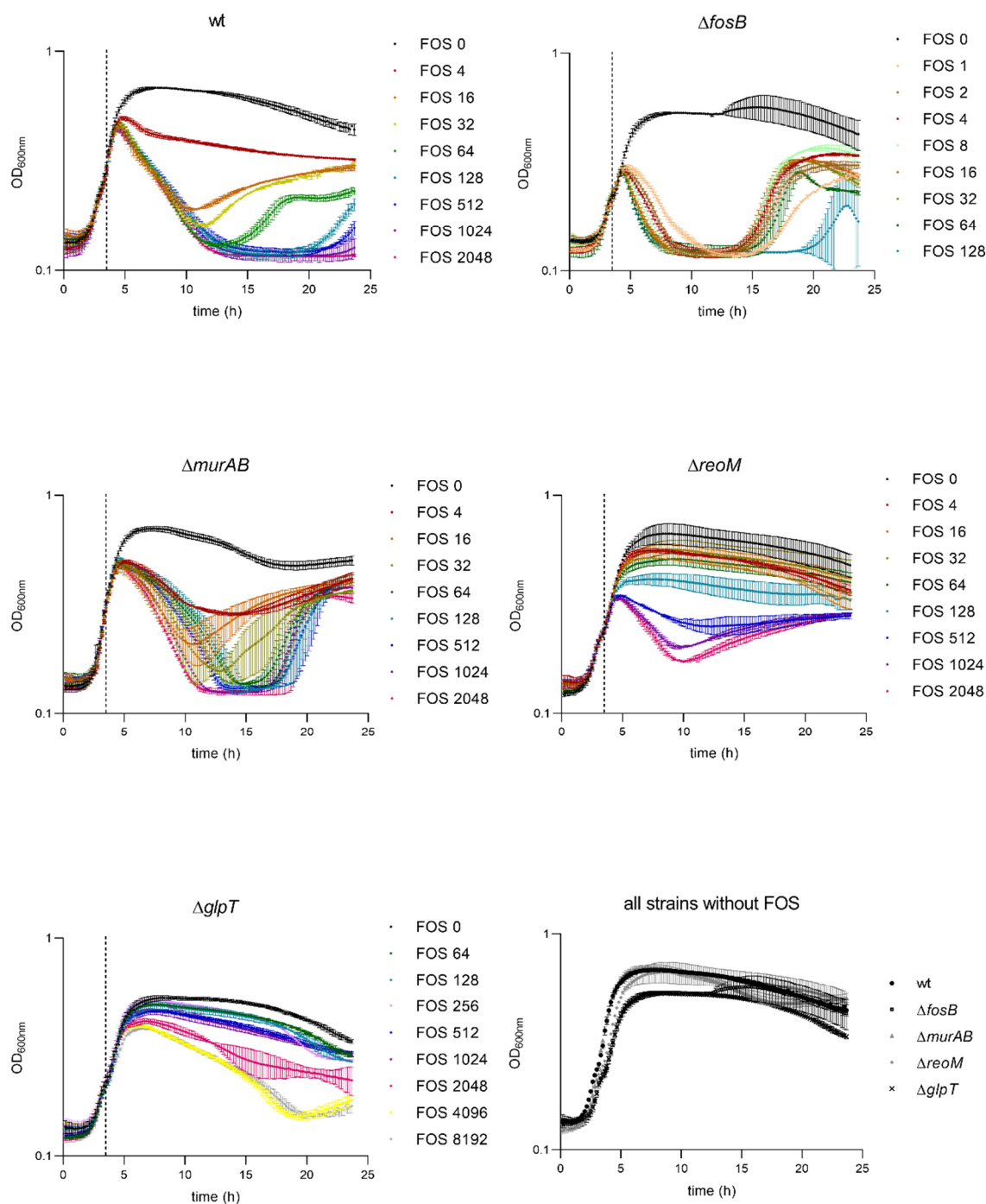


A3. *B. subtilis* growth curves in different media. Growth curves with: *B. subtilis* 168 (trpC2) = wt, with different media: lysogeny broth (LB), Mueller-Hinton-Bouillon 2 (cation-adjusted) (MHII), modified M9 [19] and MC [20] were used. Performed in quadruplicates in 24-well plates with cover shaking at 37 °C using the SPARK multimode plate reader, Tecan, by measuring OD_{600nm} every 10 min.

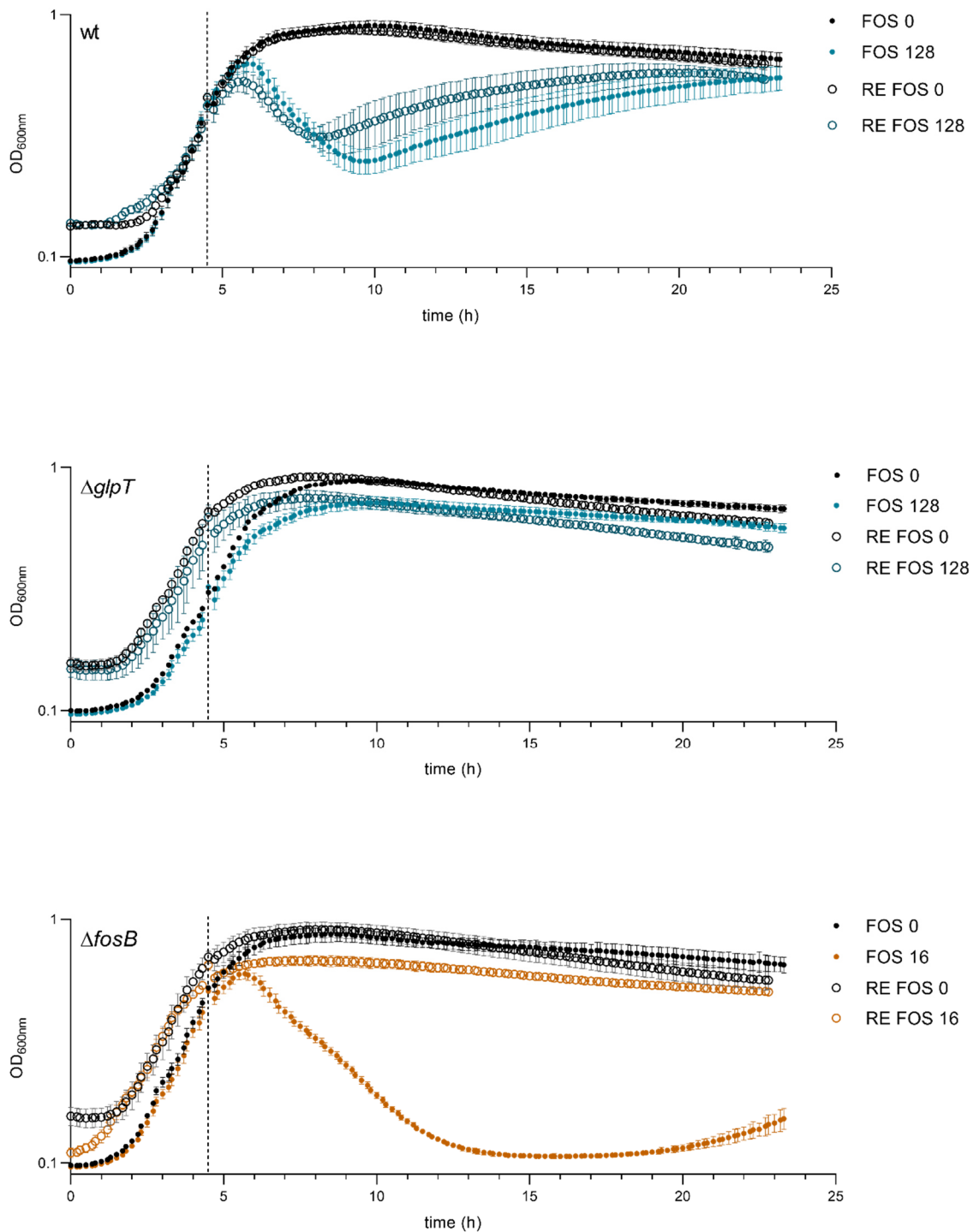
Table A3: Summary of the resistance determination of *B. subtilis* mutants against FOS on LB agar plates.

wt		
	lysis in cm	HALO lysis in cm
wt (OP)	2.5	4.0
wt RI	2.4	4.0
wt RII	2.4	4.0
wt RIII	2.4	4.0
$\Delta fosB$		
$\Delta fosB$	2.4	4.2
$\Delta fosB$ RI	2.1	2.8
$\Delta fosB$ RII	2.1	2.8
$\Delta fosB$ RIII	2.8	4.2

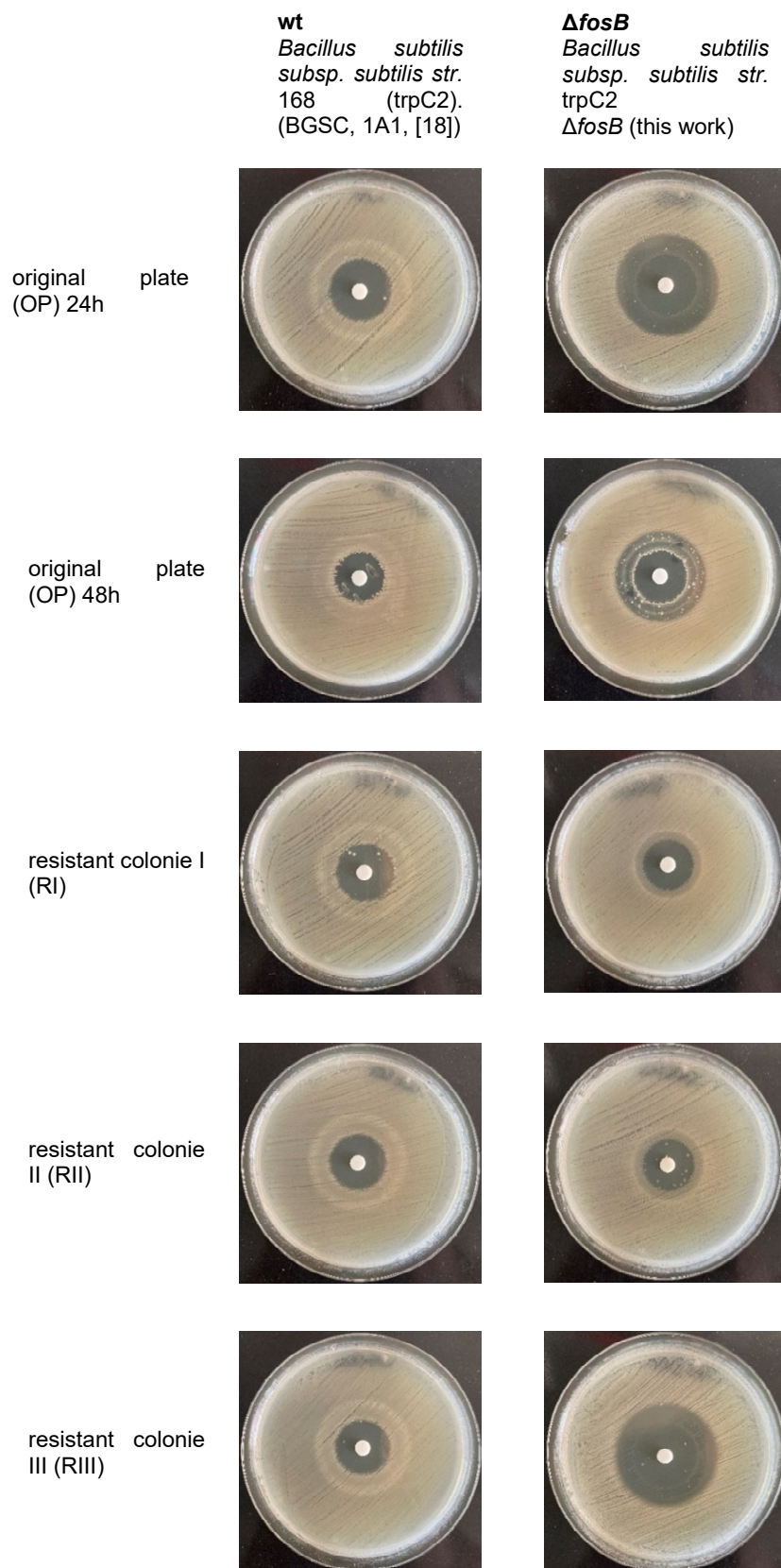
FOS-loaded 6 mm DISCS with appropriate concentrations (wt: 500 μg , $\Delta fosB$: 250 μg) were used. Cultures were plated according to CLSI guidelines, adjusted to MC-Farland 0.5 [16, 17]. A distinction is made between the non-growing area (=FOS lysis in cm (diameter)) and the growing area, which differs from the rest of the colonies (=MIC HALO). Further details can be found in the appendix (A6) on the images of the plates. Colonies (RI, RII, RIII) were picked from the non-growing areas after more than 30 h of initial incubation of the original plate (OP).



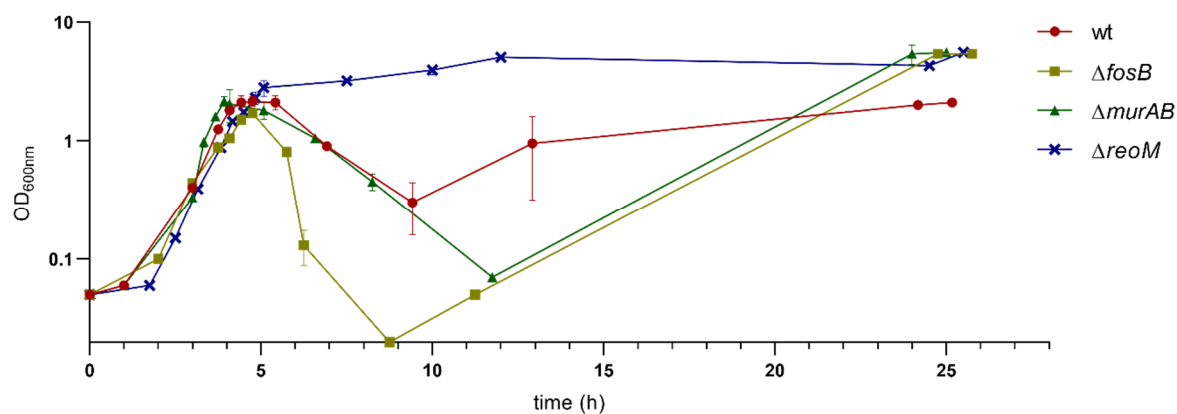
A4. Monitoring of growth behaviour and FOS sensitivity of *B. subtilis*. LB growth curves of the strains: *B. subtilis* 168 (*trpC2*) = wt, *B. subtilis* (*trpC2*) ΔfosB = ΔfosB , *B. subtilis* (*trpC2*) $\Delta\text{murAB}::\text{kan}$ = ΔmurAB , *B. subtilis* (*trpC2*) $\Delta\text{yrzL}::\text{kan}$ (*yrzL*=*reoM*) = ΔreoM , *B. subtilis* (*trpC2*) $\Delta\text{glpT}::\text{erm}$ = ΔglpT . FOS treatment with concentrations from 0-8192 $\mu\text{g mL}^{-1}$ after 3.5 h of incubation. Experiments were conducted in triplicate or duplicate in 24-well plates covered with a semi-permeable sealing membrane (Breathe-Easy, Z380059, Sigma-Aldrich) shaken at 37 °C using the EPOCH2, BioTek Absorbance Microplate Reader, Agilent Technologies, by measuring OD_{600nm} every 10 min.



A5. Monitoring the growth behaviour of *B. subtilis* to study the development of resistance to FOS LB growth curves of the strains: *B. subtilis* 168 (*trpC2*) = wt, *B. subtilis* (*trpC2*) $\Delta fosB$ = $\Delta fosB$ and *B. subtilis* (*trpC2*) $\Delta glpT::erm$ = $\Delta glpT$. FOS treatment 0, 16 and 128 $\mu g mL^{-1}$ after 4.5 h of incubation. Experiments were conducted in triplicate or duplicate in 24-well plates covered with a cover shaken at 37 °C using the SPARK multimode plate reader, Tecan, by measuring OD_{600nm} every 10 min.



A6. Resistance determination of *B. subtilis* mutants against FOS on LB agar plates. Pictures and basis for tableA1. Picked colonies are visible or marked in the original plate (OP) images for 48 h. or marked. FOS-loaded 6 mm DISCS with appropriate concentrations (wt: 500 μ g, Δ fosB: 250 μ g) were used. Cultures were plated according to CLSI guidelines, adjusted to MC-Farland 0.5 [16, 17]. Colonies (RI, RII, RIII) were picked from the non-growing areas after more than 30 h of initial incubation of the original plate (OP).



A7. Culture flask (30 mL) growth curves to determine intracellular and extracellular FOS concentration. LB growth curves of the strains: *B. subtilis* 168 (*trpC2*) = wt, *B. subtilis* (*trpC2*) $\Delta fosB$ = $\Delta fosB$, *B. subtilis* (*trpC2*) $\Delta murAB::kan$ = $\Delta murAB$, *B. subtilis* (*trpC2*) $\Delta yrzL::kan$ (*yrzL*=*reoM*) = $\Delta reoM$. Cultures were treated with FOS at concentrations of $16 \mu\text{g mL}^{-1}$ ($\Delta fosB$) or $128 \mu\text{g mL}^{-1}$ (wt, $\Delta murAB$, $\Delta reoM$) after 3-4 h of incubation. Performed in duplicates in 100 mL flasks shaken at 37 °C.

4.7 Manuscript 7

Friz S, Walter A, Franz-Wachtel M, Harbig T, Macek B, Nieselt K, Mayer C. Comprehending the responses of *Bacillus subtilis* to fosfomycin using multi-omics.

Ready for submission. Manuscript and appendix are attached.

Comprehending the responses of *Bacillus subtilis* to fosfomycin using multi-omics

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Key words: *Bacillus subtilis*, epoxide antibiotic fosfomycin, peptidoglycan proteome, transcriptome, metabolomics, LC-MS, RNA-Seq, MurA, UDP-GlcNAc, cell lysis, envelope stress, disulfide stress

ABSTRACT

The increasing incidence of antimicrobial resistance has encouraged a re-evaluation of existing antibiotics for combination therapies, with fosfomycin (FOS) emerging as a promising candidate. Known for its broad-spectrum activity against Gram-positive and Gram-negative bacteria, FOS shows synergistic effects when combined with various antibiotics of different classes. FOS has a unique mechanism of action by covalently blocking the enzyme MurA, a key step in peptidoglycan (PGN) biosynthesis. To rationalise combination therapy options, we set out to investigate the poorly understood cellular responses of *Bacillus subtilis* to FOS and the metabolic adaptations that confer FOS tolerance using a multi-omics approach (transcriptomics, proteomics, metabolomics). In two independent experimental setups, the adaptation of cells to FOS (S1) and the cellular responses to FOS-induced cell lysis (S2) were investigated. The analysis of both proteomic and transcriptomic data from the same sample allows the assessment of both transcriptional and post-transcriptional regulation and opens a variety of new avenues for analysis and conclusions. We also included a comparison between a wt and a sensitive mutant lacking the potent FOS detoxification enzyme FosB. By studying these two strains in parallel in different experimental setups, it was possible to distinguish between stress-induced, superimposed effects and stress-independent adaptations of cell metabolism to FOS. In addition, we performed metabolomics analyses to validate the conclusions drawn from the other omics techniques. The results reveal a variety of stress-induced FOS responses and metabolic perturbations, including cell envelope stress responses controlled by the extra-cytoplasmic function sigma factors SigM, V, W, X, Y and the involvement of the transcriptional regulator Spx in triggering, cell envelope, disulfide and oxidative stress responses. Remarkably, FOS induces upregulation of genes and proteins involved in lipoteichoic and teichoic acid synthesis and metabolic adaptations affecting fatty acid and iron homeostasis. In addition, because of inhibiting its primary target, MurA, FOS affects later steps of PGN synthesis, e.g. causing upregulation of PGN synthesis and downregulation of PGN degradation enzymes. Downregulation of catabolic processes and an effect on pyrimidine biosynthesis were also observed, together with potential detoxification mechanisms involving the phenolic acid decarboxylase PadC. Overall, inhibition of PGN biosynthesis and direct effects on the cell envelope remain the dominant cellular responses. However, several stress-independent effects were observed. The study thus provides a nuanced understanding of the complex cellular responses to FOS, reveals several new hypotheses and provides insights into bacterial adaptation mechanisms.

INTRODUCTION

World AMR (antimicrobial resistance) Awareness Week is an annual global campaign run by the World Health Organization to raise awareness and understanding of AMR and to draw the attention of the public, health professionals and government officials to the growing problem. This awareness is needed because of the rapid global spread of multidrug-resistant and pand-resistant bacterial *superbugs*, which cause infections that cannot be treated with existing antimicrobial drugs, including clinically important antibiotics. The high rates of resistance observed worldwide to antibiotics commonly used to treat urinary tract infections, sepsis, sexually transmitted infections and some forms of diarrhoeal diseases are very alarming as they demonstrate that we are running out of effective therapeutic options. [1]

As the rate of new antibiotic discoveries has been very low in recent years, new ways of combating the AMR crisis must be found. One option is to re-evaluate known antibiotics already in use and increase their therapeutic efficacy while reducing the development of resistance. However, this strategy requires a better understanding of the bacterial response to antibiotic action, including beyond the key target effects. An appropriate and medically eminent candidate for this type of research is fosfomycin (FOS), a long-used drug with promising therapeutic properties and often observed synergistic effects with other drugs, but which has been removed from clinical therapy due to the rapid emergence of resistance. Our goal is to better understand bacterial responses to FOS in order to improve its efficacy and rationalise its use in combination therapies.

FOS (L-cis-1,2-epoxypropyl phosphoric acid) is a phosphonic acid and forms its own antibiotic group of epoxides, a three-ring heterocyclic compound with one oxygen heteroatom. The antibiotic was first described in 1969 and isolated from cultures of *Streptomyces* species. It was later discovered that *Pseudomonas* sp. can also produce FOS, but through a different metabolic pathway. FOS is traditionally used orally as the TRIS-buffered formulation FOS tromethamine for uncomplicated urinary tract infections (UTI) or gastrointestinal infections caused by *Escherichia coli* and *Enterococcus faecalis*. Because of its broad-spectrum activity, it is also used to treat infections caused by Gram-positive and Gram-negative bacteria, especially against multidrug-resistant pathogens such as extended-spectrum (metallo)- β -lactamase-producing *Enterobacteriaceae*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* strains, usually in combination with other antibiotics. FOS has been reported to be synergistic with many antibiotics of different classes, including antibiotics targeting late steps in cell wall biosynthesis (e.g. carbapenems, cephalosporins, glycopeptide antibiotics). FOS has rare cross-resistance and has bacteriostatic and bacteriolytic effects depending on the organism. [2, 3, 4, 5, 6, 7, 8, 9, 10]

The pharmacological properties are favourable due to its low molecular weight: 138.06 g mol⁻¹, FOS has good tissue penetration, even in inflamed tissues, and its good half-life (mean half-life $\pm$ SD, 5.7 $\pm$ 2.8 h) makes it a suitable antibiotic. FOS can also be administered intravenously as fosfomycin disodium. [5, 11, 12, 13]

To get a complete picture of an antibiotic's potential, the microbiological side must be understood in addition to the medical side. A profound understanding of the cellular response of bacterial cells to antibiotics, beyond the main target effects, is mostly lacking.

FOS inhibits the key enzyme of peptidoglycan (PGN) *de novo* synthesis MurA, a UDP-*N*-acetylglucosamine-1-carboxyvinyltransferase (EC 2.5.1.7 (IUBMB)) in the cell. MurA catalyses the conversion of PEP and UDP-GlcNAc to UDP-*N*-acetyl-3-O-(1-carboxyvinyl)-D-glucosamine (UDP-GlcNAc-enolpyruvate) with the release of inorganic phosphate, the first step in *de novo* PGN biosynthesis. The inhibition of MurA by FOS is due to an unusual covalent binding of FOS to a sulfhydryl group of a cysteine in the active site region, which is crucial for the catalysed reaction. As mentioned above, this primary target of FOS cannot explain the numerous synergies between different classes and the lytic effect of the antibiotic. [14, 15].

The transport of FOS into the bacterial cell is predominantly mediated by two transporters: the glycerol-3-phosphate transport system (GlpT) and the hexose phosphate transport system (UhpT). Both transport systems are subject to catabolite suppression and are critical for FOS sensitivity in clinical and *in vivo* assays due to differential expression of these transporters. Glucose-6-phosphate induces the expression of the UhpT transporter in *Escherichia coli* and *S. aureus* via a sensor protein in association with a two-component regulatory system. In contrast, UhpT including the sensor system is absent in *P. aeruginosa* and *B. subtilis*, where GlpT, whose expression is increased in anaerobically growing cells, has the main function. [16, 17, 18]

In addition to the main target and uptake of FOS, the development of resistance is a crucial factor in understanding the cellular response. The rate of resistance development has been relatively stable in recent years at around 3 % in the relevant studies, which is a comparatively promising value. The advantage is that resistance to FOS is always associated with a high biological cost and a loss of fitness of the bacterial strain [19, 20]. However, these general conclusions have recently been questioned in a study of bacterial uropathogens [21]. As mentioned above, an important factor in the development of resistance at the cellular bacterial level is the transport of FOS. Crucial are mutations leading to different expression levels of the transporters UhpT and GlpT, the basal strain-dependent presence of the transporters or indirectly via mutations affecting catabolite repression. Furthermore, resistance is gained through rare point mutations in MurA (e.g. cysteine > aspartic acid), the loss of cysteine in the active site renders MurA FOS resistant but retains catalytic activity. This leads to natural FOS resistance, as found e.g. in *Mycobacterium tuberculosis*, *Borrelia burgdorferi* and *Chlamydiae* [22, 23]. The presence of FOS detoxification enzymes (epoxidases) is common and is mainly achieved by glutathione-S-transferases of the FosA family in Gram-negative bacteria and by the thiol-S-transferases of the FosB family in Firmicutes and other Gram-positive bacteria, requiring K⁺ and Mn²⁺ or Mg²⁺ ions, respectively. FosB requires the thiol compound bacillithiol, which is synthesised from UDP-GlcNAc, the substrate of the primary FOS target MurA. Bacillithiol and FosB (as well as glutathione and FosA) are normally crucial for resistance to oxidative stress; detoxification of FOS does not appear to be the primary task due to its enzymatic properties (e.g. FosB K_m: BSH: 4.2 mM, FOS: 17.8 mM). [2, 22]

In summary, FOS is a very important antibiotic with attractive properties for antibacterial combination therapy. This is because FOS does not exhibit cross-resistance and acts synergistically in combination with many drugs from different antibiotic classes. This characteristic is quite unique and cannot be explained by the fact that FOS inhibits only the first step of PGN biosynthesis as a PEP competitor, which should simply lead to a growth arrest.

There is evidence that FOS activates the starvation response, affects cell wall function and synthesis, and is associated with oxidative stress and altered sensitivity. This may also explain the synergistic effects. To examine which side-effects or off-targets FOS has in a bacterial cell, a holistic analysis of the cellular response is crucial. This has been achieved through 3-dimensional omics technologies with transcriptome, proteome and metabolome analyses. Using relevant, different conditions of cultures responding to FOS, a deep insight was gained. In addition to the RNA transcripts read directly from the genome (transcriptomics), the proteins translated from them (proteomics), and the metabolites produced or modified by the enzymes (metabolomics) were analysed at the same time. In this way, it was possible to identify not only effects at the omics level, such as metabolite accumulation, protein degradation or up-regulation of expression, but also, for example, regulatory processes of proteins on transcription.

For ease of availability and ideal laboratory conditions, the Gram-positive model organism *Bacillus subtilis* was used to study the bacterial cellular response to FOS. *B. subtilis* is ideal because it is the best-known Gram-positive model organism with an extremely powerful genetic toolkit. In addition, its rapid growth and low medium requirements make it easy to handle in the laboratory [24]. In the specific case of FOS effects, *B. subtilis* is additionally suitable because it possesses both the FosB detoxification mechanism, two MurA paralogs and only the GlpT transport system, which expands or limits investigations in a meaningful way. Nevertheless, the findings are generally readily transferable to other organisms.

MATERIAL AND METHODS

Bacterial strains

Bacillus subtilis subsp. *subtilis* str. 168 (trpC2). (BGSC, 1A1, [25])

Bacillus subtilis subsp. *subtilis*, trpC2 Δ fosB::erm. (BGSC, BKE17840, [25])

Bacillus subtilis subsp. *subtilis*, trpC2 Δ fosB. (this work)

Generation of a marker less Δ fosB mutant

Bacillus subtilis subsp. *subtilis*, trpC2 Δ fosB::erm were made naturally competent for transformation by the following procedure and were used to transform plasmid pDR244 [24].

20 mL MGE medium was inoculated with 250 μ L o/n culture and incubated at 130 rpm at 37 °C. Cells were harvested (4 min, 4°C, 2500 g) 120 min after the transition to the stationary phase, monitored by OD_{600nm}. The pellet was suspended in 1/10 of the supernatant volume spiked with 17 % glycerol (v/v) and stored at -80 °C. For transformation, 300 μ L of the regeneration medium (RM) was mixed with 700 ng DNA (pDR244) and incubated at 37 °C for 30 min. Subsequently, 100 μ L of expression medium (EM) was added. After 90 min at 37 °C, the cells were plated onto lysogeny broth (LB) plates containing 100 μ g mL⁻¹ spectinomycin for selection. Plates were incubated at 30 °C o/n, and the growing colonies were then heat shifted to 42 °C o/n to remove the plasmid pDR244 again, which harbours a temperature-sensitive origin of replication and allows temperature-induced expression of Cre recombinase. Successful removal of the erm^R cassette was confirmed by sequencing.

Media:

10x MM: 440 mM KH₂PO₄, 600 mM K₂HPO₄, 29 mM sodium citrate, 150 mM (NH₄)₂SO₄

MGE: 2000 μ L 10xMM, 800 μ L glucose (50%), 144 μ L L-glutamate (40%), 100 μ L ammonium iron(II) citrate (2 mg mL⁻¹), 100 μ L yeast extract (5 %), 200 μ L tryptophan (5 mg mL⁻¹), 100 μ L CAA (10%), 40 μ L MgSO₄ (1 M), 40 μ L CaCl₂ (0.5 M), filled up to 20 mL. RM: 170 μ L 10xMM, 17 μ L glucose (50%), 34 μ L MgSO₄ (1 M), 1700 μ L H₂O_{dd}.

RM: 170 μ L 10xMM, 17 μ L glucose (50 %), 34 μ L MgSO₄ (1 M), 1700 μ L H₂O_{dd}.

EM: 300 μ L yeast extract (5 %), 150 μ L CAA (10 %), 15 μ L tryptophan (5 mg mL⁻¹), 150 μ L H₂O_{dd}.

Optimised experimental setup for omics analyses with *B. subtilis*

To ensure the comparability of all omics analyses, an easy-to-use and robust experimental setup is required. Experiments are performed in lysogenic broth (LB) medium in a volume of 30 mL at 37 °C and 120 rpm with an inoculation of 0.05 OD_{600nm} of the strains *B. subtilis subsp. subtilis str. 168* (trpC2) (wt) and *B. subtilis subsp. subtilis*, trpC2 Δ fosB (Δ fosB) respectively.

Fosfomycin (FOS) was added either from the beginning (wt: 0.5 μ g mL⁻¹, Δ fosB: 0.1 μ g mL⁻¹) or in the exponential growth phase OD_{600nm} 0.5-0.8 (wt: 128 μ g mL⁻¹, Δ fosB: 16 μ g mL⁻¹). Samples were harvested OD_{600nm} 0.8 when added from the start, and 1 h after treatment when added during the exponential phase. A low FOS concentration at the beginning is named setup 1 (S1) and a high FOS concentration in the exponential phase setup 2 (S2) (Fig.1).

Transcriptome analyses

Transcriptome analysis of both S1 and S2 was performed in triplicate. The equivalent of 2 mL OD_{600nm} 0.8 cells were harvested by centrifugation (4 °C 10 min 10,000 g) at specific time points. The time points of FOS addition and sample collection in the two the setups and the corresponding OD_{600nm} are shown in Fig.1, appendix A1 and A3. Harvested samples were treated as described in the Quick-RNA™ Fungal/Bacterial Miniprep Kit (R2014, Zymo Research). Centrifugation steps were performed at 13,000 g and elution was in 25 μ L DNase/RNase-free H₂O_{dd}. A more effective and reproducible in-solution digestion was performed using recombinant, RNase-free DNase I (04716728001, Roche) instead of the unreliable in-column digestion recommended by the manufacturer. 25 μ L of purified RNA was incubated together with the corresponding 10x incubation buffer: 5 μ L DNase I and 15 μ L DNase/RNase-free H₂O_{dd} at 37 °C for 20 min. For inactivation, 2 μ L of 0.2 M ethylenediaminetetraacetic acid (EDTA, pH 8.0) was added and the solution was heated to 75 °C for 10 min.

For purification and concentration, samples were treated with the RNA Clean & Concentrator-5 Kit (R1017, Zymo Research) as described. In this case, centrifugation was also carried out at 13,000 g and 15 μ L DNase/RNase-free H₂O_{dd} was used for elution. The RNA samples were sequenced by the NGS Competence Centre Tuebingen (NCCT, Institute of Medical Microbiology and Hygiene, Tuebingen 72076, Elfriede-Aulhorn-Straße 6, Germany).

Total RNA was quantified using a Qubit RNA BR Assay Kit (Thermo Scientific), and RNA integrity was checked using an Agilent 2100 BioAnalyzer with a Pico kit. A total of 100 ng RNA per replicate was subjected to rRNA depletion. Illumina™ Stranded Total RNA Prep Ligation with Ribo Zero Plus Kit was used for rRNA depletion and cDNA library construction according to the manufacturer's instructions. Libraries were sequenced as single reads (151 bp read length) on a MiSeq v3 (150 cycles) Flow Cell (Illumina) at an average depth of 19 million reads per sample, demultiplexed with bcl2fastq (v2.19.0.316), quality checked with fastp (v0.20.1) and visualised with MultiQC (v1.7). The sequenced samples were bioinformatically processed by the Institute of Bioinformatics and Medical Informatics, Eberhard Karls University of Tuebingen, Tuebingen 72070, Sand 14, Germany. Sequencing statistics, including the quality per base and adapter content assessment of the resulting transcriptome sequencing data, were conducted using FastQC v0.11.8 [25].

All read mappings were performed against the assembled *B. subtilis* strain (RefSeq ID NC_000964.3). All samples were mapped using HISAT2 v2.1.0 [27]. As parameters, spliced alignment of reads was disabled, and strand-specific information was set to reverse complemented (HISAT2 parameters --no-spliced-alignment and --rna-strandness "R"). The resulting mapping files in SAM format were converted to BAM format using SAMtools v1.9 [28]. Mapping statistics, including strand specificity estimation and percentage of mapped reads, were performed with the RNA-Seq module of QualiMap2 v2.2.2-a [29]. Gene counts for all samples were calculated using featureCounts v1.6.4 [30], with the selected feature type set to transcript records (featureCounts parameter -t transcript). A quality check for ribosomal rRNA was performed using a custom script based on the absolute counts of annotated rRNAs. Principal component analysis (PCA) was performed using the DESeq2 package v1.28.1 to assess the variability of replicates of each time series [31]. All RNA-Seq Illumina read files and the raw data were made available in the digital Zenodo digital appendix Z [32, A19]. To calculate genes differentially expressed between the two strains and treatments, DESeq2 was applied to the absolute gene counts as computed with featureCounts. For differences between the two strains and conditions, genes with an adjusted p-value (FDR) <0.05 and an absolute log2 fold change (FC) >1 were reported as differentially expressed.

Proteome analyses

For proteomic analysis, after numerous preliminary experiments in different setups, both S1 and S2 (Fig.1) were performed in quadruplicate. The equivalent of 15 mL OD_{600nm} 0.8 was harvested by centrifugation (4 °C 10 min 5000 g) at the set up specific time points. The time points of FOS addition and sample collection in the two the setups and the corresponding OD_{600nm} are shown in Fig.1, A1 and A3. Protein isolation was performed according to the protocol (Protein extraction with SDS buffer (vers. 1.0), Philipp Spät, 01.01.2014 developed by MPI FASP protocol) of the Proteome Center Tuebingen, Eberhard Karls University of Tuebingen, Tuebingen 72076, Auf der Morgenstelle 15, Germany, which also performed the subsequent analysis of the purified protein samples.

Cell pellets stored overnight at -80 °C were suspended in 100 µL phosphate buffered saline (PBS) containing 200 µg mL⁻¹ lysozyme (pH 6.5) and incubated at 37 °C for 20 min. Addition of 1 mL (for 20-50 mg cells) of SDS buffer (4% w/v sodium dodecyl sulfate (SDS), in 100 mM tris(hydroxymethyl)aminomethane (Tris)/HCl; pH 8.0) followed by incubation at 95 °C for 10 min.

After cooling the samples on ice, the DNA is comminuted by sonication (30-300 s until viscosity is aqueous, Branson Sonifier 250 with 5 mm micro tip; output control 5, duty cycle 40 %). The cysteine disulfide bonds are reduced (10 mM dithiothreitol (DTT, stock: 1 M)) and the reduced cysteine thiol groups are alkylated (5.5 mM iodoacetamide (IAA, stock: 0.55 M)), each for 45 min at RT with shaking (650 rpm). From the supernatant after centrifugation (15 min, 3345 g in 15 mL Falcons), proteins were precipitated by adding 9 times the sample volume of a solution of 8:1 v/v acetone/methanol by cooling at -20 °C and vortexing. The precipitated proteins were washed 3 times with twice the sample volume of 80% v/v acetone aq. at RT. The protein pellet was air dried without complete drying-out and finally soaked in denaturation buffer (6 M urea, 2 M thiourea in 10 mM Tris/HCl; pH 7.5) for rehydration. 10 µg of each sample was digested in solution with trypsin as previously described [33]. Acidified peptides were purified [34] and labelled on C18 stage tips using TMTpro™ 16plex (Thermo Scientific) according to the user manual. Briefly, bound peptides were washed with 50 µL of 50 mM HEPES (pH 8.5) and TMT16 reagents reconstituted in CH₃CN were added (4.1 µL reagent to 10 µg peptide sample). After an incubation period of one hour at room temperature, labelled peptides were eluted with 50 µL of 80 % CH₃CN and the reactions were finally quenched by adding 0.8 µL of 5 % hydroxylamine for 15 min (700 rpm, RT). TMT labelling efficiency tests were performed, and samples were pooled in equal proportions. Acetonitrile was evaporated, and 100 mg of peptide mixtures were further fractionated using a Pierce High pH Reversed-Phase Peptide Fractionation Kit (Thermo Scientific). A total of nine fractions were collected by stepwise elution of 5-50 % CH₃CN in 10 mM NH₄OH. CH₃CN was evaporated from the acidified samples and the peptides were further purified using C18 stage tips [33].

Peptide fractions were analysed on an Easy-nLC 1200 UHPLC system coupled to an Orbitrap Exploris 480 quadrupole mass spectrometer via a nanoelectrospray ion source (all Thermo Scientific). Separations were performed on a 20 cm analytical HPLC column (75 µm ID PicoTip fused silica emitter (New Objective); in-house packed with ReproSil-Pur C18-AQ 1.9 µm fused silica beads (Dr. Maisch GmbH)) using 87 min fraction-specific stepwise gradients of 10-33-50-90 % HPLC solvent B (80 % CH₃CN in 0.1 % formic acid) in solvent A (0.1 % formic acid) at a flow rate of 200 nL min⁻¹ [35]. In each scan cycle the 12 most intense precursor ions were selected for HCD fragmentation using stepped normalised collision energies of 27/29/31 %. Masses were excluded from further fragmentations for 30 s. MS and MS/MS spectra were recorded at 60,000 and 30,000 resolution respectively. AGC was set to standard and fill time to automatic, with the TurboTMT feature enabled. The acquired MS data were further analysed using MaxQuant software version 2.4.2.0 [36] with the integrated Andromeda search engine [37]. Spectra were searched against a *B. subtilis* database from UniProt (retrieved 16 December 2022; 4,271 entries) and a database of 286 commonly observed contaminants. Full tryptic specificity was required and up to two missed cleavages were allowed. Protein N-terminal acetylation and methionine oxidation were defined as variable modifications, whereas cysteine carbamidomethylation was defined as fixed or variable modification. The latter was defined when FOS and its product fosfomycin-aldehyde (FOS-aldehyde) after elimination of phosphonic acid were also set as variable modifications on cysteine residues. At the MS/MS reporter ion level, the TMTpro 16plex tags on lysines and peptide N-terminals were selected and correction factors were applied.

The target decoy strategy [38] was used to calculate the false discovery rate (FDR) at the protein, peptide and modification site level and the threshold was set at 0.01 % FDR. Precursor and product ion mass tolerances were set at 4.5 ppm and 20 ppm, respectively.

Downstream statistical analysis was performed using Perseus software version 1.6.15.0 [39]. Potential contaminants, reverse hits and those identified by site only were filtered from the protein dataset. Logarithmised TMT reporter ion intensities of proteins were normalised by width adjustment, and proteins that did not have 100 % values in at least one group were removed. Student's t-test was used to compare the means of logarithmised reporter ion intensities between different conditions. Based on Benjamini-Hochberg ($s = 0$; $FDR \leq 0.01$), proteins were defined as regulated with statistical significance. All raw data were made available in the digital Zenodo digital appendix Z [32, A19].

Metabolome analyses

For metabolomic analyses, both S1 and S2 were performed in triplicate. In contrast to the transcriptomic and proteomic analyses, which were performed in collaboration, the metabolomic analyses were performed in-house. The focus was on the development and optimisation of extraction and LC-MS methods as well as a convenient workflow including bioinformatic processing of the data. The results of this development work are briefly described below.

The appropriate amount of cells (usually the equivalent of 20 mL OD_{600nm} 0.8) was cooled with liquid nitrogen (three immersions for 3-5 s each, without freezing). Afterwards, the cell sample has an estimated temperature of 5 °C. The cooled sample was passed through a cooled filter system (XX1014700, Millipore Classic Glass Filter Holder - Kit 47 mm, glass frit membrane support, 300 mL funnel, S-Pak filter, 0.45 µm, 47 mm, MERCK, all cooled at 4 °C for at least 30 min) in less than 5 s. The filter was washed with 5 mL of cooled sterile isotonic saline. The filters were immediately placed in 5 mL of 60 % ethanol (50 mL Falcon), frozen in liquid nitrogen and stored at -80°C for at least overnight. The cells were disrupted by thawing (room temperature), shaking and vortexing, 10-15 times each. After centrifugation at 4 °C for 5 min at 10000 g, 4 mL of supernatant was removed, and the extraction was repeated with 2 mL of cooled water. This resulted in a 6 mL sample, which was dried in a SpeedVac System at 30 °C and then dissolved in H₂O_{dd}. [40]

Measurements were performed using an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) connected to an ACQUITY ultra-performance liquid chromatography (UPLC) system (Waters Corporation). Separation in the UPLC was carried out using a Phenomenex C18-Gemini column (150 × 2 mm, 3 µm, 110 Å, Phenomenex) at 37 °C with 0.1 % formic acid and 0.05 % HCO₂NH₄ (A1) and CH₃CN (B1) buffer system. A single run (injection volume of 5 µL) was performed with a flow rate of 0.2 mL min⁻¹ and a two-step gradient: after 2.5 min of equilibration with 100 % A1, a 1 min gradient up to 5 % B1 is followed by a 4 min gradient up to 70 % B1. After 2 min at 70 % B1, a re-equilibration step of 2.5 min follows with a flow rate of 4 mL min⁻¹ follows. Analysis of the mass to charge ratios was performed in the positive ion mode in the range of 50-200 m/z⁻¹ and 150-1200 m/z⁻¹.

The MS settings were as follows: heater temperature 300°C, sheath gas/aux gas/sweep gas flow rate 30/10/0 arb, I spray voltage 4 kV, capillary temperature 275 °C, S-lens RF level 50%, multipole 00 offset -2.28 V, lens 0 voltage -2.78 V, multipole 0 offset -12.00 V, lens 1 voltage -7.00 V, multipole 1 offset -15.56 V, multipole RF amplitude 800.00 V PP, front lens -12.00 V. Raw data MS files were converted to mzML format using msConvert [41]. MS data processing was performed using UmetaFlow GUI [42, 43]. For the untargeted metabolomics workflow default settings were used with the exception of a noise intensity threshold of 1000 in the feature detection step. Four replicates of blank samples were used to filter out blank features. A retention time range of 40 s around the peak apex (+/- 20 s) and a mass tolerance of 10 ppm were used. The meta data template was filled with meaningful attributes for statistical analysis. The statistical analysis was performed using the application for statistical analysis of metabolomics data [43]. Blank metabolites were removed with a threshold of 0.3 (mean metabolite intensities in samples need to be greater than 30 % of the mean blank intensity), missing values were imputed with random values between zero and the lowest measured intensity (limit of detection), and data were scaled and centred. Parametric assumptions were tested with the Shapiro test for normal distribution and the Levene test for equal variance between the treatment and control groups. Significantly differentially regulated metabolites were detected by one-way ANOVA and confirmed by Tukey's post hoc test.

Multi-omics clustering comparison of transcriptome and proteome to identify common trends using the Omics Trend-comparing Interactive Data Explorer (OmicsTIDE)

The comparison of multi-omics data is not trivial and only possible with complex tools like OmicsTIDE [44], which was developed by the collaborators of this project. To provide the necessary input, quantile normalisation was applied separately to transcriptomics data (raw gene counts) and proteomics data (corrected reporter ion intensities). For both datasets, replicates were merged. The tool generates trends across the omics methods in clusters, which are visualised in different ways (e.g. profile plots) and connected by Sankey diagrams. An interactive analysis is then possible, providing deep insights into the relationships between the transcriptome and proteome. S1 and S2 were compared independently with a cluster depth (k) of 3 and 5. Variance filtering is not necessary as the data is already processed and filtered by the internal omics analyses. OmicsTIDE is available as a web tool (<http://omicstide-tuevis.cs.uni-tuebingen.de/>) or as a GitHub repository (<https://github.com/Integrative-Transcriptomics/OmicsTIDE2.0>). The formatted input data can be found under Zenodo digital appendix: Z1, [32, A19].

A data management approach based on SubtiWiki (status 2023-10-17) was also used, making use of the existing exports of the *B. subtilis* database [45]. The information from the "categories", "interaction", "operons" and "regulations" exports (<http://subtiwiki.uni-goettingen.de/v4/exports>), as well as all generated transcriptome and proteome results (S1 and S2 for wt and $\Delta fosB$ mutant, respectively) were added to the gene database of *B. subtilis* (Z1, [32, A19]). This provides the ability to filter the genes by regulators, pathways, operons and protein and gene actions, and additionally the option was created to filter this data collection by BSU gene identifier (ordered locus names, e.g. BSU36760, *murAA*) and protein identifier (UniProt primary accession, e.g. P70965 MURA1_BACSU).

Statistical overrepresentation test using the PANTHER classification system

PANTHER (<https://pantherdb.org/>, version 18.0 (released 2023-08-01)) was used to statistically analyse individual groups of the omics analyses [46, 47, 48]. Statistical overrepresentation analysis was used to determine whether a biological process with more genes than statistically expected was present in a selected group (e.g. proteins that are upregulated in the wt by FOS). The analysis was performed using the annotation dataset:GO biological process complete and the provided genome of *B. subtilis* for selected groups of proteins or transcripts from the respective analyses. Fisher's exact significance test and correction by false discovery rate (FDR) calculation and Bonferroni correction were performed. significant results (p-value<0.05) were presented and discussed. If this is not the case, it is mentioned in the text.

RESULTS AND DISCUSSION

Development of stable experimental setups for omics analysis with *Bacillus subtilis*

To comprehensively assess the cellular responses of *B. subtilis* to fosfomycin (FOS), global changes at the transcript (transcriptomics) and protein (proteomics) levels were investigated in parallel. As described in more detail in the materials and methods section.

To fully understand the cellular response of *B. subtilis* to FOS, and to study both the adaptation to FOS and the lytic effect of FOS, more than one experimental setup is required.

The adaptation to sub-MIC concentrations of FOS was investigated in setup 1 (S1), while the effect of FOS on the onset of cell lysis in exponential phase cultures was investigated in setup 2 (S2). After preliminary experiments in cell culture plates, both setups were optimised in 30 mL liquid culture. Furthermore, all analyses were performed in parallel with *B. subtilis* wt (wt) and *B. subtilis* $\Delta fosB$ ($\Delta fosB$) mutant strains. This provides additional information on the role of the FOS detoxification mechanism (thiol-S-transferase FosB) and excludes the influence of absolute FOS concentrations, since very different FOS concentrations in wt and $\Delta fosB$ mutant strains lead to similar growth inhibition phenotypes.

The ideal conditions were determined by observing the general growth behaviour of *B. subtilis* under FOS treatment (appendix: A4). After optimisation, 0.5 $\mu\text{g mL}^{-1}$ for the wt and 0.1 $\mu\text{g mL}^{-1}$ for the $\Delta fosB$ mutant was used for S1, where the adaptation of the cells to FOS could be studied (Fig.1). Addition of higher concentrations of FOS (wt: 128 $\mu\text{g mL}^{-1}$, $\Delta fosB$: 16 $\mu\text{g mL}^{-1}$) led to a lytic effect of the strains and it became possible to investigate the influence or cause of bacterial lysis (S2, Fig.1). The samples for the transcriptome and proteome analyses were taken from the identical biological culture (A1, A3). Due to the volume of samples required and the way they were collected, a separate culture with an identical experimental setup was required for metabolomic analyses (A2).

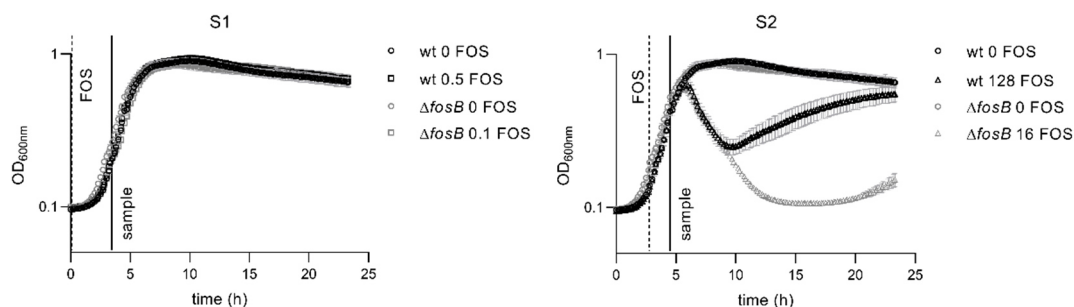


Figure 3: Experimental setup for multi-omics studies on the cellular responses of *B. subtilis* to FOS. Shown are S1 and S2 of *B. subtilis* of wt and $\Delta fosB$ with FOS concentrations (S1/S2: wt: 0.5/128 $\mu\text{g mL}^{-1}$, $\Delta fosB$: 0.1/16 $\mu\text{g mL}^{-1}$) to study the lytic effect (S2) and the adaption of *B. subtilis* to FOS. FOS addition (dashed line) and sample harvest time (straight line) are marked. Performed in triplicate or duplicate in 24-well plates covered with a semi-permeable sealing membrane (Breathe-Easy, Z380059, Sigma-Aldrich) shaking at 37 °C using the EPOCH2, BioTek Absorbance Microplate Reader, Agilent Technologies, by measuring OD_{600nm} every 10 min. The upscaled setups and the growth curves analysed in the multi-omics are shown in the appendix (A1, A3).

Global analysis of FOS-treated *B. subtilis* side by side using transcriptomics and proteomics

The genome of *B. subtilis* 168 consists of 4,214,810 base pairs containing 4,100 protein-coding genes (CDS) [49]. Due to the diverse functions of non-coding RNA in the bacterial cell, the number of sequenced RNA transcripts exceeds the number of mRNAs. In the transcriptome analyses, more than 4,300 RNAs were sequenced and identified for both setups. Remarkably, at least 2,500 proteins were identified in the proteome analyses for both sets. Principal component analysis (PCA) for the transcriptome and proteome showed that the corresponding replicates clustered as expected (A5, A6). Overall, it can be seen that the investigated conditions differ less in S1 than in S2. This was shown by the clearly different growth effects in S2 and only minor changes in growth behaviour in S1 (Fig.1). In principle, the study design combining S1 and S2 and the inclusion of wt and $\Delta fosB$ mutant allows an analysis that covers complementary aspects required for a comprehensive understanding of the cellular response of *B. subtilis* to FOS.

In addition to the setup specific studies on adaptation to FOS (S1) and the lytic effect of FOS (S2), the design allows studies and results independent of the FOS concentration by using two strains with different sensitivities (wt and $\Delta fosB$). The $\Delta fosB$ mutant showed approximately 5-fold higher FOS sensitivity compared to the WT, with an identical effect per setup by adjusting the concentrations used (Fig.1, A3, A4). Moreover, the effects of FOS can be analysed independently of the time of treatment, since the treatment was carried out at different times (S1: from the beginning, S2: at the exponential growth phase, Fig.1). It is also possible to evaluate the effect of the detoxification protein FosB (both with and without FOS treatment) compared to wt. Higher concentrations of FOS than those used for S1 will result in non-growing cultures if added from the beginning.

To analyse these global and general effects in the context of transcriptome and proteome analysis, heatmaps were used. Fig.2 and 3 show the heatmaps of the transcriptome and proteome analyses of the two setups, respectively, both comparing wt and $\Delta fosB$ mutant with and without FOS. For the transcriptome (Fig.2), the variant genes are shown as individual replicates for each set. For the proteome (Fig.3), the significant proteins tested by ANOVA (false discovery rate (FDR) 0.01) are presented per set as means (quadruplicates) of the individual conditions (post hoc Tukey's, FDR 0.01).

This resulted in the best presentation for both conditions, ensuring a balance between maximum information and clarity.

Heatmaps provide an overview and relative comparison of omics analyses. Particularly useful is the clustering of genes/proteins into groups of similarly relatively regulated entities. A full list of genes/proteins belonging to different clusters within the heatmaps can be found in the appendix (A7 and A8) for the transcriptomic and proteomic analyses respectively.

The effect of FOS on *B. subtilis* wt and $\Delta fosB$ differs significantly between the different setups. These differences were identical in the transcriptome and proteome analysis (in the corresponding ratio). In the S1 heatmaps (Fig.2, S1 and Fig.3, S1), the clustering of the entire setup showed that the differences in these datasets were greater between wt and $\Delta fosB$ than between the FOS treatment and the respective control of a particular strain. While in the transcriptome (Fig.2) no clusters could be identified that were regulated in wt and $\Delta fosB$ in an identical FOS-dependent manner, in the proteome (Fig.3) the clusters B, C, K and M (Fig.3, A8) showed an identical FOS-dependent regulation of proteins in wt and $\Delta fosB$. For S1, there was a slight difference between the transcriptome and proteome in the overall heatmap comparison. The separation between strains described above is mixed in the transcriptome analyses (Fig.2), as the FOS-treated sample of the $\Delta fosB$ mutant behaves similarly to an untreated sample. More specific comparisons of two conditions within a strain did not confirm this effect.

In contrast, the S2 heatmaps (Fig.2, Fig.3) show a clustering, as expected from the experimental setup, with the differences mainly due to the FOS treatment (except for clusters F and I in the transcriptome study, Fig.2, and clusters A,B and H-K in the proteome study, Fig.3). Most transcripts (Fig.2) are either upregulated (clusters A-E) or downregulated (clusters G and H) in the presence of FOS. Similarly, the regulated proteins are upregulated in clusters K-O and downregulated in clusters D-G in the presence of FOS treatment, which also represents the clear majority of regulated proteins. A detailed consideration of these regulated transcripts and proteins, which are crucial for understanding the lytic effect of FOS in *B. subtilis*, is discussed in the following section.

The described intentional differences between the setups to address the questions of cellular response of *B. subtilis* to FOS and the influence of FosB at different levels (different concentrations and time points, see above) are also evident in the PCA analyses (A5, A6) and in the comparison of up- and downregulated hits per strain and setup. The Venn diagram comparison of the up- and downregulated transcripts (A9A and A9B) in S1 and S2 (A10A and A10B) clearly shows the described effect that in S2 most transcripts overlap between wt and $\Delta fosB$. In S1, the overlap only accounts for a small proportion. This is also confirmed by the Venn diagrams of the proteome analysis (S1: A11, S2: A12).

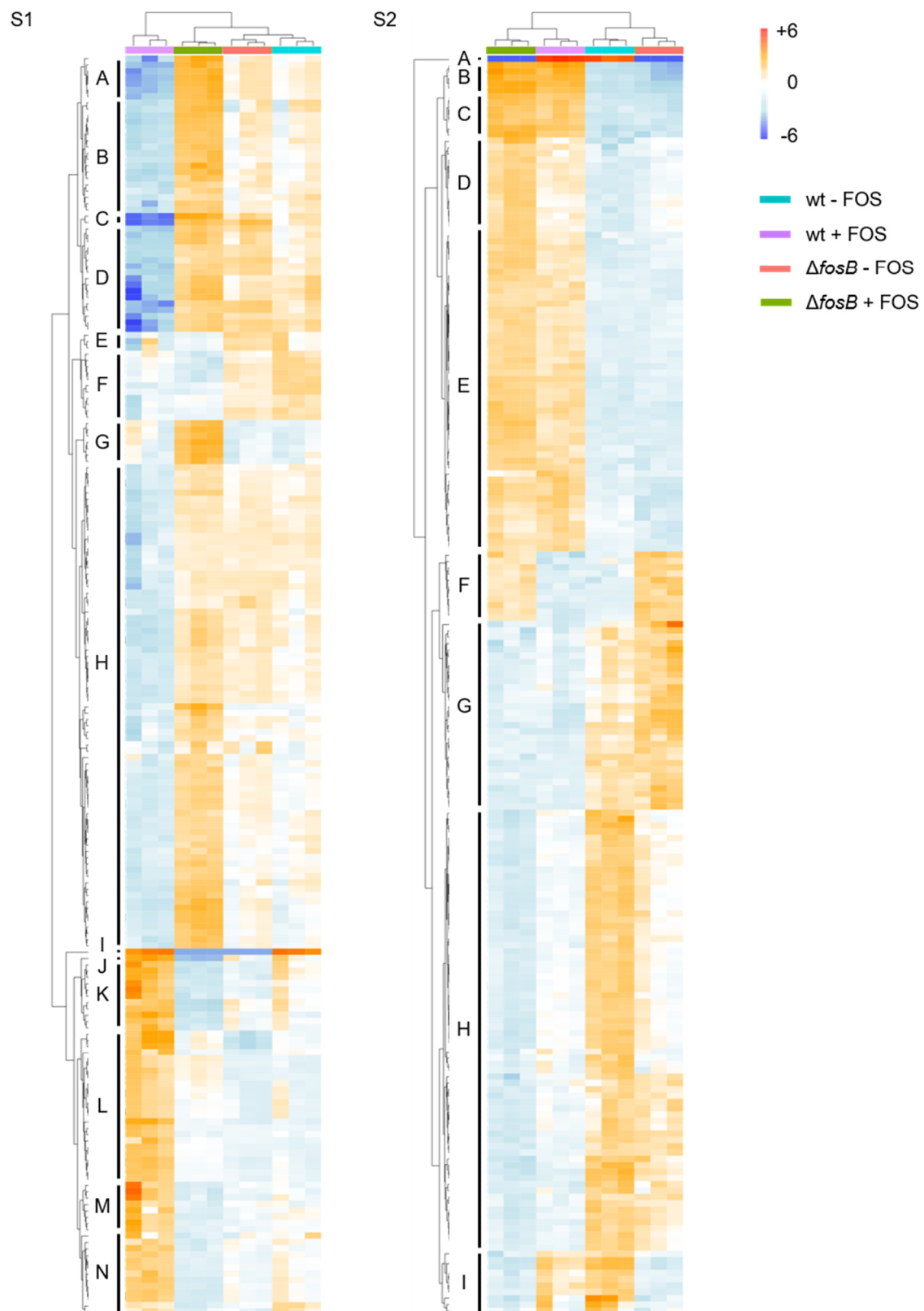


Figure 2: Heatmaps of the transcriptome analyses. Shown are the significant variant transcripts (genes) (in three replicates) of the conditions (wt - FOS: turquoise, wt + FOS: violet, $\Delta fosB$ - FOS: red, $\Delta fosB$ + FOS: green) for S1 and S2. The clustering (label: S1 A-N; S2 A-I; BSU identifier listed in A7) of similar behaving transcripts and the four conditions (colour code) are shown on the left side of the heatmap. The relative transcript levels are indicated (+6. red to -6 blue, colourless no changes).

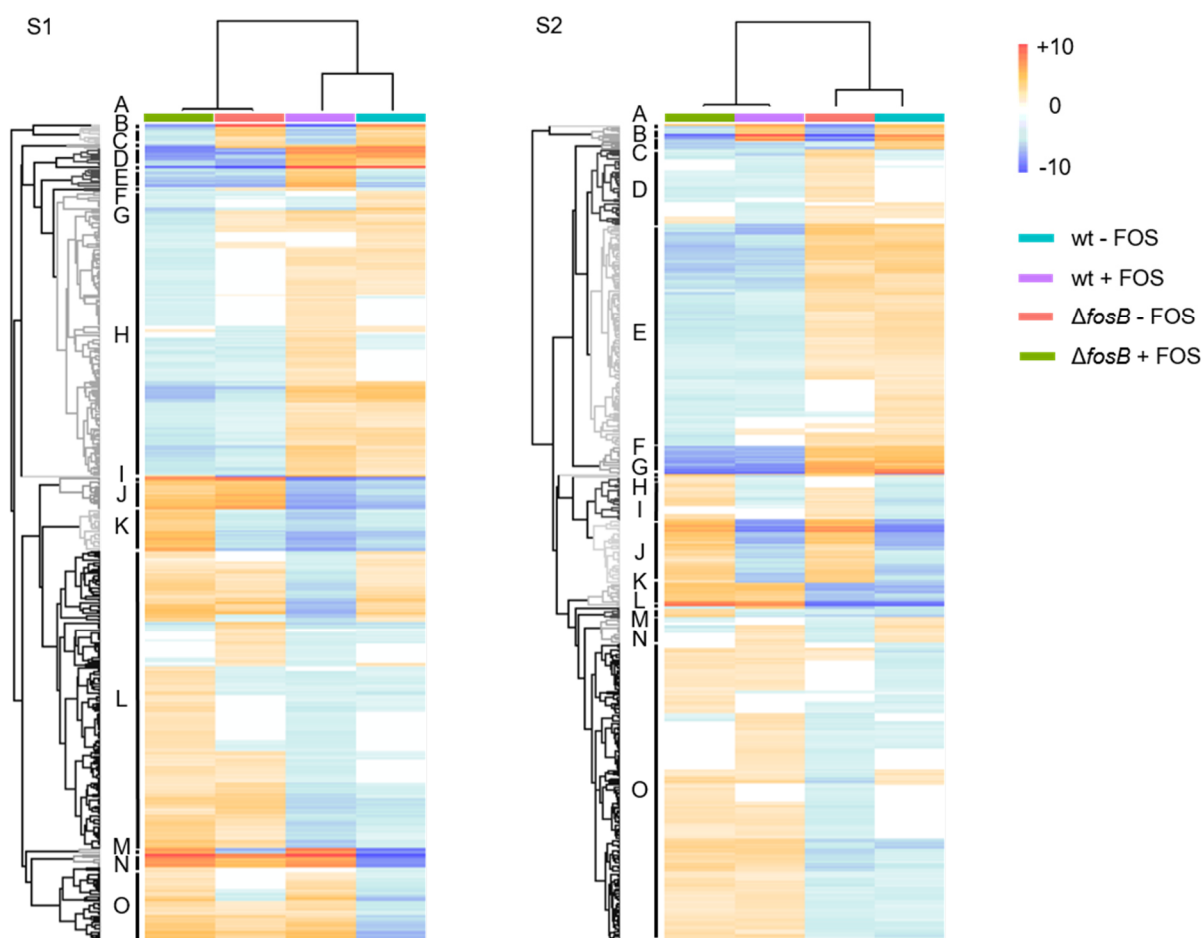


Figure 3: Heatmaps of the proteome analyses. Shown are the significant variant proteins (mean of four replicates) of the conditions (wt - FOS: turquoise, wt + FOS: violet, $\Delta fosB$ - FOS: red, $\Delta fosB$ + FOS: green) for S1 and S2. The clustering (label: S1 A-O; S2 A-O; BSU identifier listed in A8) of similar behaving transcripts and the four conditions (colour code) are shown on the left side of the heatmap. The relative transcript levels are indicated (+10. red to -10 blue, colourless no changes).

Investigation of the lytic effect on *B. subtilis* by FOS using a multi-omics approach

The key question for understanding the cellular effects of FOS is how FOS, as a cell wall inhibitor of MurA, induces a lytic effect upon *B. subtilis*. This question can be answered by looking at setup 2 (S2), where a clear lytic effect is present (Fig.1).

To independently confirm the described FOS effect (see introduction), a metabolomics study was performed with the experimental setup 2. Using HPLC-MS, it was shown that the substrate of MurA, the primary target of FOS, UDP-GlcNAc, accumulates (A13). At the same time, lower levels of the subsequent benchmark PGN precursors UDP-MurNAc (MurB substrate) and UDP-MurNAc pentapeptide (MurF substrate) were identified as a result of FOS treatment (A13). This clearly demonstrated the primary effect of FOS on *B. subtilis*.

In order to obtain a comprehensive result of the cellular response of *B. subtilis* to FOS leading to lysis, it is necessary to analyse and compare the results of the transcriptome and proteome analyses in different ways and with different focus.

The primary and initial focus was on the direct comparison between cultures treated with FOS and their controls without FOS for the wt and $\Delta fosB$ mutant, respectively. The fold changes in the transcriptome (log2fold change >1, p-value <0.05) and proteome (p-value <0.05) were visualised with scatter plots (Fig.4, Fig.5, Fig.6, Fig.7). The focus was on the identification and evaluation of the most differentiating hits. Venn diagrams were used to assess the overlap between wt and $\Delta fosB$ (A10, A12). In addition to this evaluation of the FOS-dependent hits with the highest differentiation, a level completely independent of the fold change was integrated by analysing the statistically overrepresented pathways using PANTHER (GO biological process complete, see methods section). The biological process classification of a defined group of hits was compared to the *B. subtilis* reference and analysed to determine whether certain pathways were statistically overrepresented in this defined group of results (FDR P <0.05). The more analytical, descriptive approach of this section has been complemented by an explanatory summary at the end. In order to take all aspects into account, the results have been interpreted and evaluated in the overall context of the summary.

Transcriptome investigation of the lytic effect of FOS on B. subtilis (S2)

In the transcriptome analyses of S2, 458 transcripts in the wt and 537 transcripts in the $\Delta fosB$ mutant were upregulated and overrepresented under the influence of FOS compared to the respective untreated control (Zenodo digital appendix: Z1 [32, A19], appendix: A10). Of these, 330 hits overlapped, representing the clear majority. In the overview, metabolic pathways were statistically overrepresented among the upregulated transcripts in the wt and $\Delta fosB$ mutant (PANTHER, GO biological process complete). These include carbohydrate catabolism, protein metabolism and other biosynthetic processes such as those of small molecules and organic substances (Z2, Z3, [32]). On the other hand, 467 transcripts were downregulated in the wt mutant and 569 in the $\Delta fosB$ mutant, with 319 hits overlapping (Z1, A10). Among these, secondary pathways such as biosynthesis of (peptide) antibiotics, toxin metabolism and biosynthesis and especially bacteriocin biosynthesis were statistically overrepresented among the downregulated hits during FOS treatment (PANTHER, GO biological process complete, Z4, Z5, [32]). The downregulated transcript hits in wt also included statistically upregulated pathways of pyrimidine biosynthesis and in particular *de novo* UMP biosynthesis (PANTHER, GO biological process complete, Z4, [32]). Despite the generally high overlap and consistency between wt and $\Delta fosB$ mutant in the S2 transcriptome analyses (Fig.2, A10), a detailed examination of the strains is crucial to identify the outstanding hits in each strain. To facilitate this, a scatter plot is shown in Fig.4, which graphically illustrates the influence of FOS on *B. subtilis* wt by depicting fold changes. Significantly upregulated hits that occur at least twice as often as in the reference (log2fold change >1) are shown in red on the right-hand side; identically, downregulated transcripts are shown on the left-hand side. Selected important genes are annotated and grouped into incomplete categories for simplicity and to highlight particular hits.

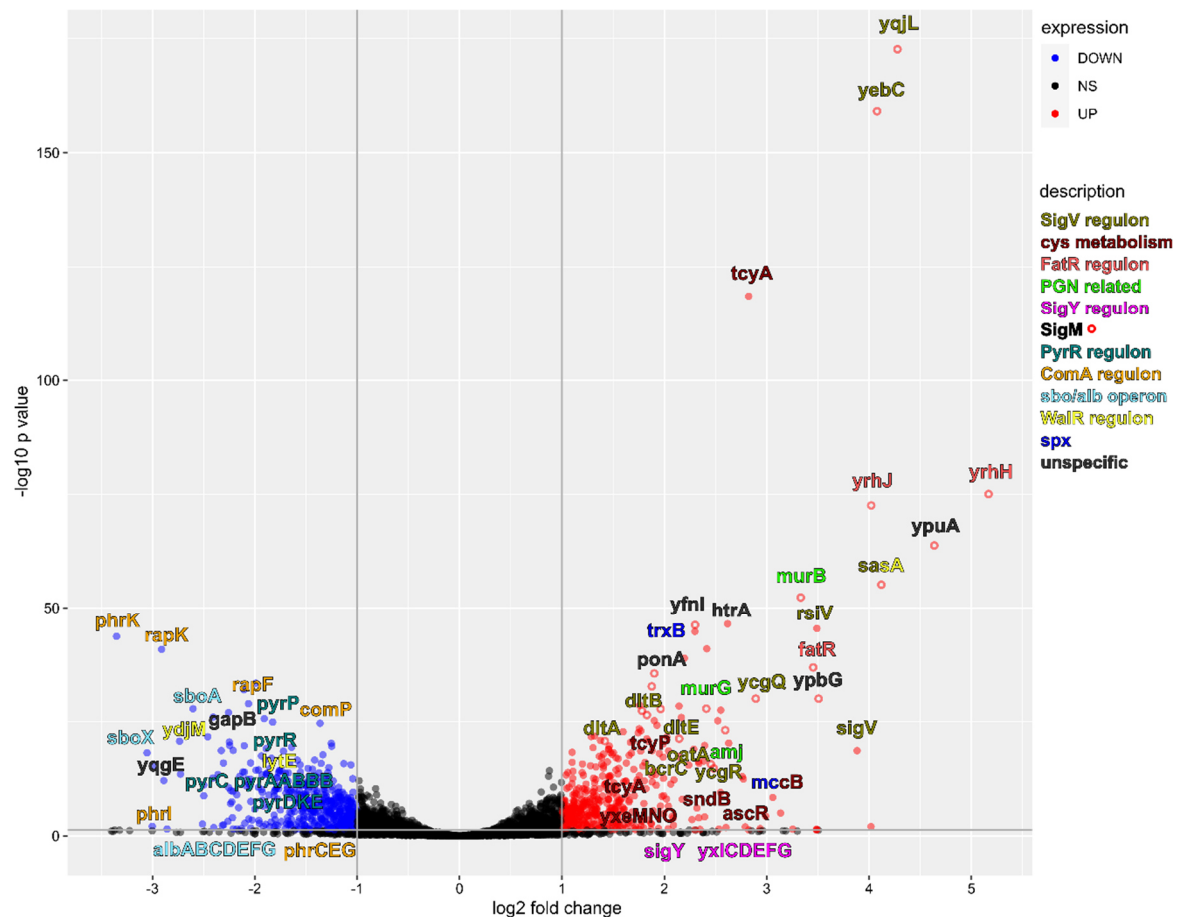


Figure 4: Scatter plot of the S2 transcriptome comparison of FOS treated and untreated *B. subtilis* wt. Shown are the differentially expressed genes of the conditions: wt without and with FOS. FOS treatment :128 $\mu\text{g mL}^{-1}$ after 3.5 h incubation, in the exponential growth phase (Fig.1, A3). Upregulated (UP) significant transcripts ($\log_2\text{fold change} > 1$, $p\text{-value} < 0.05$) are shown in red on the right side; downregulated (DOWN) transcripts are shown in blue on the left side. NS = not significant.

Of the 458 upregulated transcripts (A10, Z2 [32]), more than 52 are associated with cell envelope stress (controlled by the extracytoplasmic function (ECF) sigma factors Sig V, Y, M, W, X). In S2, in contrast to S1, the cells showed a strong stress response, probably related to the effect of FOS on PGN biosynthesis, resulting in a weakened cell wall and ultimately cell lysis. Particularly striking was the number of upregulated genes belonging to the SigV regulon (15 of 21), which confers resistance to cell wall lytic enzymes, and those belonging to the SigY regulon (5 of 6), which is in general upregulated in response to nutrient stress, production and resistance to the antibiotic subblancin, and is associated with Sp β prophage function. (Fig.4, Z1) [50, 51]. The SigV regulon, induced for example by lysozyme, confers resistance to lytic enzymes. SigM, responsible for adaptation to inhibitors of PGN synthesis, regulates 89 genes, 45 of which are upregulated under FOS [52]. Similarly, 18 of the 40 genes regulated by SigX, which are expressed to protect against antimicrobial peptides, were upregulated under FOS treatment. Also relevant to the cell wall is SigW, which basically ensures adaptation to membrane-active compounds. Compared to the other sigma factors, SigW was the least regulated cell wall stress regulator with 18 of 77 upregulated genes. Apparently, the sigma factors overlap so that a prominent gene such as *yqjL* (BSU23830) is regulated by SigM, W, V as a putative hydrolase involved in oxidative stress resistance. (Fig.4, Z1) [53]

The FatR regulon ((*yrhH* (BSU27180)) *fatR* (BSU27170)-*yrhJ* (BSU27160)), which was the most upregulated regulon in the S2 transcriptomics results, is controlled by SigM, W, X. FatR is a transcriptional repressor that is derepressed in the presence of particular fatty acids, e.g. unsaturated oleic acid, and causes an adaptive response to toxic fatty acids [54]. Two genes in this operon involved in energy balance related to lipid metabolism, *yrhH* (a potential methyltransferase that hydroxylates fatty acids, BSU27180) and *yrhJ* (NADPH-cytochrome P450 reductase, BSU27160), were highly upregulated under FOS treatment [55]. Interestingly, the FatR regulon is located next to the SigV operon, a second FOS-induced gene cluster. Hypotheses about interactions, e.g. that *fatR* expression is reduced in a *sigV* deletion mutant, cannot be substantiated.

The enzymes *murB* (UDP-*N*-acetylenolpyruvoylglucosamine reductase, BSU15230), which are directly involved in the peptidoglycan synthesis, *murG* (UDP-*N*-acetylglucosamine-*N*-acetylmuramyl- (pentapeptide) pyrophosphoryl-undecaprenol *N*-acetylglucosamine transferase, BSU15220) and *amJ* (*murJ* alternative, lipid II flippase, BSU04230) were significantly upregulated (Fig.4, Z1). In addition, a considerable number of genes associated with the biosynthesis or acquisition of cysteine were upregulated. These include the *tcy* operon (cystine and diamonpimelate transporter) or *sndB* (BSU39470), a *N*-acetylcysteine deacetylase involved in the detoxification of S-(2-succino)cysteine. Many of these genes are regulated by CymR (BSU27520) a pleiotropic regulator of sulfur metabolism [56].

In addition, many genes regulated by Spx were upregulated by FOS treatment (for clarity and mean log2fold changes, the hits in Fig.4 are not specifically labelled). Spx (BSU11500) regulates 50 genes (of which 25 are upregulated), particularly those related to heat and paraquat stress. Many of the hits overlapped with those of the Cys group described above, but others such as *trxB* (BSU34790, thioredoxin reductase (NADPH)), *msrAB* (BSU21690/BSU21680, methionine regeneration and restoration of protein function after oxidative damage) or *bshC* and *bshB2* (BSU15120/BSU19460, bacillithiol biosynthesis) were also visible. These and many other genes upregulated by Spx are associated with resistance to oxidative stress [57].

Looking at the downregulated transcripts under FOS treatment in the wt comparison (Fig.4, Z4 [32]), there are fewer outstanding hits in terms of fold change differences, but two operons were downregulated almost without exception. On the one hand, 9 of the 10 *pyr* operon genes involved in pyrimidine biosynthesis were downregulated. And the *sbo/alb* operon (production of anti-listerial bacteriocin (subtilisin)) was also completely downregulated. Other downregulated biosynthetic pathways included the RAP phosphatases *rapK* (BSU18910) and *rapF* (BSU37460) which regulate ComA (BSU31680, regulation of genetic competence and quorum sensing) and the associated regulators *phrK* (BSU18920), *phrI* (BSU05020), *phrC* (BSU03780) and *phrE* (BSU25840) [58]. Remarkably, genes important for cell wall metabolism and regulated by WalR (BSU40410) were downregulated. Mainly hydrolases such as *lytE* (BSU09420); *cwlO* (BSU34800), *yocH* (BSU19210), *ydjM* (BSU06250) and the cell division protein *ftsA* (BSU15280). PGN synthesis inhibited by FOS inevitably leads to a weaker PGN and the cell tries to compensate by downregulating degradation. The comparison just described is shown identically in Fig.5, but in this case with the $\Delta fosB$ mutant. Although the vast majority of up- and downregulated transcripts overlap (A10), differences were also identified.

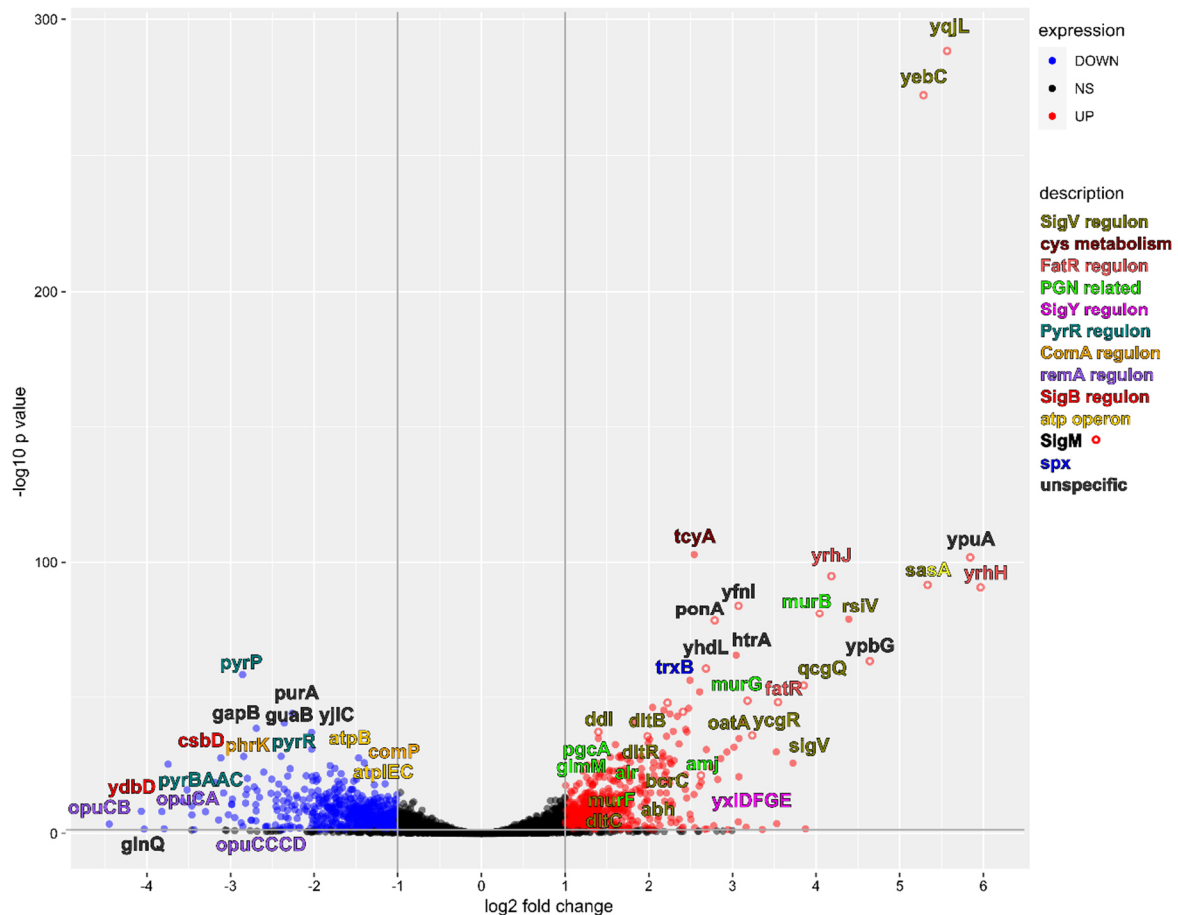


Figure 5: Scatter plot of the S2 transcriptome comparison of FOS treated and untreated *B. subtilis* $\Delta fosB$. Shown are the differentially expressed genes of the conditions: $\Delta fosB$ without and with FOS. FOS treatment: $16 \mu\text{g mL}^{-1}$ after 3.5 h of incubation, in the exponential growth phase (Fig.1, A3). Upregulated (UP) significant transcripts ($\log_2 \text{fold change} > 1$, $p\text{-value} < 0.05$) are shown in red on the right side; downregulated (DOWN) transcripts are shown in blue on the left side. NS = not significant.

Of the 537 upregulated transcripts (A10, Z4 [32]), 68 hits associated with cell envelope stress were upregulated in the $\Delta fosB$ mutant under FOS treatment.

Similar to the wt comparison described above, the corresponding sigma factor regulated hits were also highly represented individually. SigV (17 of 21) and SigY (7 of 7) were the most upregulated, SigM (55 of 89) and SigX (19 of 20) were highly upregulated and SigW (15 of 77) was the least upregulated. The influence of SigM (Fig.5, Z4 [32]), which also overlapped and included many of the highly upregulated hits, was particularly evident in the $\Delta fosB$ mutant, with 10 more upregulated hits than in wt (55 of 89). SigX, SigV and SigY again played a major role, and the cysteine metabolism genes (not all labelled) were also present, as well as those of the FatR regulon (Z4 [32]). Genes related to PGN synthesis, the UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamyl-*meso*-2,6-diaminopimeloyl-D-alanyl-D-alanine synthetase *murF* (BSU04570), the D-Ala-D-Ala precursor enzymes (*alr* BSU04640, *ddl* BSU04560) as well as *pgcA* (BSU09310) and *glmM* (BSU01770), which are important in the early phase of synthesis, were upregulated. The *pyr* regulon was downregulated in the $\Delta fosB$ mutant as in the wt (Z5, [32]). In addition to the wt hits, RemA, transcriptional regulator of the extracellular matrix genes, regulated genes (7 of 29) and also genes from the *atp* operon (4 of 9), negatively stringent response regulated, which are important for ATP synthesis, were downregulated.

The aforementioned overlap between the two *B. subtilis* strains wt and $\Delta fosB$ was highlighted by the evaluation of the scatter plot analyses. The upregulated genes and pathways were largely identical, especially the most upregulated genes, as shown in Fig.4 and Fig.5, but the analysis was also ensured by statistical overrepresentation of pathways (Z2, Z4 [32]). In addition to this systematic analysis, some other interesting hits were found among the transcripts with the highest increase or decrease that could not be classified into either pathways or associated operons. These include the upregulated SigM-related genes *ponA* and *yfnI* for wt and $\Delta fosB$. PonA is a bifunctional enzyme (BSU22320, glucosyltransferase/transpeptidase) that is a major class A penicillin-binding protein 1A/1B and thus significantly involved in cell shape [58]. YfnI (BSU07260) is the minor lipoteichoic acid synthase [59]. Important but not SigM regulated is the serine protease HtrA (BSU12900), which is regulated by CssR (control of cellular responses to protein secretion stress) [60]. In order to take all aspects into account, the results were interpreted and evaluated in the overall context in the summary (see below).

Proteome investigation of the lytic effect of FOS on B. subtilis (S2)

A strong advantage of the combined omic approach used in this study is the comparability: the transcriptomics results (described above) can be directly related to the proteomics results (described below), as the samples originate from the same biological experiment. In general, a high degree of overlap was observed. Many of the up- or downregulated transcripts were appropriately regulated at the protein level, as described below. In some cases, the expected differences due to transcriptional and post-transcriptional regulatory features (e.g. Spx stress response) also occurred.

In the proteome analyses of S2, 299 proteins in wt and 189 proteins in the $\Delta fosB$ mutant were up- and overrepresented under the influence of FOS compared to the respective untreated control (Z1 [32], A12). Of these, 84 hits overlapped. A quarter of these were stress proteins of the cell envelope (controlled by SigM, V, W, X, Y). The lytic effect and cellular stress were clearly visible. Interestingly, no SigY regulated protein was found (wt and $\Delta fosB$ mutant independent), whereas up to half of the proteins controlled by the other sigma factors were upregulated (Z6, Z7 [32]) [61].

Although the number of significant overlapping proteins was not the majority in wt and $\Delta fosB$ mutant, the relevant hits were in this group. The most upregulated protein in wt, as in the $\Delta fosB$ mutant, was Spx (BSU11500), a transcriptional regulator of 52 genes that plays an important role in preventing protein aggregation during heat stress and protecting against paraquat stress (oxidative stress) (Fig.6, Fig.7, Z6, Z7 [32]). Spx is regulated by SigW, X, M and others. Consequently, other Spx-regulated proteins were found among the upregulated proteins in wt and $\Delta fosB$ mutant (wt:7, $\Delta fosB$:8, Z1 [32]). Spx is proteolytically regulated by the adaptor protein YjbH and the protease ClpXP. Oxidative and proteolytic stress leads to aggregation of YjbH and rapid accumulation of Spx. In addition, Spx SigM is regulated and transcriptionally upregulated during cell wall stress together with *yirB*, which acts as an anti-adaptor for YjbH, thereby stabilising Spx. In addition, *spx* can be transcriptionally upregulated by the redox status of the cell (formation of a disulfide bond by oxidation of cysteines at the N-terminus). [57, 62, 63, 64]

The proteins of lipoteichoic acid biosynthesis (LTA) were prominent in Fig.6 and Fig.7. In addition to the two main synthases LtaS (BSU07710) and YfnI (BSU07260), the primase YvgJ (BSU333609) with potential synthase function was one of the most upregulated and significant proteins [59, 65]. Besides LTA, other proteins involved in fatty acid metabolism were upregulated. On the one hand, there was the *fat* regulon (*fatR* BSU27170, *yrhJ* BSU27160, *yrhH* BSU27180). On the other hand, almost the entire 10-gene regulon (*wt*:6, Δ *fosB*:5, Z1 [32]) was among the highly upregulated proteins, especially the synthases (FabHB BSU11330, FabHB BSU10170, FabF BSU11340) and the transacylase (FabD BSU15900) [55, 66].

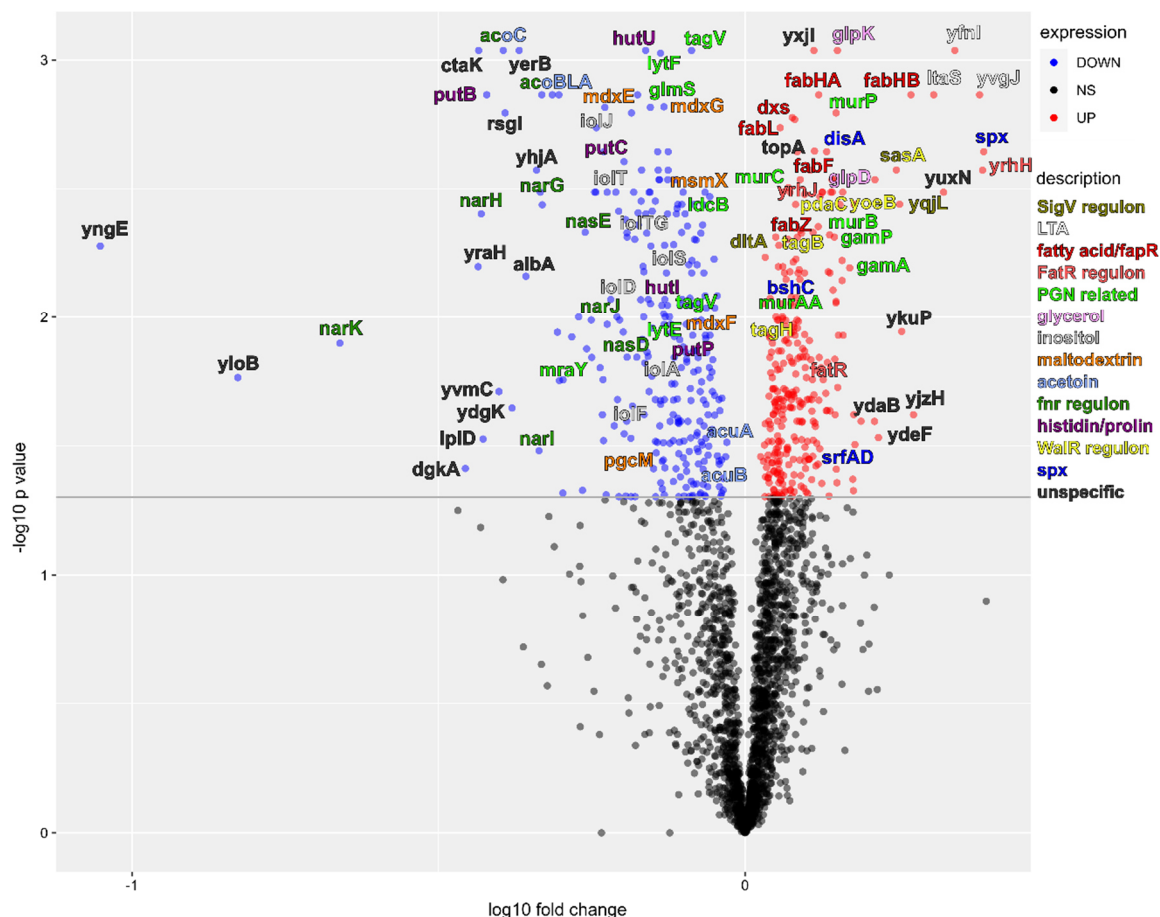


Figure 6: Scatter plot of the S2 proteome comparison of FOS treated and untreated *B. subtilis* wt. Shown are the detected proteins present in different amounts in the conditions: wt without and with FOS. FOS treatment: 128 $\mu\text{g mL}^{-1}$ after 3.5 h of incubation, in the exponential growth phase (Fig.1, A3). Upregulated (UP) significant transcripts ($p\text{-value} < 0.05$) are shown in red on the right side; downregulated (DOWN) transcripts are shown in blue on the left side. NS = not significant.

Another relevant group that was upregulated under FOS treatment in both wt and $\Delta fosB$ mutant was related to PGN and the cell wall. Some PGN *de novo* synthesis genes (MurAA BSU36760, MurB BSU15230, MurC BSU29790 (wt only)) were upregulated, which contain the FOS target protein or proteins directly related to MurAA. In addition to the PGN synthesis proteins, there were also associated proteins such as the glucosamine and *N*-acetylmuramic acid permeases GamP (BSU02350) and MurP (BSU01680) or the glucosamine-6-phosphate deaminase GamA (BSU02360) or the phosphoglucosamine mutase GlmM (BSU01770). A (stress) response of the cell wall and an attempt by the cell to compensate for the unavailability of precursors was evident. [67, 68]

In addition to the overlapping proteins, there were also proteins that were only upregulated in wt under FOS treatment (S2), and the corresponding pathways were statically overrepresented here (Z6, [32]). In particular, the alditol metabolism, here the glycerol-3-phosphate dehydrogenase GlpD (BSU09300) and the glycerol kinase GlpK (BSU09290) were strongly overrepresented (Z6 [32]) and these proteins also showed high significant fold changes (Fig.6) [69]. In addition, not only LTA proteins but also the metabolic pathway of proteins related to teichoic acid biosynthesis were significantly upregulated. A group that can be summarised under RNA modifications (tRNA modifications, tRNA and rRNA processing) was statistically overrepresented in the wt under FOS conditions (without high fold changes of individual proteins) (Z6 [32]). In contrast, when the $\Delta fosB$ mutant was analysed individually, pathways associated with pyrimidine biosynthesis (Z7 [32]) were significantly overrepresented. In particular, some proteins regulating the stringent response were upregulated, as well as proteins of the Css regulon (HtrA BSU12900, HtrB BSU33000, CssS BSU33020), which is associated with protein secretion stress and Spx. [60, 70]

When analysing the proteins downregulated by FOS treatment in S2, there was also a clear overlap between wt and the $\Delta fosB$ mutant. 278 proteins in wt and 188 proteins in the $\Delta fosB$ mutant were downregulated under the influence of FOS, with an overlap of 134 hits (Z1 [32], A12).

The proteins with the highest downregulated fold change are shown in Fig.6 and Fig.7. Among these proteins, some pathways were also significantly overrepresented in both wt and $\Delta fosB$ mutant (Z9, Z10 [32]). Most downregulated proteins with statistically overrepresented pathways were associated with catabolic processes. For example, the utilisation of alcohols (inositol, acetoin, polyol of inositol) or amino acids (histidin (mainly the degradation enzymes to glutamate and formates, HutU BSU39360, HutH BSU39350), proline). The utilisation and uptake of maltodextrin was downregulated in relation to the fold changes and the pathway was statistically overrepresented. Specifically downregulated in wt was the *fnr* regulon (regulation of anaerobiosis, fermentation and overflow metabolism), which includes the acetoin (*aco*) operon, which is completely upregulated except for one protein (Z8 [32]). In the $\Delta fosB$ mutant, in addition to the described catabolic processes, arginine utilisation is clearly overrepresented among the downregulated proteins (Z9 [32]). In order to take all aspects into account, the results were interpreted and evaluated in the overall context in the summary.

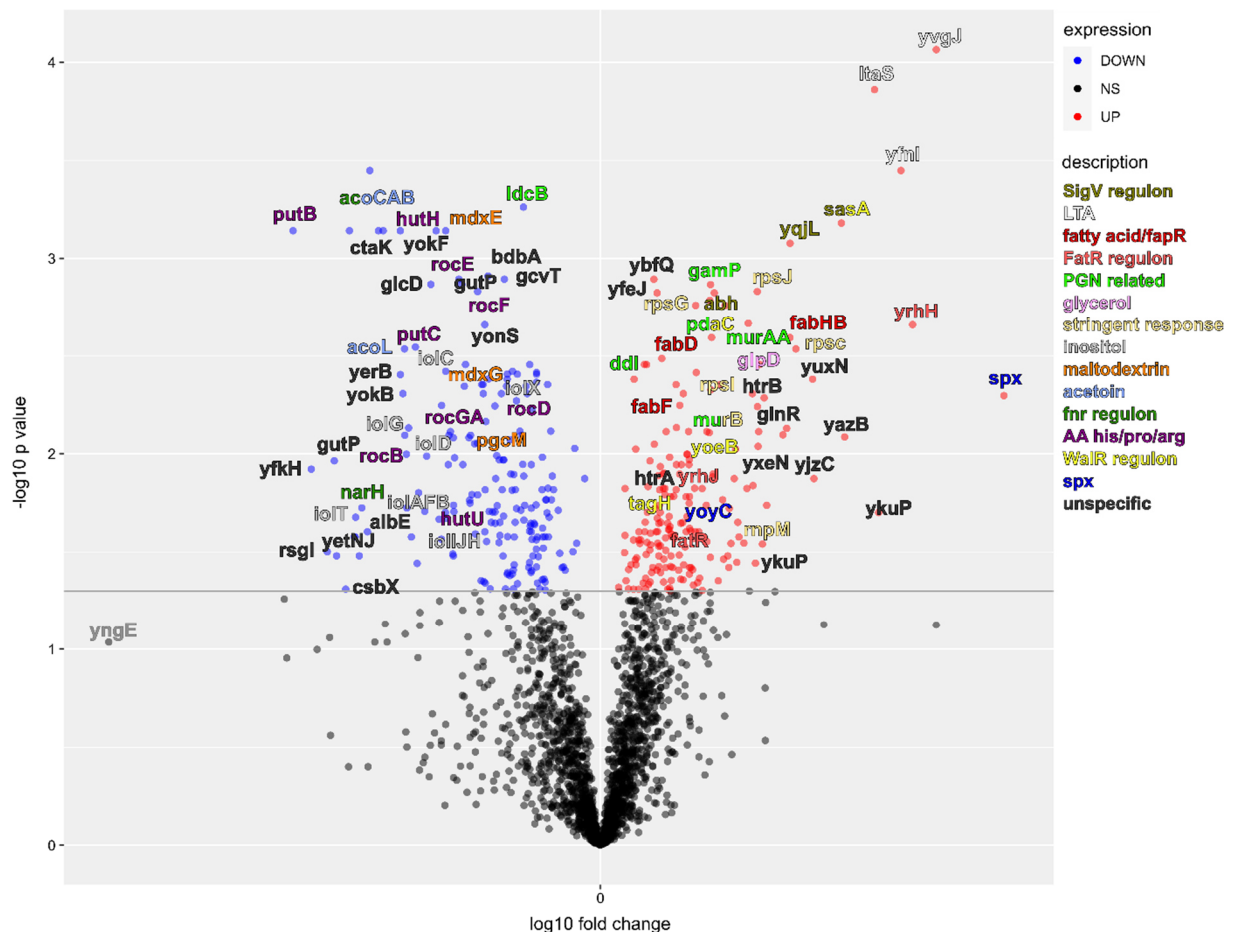


Figure 7: Scatter plot of the S2 proteome comparison of FOS treated and untreated *B. subtilis* $\Delta fosB$. Shown are the detected proteins present in different amounts in the conditions: $\Delta fosB$ without and with FOS. FOS treatment: $16 \mu\text{g mL}^{-1}$ after 3.5 h of incubation, in the exponential growth phase (Fig. 1, A3). Upregulated (UP) significant transcripts ($p\text{-value} < 0.05$) are shown in red on the right side; downregulated (DOWN) transcripts are shown in blue on the left side. NS = not significant.

*Comprehensive analysis of transcriptomic and proteomic analyses to investigate the lytic effect of FOS on *B. subtilis**

Combined analysis of the transcriptome and proteome is a powerful and important approach, especially when, as in this case, the samples for both technologies originate from the same biological culture. This comparison is challenging because the cellular state at exactly the same time (which is experimentally provided by identical cultures) does not necessarily show the same effect. There are regulations at different levels of the cell and the time lag between transcription and protein biosynthesis has to be taken into account. Furthermore, absolute comparability is not given, as method-specific differences cannot be reduced to the same denominator without error. With the OmicsTIDE tool, cross-method clustering is possible, which makes the promising comparison of transcriptome and proteome results possible despite all the challenges [44].

OmicsTIDE compared the S2 trends (up- or downregulated) for the wt and the $\Delta fosB$ mutant simultaneously (see method section for details). This four-way trend (wt and $\Delta fosB$ mutant each with and without FOS) of a protein (Fig.8, left) was compared to the identical four-way trend of a gene (Fig.8, right) and clustered according to similarity, depending on the settings. The visualisation as a Sankey diagram (Fig.8) provides a good overview of the similarities and differences between the technologies.

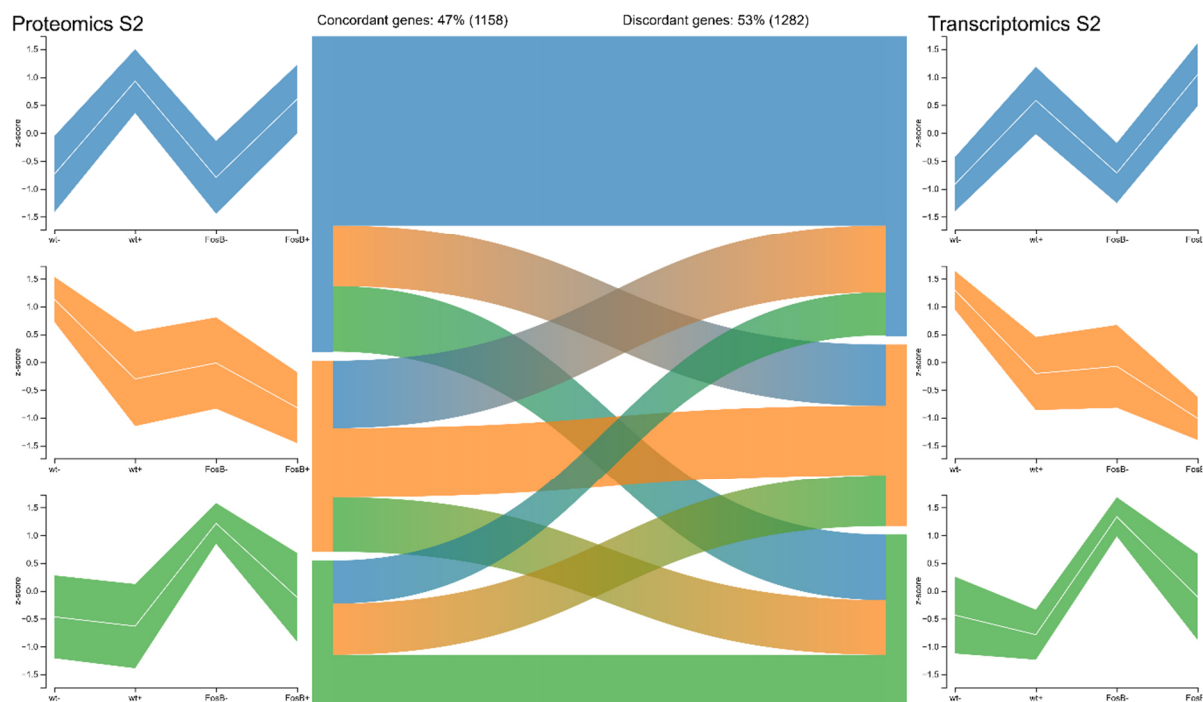


Figure 8. Sankey diagram of the omics trend-comparing analysis of proteome and transcriptome data from S2. Transcriptome and proteome analyses of the strains: *B. subtilis* 168 (*trpC2*) wt, *B. subtilis* (*trpC2*) $\Delta fosB$. FOS treatment with $128 \mu\text{g mL}^{-1}$ (wt) and $16 \mu\text{g mL}^{-1}$ ($\Delta fosB$) for S2 (after 3.5 h of incubation, in the exponential growth phase). Analysis of the intersecting hits with 3 clusters ($k=3$), shown as centroid profile plots in the individual conditions. Data: Z10, Z11 [32].

All results from each technology were compared independently of fold changes using trend clustering (see method section for details). For S2, 2440 hits were identified that could be detected as both protein and transcript. Proteins (5) or transcripts (1856) for which the corresponding gene or protein could not be identified by the other technology were not considered. Of the matched pairs, 1158 pairs were concordant (identical trend pattern in proteome and transcriptome, 47 %) and 1282 pairs were discordant (different trend pattern in proteome and transcriptome, 53 %).

The blue trend in Fig.8 shows protein-gene pairs that were upregulated under FOS treatment in both omics analyses, in the wt and in the $\Delta fosB$ mutant (29 %, Z12 [32]). In contrast, the orange and green clusters each represent downregulation, with a stronger trend difference for either the wt (orange, Z13 [32]) or the $\Delta fosB$ mutant (green, Z14 [32]).

The most meaningful cluster for the analysis of the cellular responses of *B. subtilis* to FOS is, as described, the intra-cluster of blue (Fig.8). If this cluster was restricted to only significant ($p\text{-value} < 0.05$) hits in both technologies, 37 highly relevant protein-gene pairs remain (Z15 [32]).

The relevance of this group and the quality of the analysis as well as the accuracy of the datasets were further demonstrated by the fact that a comparison of the overlapping hits of the individual analyses using Venn comparisons (A10A and A12A) left the identical 37 hits.

15 of these hits can be associated with cell envelope stress and 13 of the 37 were SigM controlled (Z15 [32]) [52]. Among them, the transcripts and proteins of fatty acid and lipid metabolism were strongly overrepresented (*cypB* BSU27160, *fabHB* BSU10170, *fabF* BSU11340, *ypbG* BSU22980) [55, 66]. Many of the 37 hits also fell into the category of cell wall and peptidoglycan organisation (*ltaS1* BSU07260, *murB* BSU15230, *yusF* BSU32780, *topA* BSU32780, *ywqF* BSU36230, *pbpA* BSU25000). Individual up-regulated hits were associated with teichoic acid (*tagH* BSU35700), glycerol-3-phosphate (*glpD* BSU09300), salicylic acid (*dhbC* BSU31990) or siderophore synthesis (*dhbA* BSU32000, *dhbC* BSU31990) (Z15 [32]) [59, 65, 67, 68, 69, 71].

To analyse the downregulated hits in detail, a more detailed clustering (k=5) was performed (A14). This more specific clustering of trends results in the green cluster (A14, Z16 [32]) with a group of protein-gene pairs that are downregulated under FOS in the wt and in the $\Delta fosB$ mutant. Restricting this cluster to significant (p-value <0.05) hits in both technologies resulted in 33 highly relevant protein-gene pairs (Z17 [32]). Among these 33 candidates were hits from the category cell wall degradation (*lytF* BSU09370, *lytA* BSU35640, *yrvJ* BSU27580, *wprA* BSU10770), catabolic processes, utilisation of sugars, peptides and amino acids (*malL* BSU34560, *pgcM* BSU34550, *gutB* BSU06150, *gutP* BSU06160, *appF* BSU 11370, *appC* BSU11400, *ald* BSU31930, *yrvJ* BSU27580, *putC* BSU03210, *rocB* BSU37770, *kbl* BSU17000, *iolJ* BSU39670) and hits related to the biosynthesis of antibacterial compounds (*pksS* BSU17230, *pksD* BSU17110). [66, 72, 73, 74, 75, 76]

In order to take all aspects into account, the results were interpreted and evaluated in the overall context in the summary. An obvious and expected effect of the addition of FOS levels leading to a lytic effect in *B. subtilis* was a stress response focused on cell envelope stress, and these stress responses were clearly identifiable. The numerous hits in other pathways, operons and regulatory networks lead to many other hypotheses about the cellular effects of FOS (see summary). One way to identify effects that are due to FOS and not due to other stress response that may have been previously induced by FOS is to consider S1.

Investigating the adaptation of *B. subtilis* to FOS using a multi-omics approach

The concentrations of FOS used in setup 1 allow the investigation of cellular effects of *B. subtilis* wt and $\Delta fosB$ mutant, which have no physiological effect and do not lead to cell lysis (Fig.1). Consequently, these effects alone are of limited significance, as an antibiotic used alone is only relevant if it leads to lysis or at least inhibits growth.

For this reason, the comparison between S1 and S2 is of particular interest, as the addition of an analysis without superimposed stress reactions makes the overlapping hits in both setups extremely relevant and provides valuable information on the cellular effects and interactions of FOS to which the cell adapts on the one hand, but which are also involved in cell lysis on the other.

In order to properly compare S1 with S2, a brief description of the transcriptome and proteome analysis of S1 is necessary. The procedure was identical to that described above for S2.

Transcriptome investigation of B. subtilis adaptation to FOS (S1)

Comparing the transcriptome analyses of S1 and S2, not only the total number of differentially expressed genes for wt (237 upregulated and 399 downregulated) and $\Delta fosB$ mutant (146 upregulated and 138 downregulated) was significantly lower by half, but also the overlapping hits (upregulated: 11, downregulated: 23) were only a minor part, compared to S2 (A9, A10). The less clear difference between the conditions treated with FOS compared to the respective controls, as already described for Fig.2, makes a comparison between the same conditions without FOS and with FOS between wt and $\Delta fosB$ mutant essential. This is described in A15.

As expected, among the upregulated transcripts, no hits were associated with a stress response, neither in wt nor in the $\Delta fosB$ mutant (Z18, Z19 [32]). Among the 16 upregulated S1 transcripts overlapping between wt and $\Delta fosB$ mutant (Z18, Z19 [32]), genes for ribosomal proteins and mannitol utilisation were predominantly found, in addition, a SigW-regulated gene *yrhH* (BSU27180) (adaptation to membrane-active compounds and cell wall-active antibiotics) and the statistically most overrepresented genes *leuB* (BSU28270) and *leuC* (BSU28260), which are involved in leucine biosynthesis, were found. In addition, transcripts involved in pyrimidine biosynthesis and utilisation were strongly upregulated and statistically overrepresented in the wt (Z18 [32]). In contrast, transcripts involved in the metabolism of branched-chain amino acids (leucine, valine, isoleucine), secondary alcohols, fatty acid biosynthesis or related pathways, but also catabolic processes (acetoin, histidine, ketone) were upregulated in the $\Delta fosB$ mutant (Z19 [32]).

The metabolic adaptation of *B. subtilis* to FOS treatment was not only indicated by the described upregulated transcripts, but also by the downregulated transcripts. Among the downregulated hits in wt were many pathways regulated by CcpA (Carbon Catabolite Control Protein A), such as the transport and degradation of maltose and maltodextrin, but also amide, inositol and peptide degradation pathways. The biosynthesis of amino acids (e.g. arginine) was also downregulated (Z20 [32]). Among the downregulated transcripts in the $\Delta fosB$ mutant, no pathways were significantly overrepresented, only 3 genes of the melibiose utilisation and transport pathways, which are also present in the wt, stood out (Z21 [32]).

Proteome investigation of B. subtilis adaptation to FOS (S1)

In the proteomics analyses, S1 behaves identically to the transcriptome analyses. Compared to S2, significantly fewer differentially occurring proteins were identified for the wt (126 upregulated and 151 downregulated) and the $\Delta fosB$ mutant (91 upregulated and 58 downregulated) and the overlap was also lower (upregulated: 20, downregulated: 22) (A11, A12).

As for the transcriptome analyses, the comparison between the same conditions without and with FOS between wt and $\Delta fosB$ mutant is described in A16.

Proteins associated with cellular stress could not be detected in S1, neither in wt nor in the $\Delta fosB$ mutant. Among the highly upregulated proteins in the wt, many proteins were associated with cellular sporulation and metabolic pathways were statistically overrepresented. In addition, pyrimidine biosynthesis and utilisation was significantly overrepresented (Z22 [32]) [77]. There were no significantly overrepresented, overlapping pathways between wt and $\Delta fosB$ mutant, although pyrimidine biosynthesis proteins were found as not significant hits in the $\Delta fosB$ mutant. The highly upregulated and statistically overrepresented proteins and pathways in the $\Delta fosB$ mutant were associated with carbohydrate metabolism (but also organic acids or inositol), the carbohydrate transport (especially the phosphoenolpyruvate-dependent sugar phosphotransferase system), and alcohol and polyol catabolism and metabolism.

In contrast, all significantly overrepresented pathways of downregulated proteins overlapped between wt and $\Delta fosB$ mutant (Z24, Z25 [32]). The hits were related to amino acid metabolism, in particular proline, glutamine and arginine. In addition, the pathways of inositol metabolism and catabolism, fatty acid catabolism and the tricarboxylic acid cycle were over-represented (Z24, Z25 [32]).

Comprehensive analysis of transcriptome and proteome analyses to investigate the lytic effect in combination with the adaptation of *B. subtilis* to FOS.

To analyse the protein-gene pairs that are identically regulated in both setups, in the wt and in the $\Delta fosB$ mutant, the OmicsTIDE [44] analysis had to be performed with S1 identical to S2.

Although the basic conditions in terms of intersecting hits (2698, non-intersecting: 6 proteins, 1613 transcripts) or concordant pairs (45%) hardly differ from S2, the clustering shows the effect already described for Fig.2 and Fig.3, that those of S1 were internally significantly more similar than those of S2 and thus fewer unique clusters arise (A17). Clearly upregulated (violet, A18) or downregulated (red, A18) protein-gene pairs could only be identified with more precise clustering (k=5) (A18). Compared to S2, the difference between the two setups is again obvious.

While in S2 there was a high number of hits between the identical trends (e.g. blue, A14), in S1 the majority of the up- or downregulated pairs (red and violet, A18) are not paired with the corresponding identical cluster in the other omics analysis. A comparison of the protein-gene pairs upregulated under stress conditions (S2) (Fig.8, blue-blue (Z12 [32]) with the upregulated hits of the stress-independent cell response (A18, violet-violet, Z29 [32]) shows hits that are upregulated in *B. subtilis* by the influence of FOS independently of stress responses and thus characterise the cellular response of adaptation in combination with cell lysis (Z30 [32]). 13 hits resulted from this analysis. The multiple fatty acid related hits were relevant (*fabHB* BSU10170, *fabL* BSU08650), as were the teichoic acid related hits (*dltE* BSU38540). There were also hits from methionine salvage (*mtnE* BSU13580), cell redox related (*ydbP* BSU04550) and thiamine acquisition (*tenA* BSU11650) [55, 66, 78]. Many hits were uncharacterised (Z30 [32]).

Similarly, looking at the downregulated clusters of S1 (A18, red-red, Z28 [32]) and S2 (A14, green-green, Z14 [32]) resulted in 6 hits. These hits include protein quality control (*wprA* BSU10770), citrulline utilisation (*rocB* BSU37770) and an electron transport chain regulator (*resD* BSU23120). [74, 75]

Another approach to study the cellular response of *B. subtilis* to FOS is to compare the two setups within an omics technology. In the transcriptome, only one transcript overlapping in S1 and S2 was upregulated (a potential methyltransferase that hydroxylates fatty acids, *yrhH* BSU27180) and two transcripts were downregulated. A nitrate reductase gene (*narG* BSU37280) and a gene involved in the regulation of anaerobic genes (*arfM* BSU37290). In the proteome, there was one upregulated protein (uncharacterised, YizC BSU11139) that overlapped between S1 and S2. 9 proteins were downregulated, including, remarkably, 5 from the arginine/ornithine utilisation category (RocA BSU37780, RocD BSU40340, RocE BSU40330, RocF BSU40320, RocG BSU37790), one from acetoin utilisation (AcoB BSU08070) and a general stress protein (YwsB BSU35970). *AcoB*, *narG* and *arfM* are all under the control of Fnr [79, 80, 81].

Based on the analytical investigation of the cellular response of *B. subtilis* to FOS under lytic and stress-free conditions carried out in the previous sections, a comprehensive picture of the hypotheses will now be drawn.

Summary of cellular responses of *B. subtilis* to FOS revealed by multi-omics

The presented multi-omics data allow the assessment of the cellular response of *B. subtilis* to FOS at different regulatory levels, providing a global view. The quality of the samples appears to be excellent, as the coverage of the *B. subtilis* proteome and transcriptome was comprehensive. The behaviour of the replicates clearly demonstrates the validity of the samples and setups.

The choice of setups allows a clear distinction between the stress-induced lytic FOS response (S2), independently confirmed by metabolomics results such as the accumulation of UDP-GlcNAc, the substrate of the primary target MurA inhibited by FOS, and the metabolic adaptation to FOS (S1). Included, but with potential for further conclusions, was the timing of FOS addition, which differs significantly (S1 from the start, S2 in the exponential growth phase). The fact that the proteome and the transcriptome were analysed from the identical biological culture provides extremely powerful results. The extension by the *B. subtilis* $\Delta fosB$ mutant provides another set of replicates and the opportunity to analyse the potent FOS detoxification mechanism (fivefold difference in sensitivity) of the thiol S-transferase FosB. The results of each omics technology clearly confirmed the expected characteristics of the setups through heatmaps and scatter plots. In addition, the OmicsTIDE and Venn analyses showed how comparable and overlapping the technologies and setups were in most cases, allowing differentiated methodological and setup specific conclusions to be drawn.

FOS causes cell envelope stress under the influence of SigM, Y, W, X, Y

The transcriptome, proteome and metabolome clearly show that FOS induces cell envelope stress. The metabolomics studies (A13) showed the accumulation of UDP-GlcNAc, the substrate of irreversibly inhibited MurA, and a decrease in the levels of downstream peptidoglycan precursors, independent of the other technologies. Most of the transcripts and proteins upregulated in S2 were regulated by SigM, V, W, X, Y, with the exception of SigY, which was almost completely upregulated, but only in the transcriptome, the genes and proteins regulated by the other sigma factors were present in significant numbers in both the transcriptome and the proteome.

It was therefore a comprehensive cellular stress response involving all the sigma factors mentioned, in contrast to some publications that only attribute an influence on SigM, W and X to FOS. In particular, lysozyme activated SigV was not only almost completely upregulated (especially in the transcriptome), but also represented the most highly upregulated hits. [50, 53, 82, 83, 84]

B. subtilis regulates the cellular FOS stress response via Spx, thereby initiating an oxidative stress response

The transcriptional regulator Spx, which is mainly upregulated during heat, disulfide and paraquat stress (oxidative stress), controls 52 genes. FOS activates the cellular Spx response, as previously described and clearly confirmed by these experiments. Spx was the most upregulated protein in the proteomics analyses and also in the transcriptome, about half of the Spx-regulated transcripts were upregulated. As described above and shown by the results, Spx is stabilised in the cell under FOS and thus has a considerable influence. Spx itself is regulated by SigM and SigW but accumulates under these conditions mainly due to lack of degradation. In addition to the activation of Spx by sigma factors, Spx can also be activated directly by the redox status of the cell, as described above. Regardless of whether Spx induces an antioxidant response or whether oxidative stress additionally activates Spx, a cellular response to oxidative or disulfide stress could be identified under the influence of FOS. The fact that the redox status of the cell is out of balance or that the cell senses oxidative stress was indicated by the fact that the number of genes associated with oxidative stress was very high under Spx, but also by the fact that others, such as *yqjL* (general stress protein, BSU23830), were among the most differentially expressed hits in both technologies. In particular, the oxidoreductase thioredoxin plays an important role, as not only did the Spx-regulated *trxA* (BSU28500) and *trxB* (BSU34790) stand out, but excitingly, *ydbP* (BSU04550) was even more significant as it was upregulated in both S1 and S2. *B. subtilis* adapts its metabolism and needs reducing agents to cope with FOS. [52, 57, 62, 63, 64, 85, 86, 87, 88]

FOS stress leads to differential regulation of various players associated with the PGN

Further investigation of the described cell envelope and Spx stress response to FOS necessarily leads to cell wall and peptidoglycan stress. With partially overlapping, but also independently differentially expressed hits, the influence of FOS on PGN synthesis, but also on cell wall homeostasis including degradation, became clear based on the group of the regulated peptidoglycan-related hits. Both in the proteome and in the transcriptome, important enzymes of the PGN *de novo* synthesis were upregulated (see previous analysis for details), the most important hit being the UDP-*N*-acetylenolpyruvoylglucosamine reductase MurB (BSU15230). In the proteome, the number of upregulated PGN *de novo* synthesis was higher, including the FOS target MurAA (BSU36760) and related enzymes such as the glucosamine-specific permease GamP (BSU02350), *N*-acetyl-muramic acid permease MurP (BSU01680), glucosamine-6-phosphate deaminase GamA (BSU02360) or the class B penicillin-binding protein PBP 2A PbpA (BSU25000). *B. subtilis* attempts to compensate for cell wall problems by importing and synthesising PGN precursors. This effort was further evidenced by the attempt to reduce PGN degradation. [67, 68, 89, 90, 91, 92]

Prominent autolysins (*lytE*, BSU09420, *lytF* BSU09370, *yocH* BSU19210), peptidases (*cwlS* BSU19410, *ldcB* BSU19620) and amidases (*yrvJ* BSU27580) are downregulated in both omics studies. Interestingly, the secreted quality control protease WprA was also an overlapping hit with S1 and one of the few common downregulated hits for both omics studies in S1 and S2 and thus stress-independent regulated. [67, 68, 89, 90, 91, 92]

FOS leads to increased lipoteichoic acid (LTA) and teichoic acid (TA) synthesis

TA, especially LTA, are of particular importance in FOS treatment. In the proteome, three of the four proteins of the LTA synthase family were among the most upregulated hits (*ltaS* BSU07710, *yvgJ* BSU33360, *yfnI* BSU07260). Not only the YfnI synthase, which is regulated as a stress enzyme by SigM, but also the LtaS homeostasis synthase and the YvgJ primase, which are not only regulated by a stress response. The ABC transporter for teichoic acid translocation (ATP-binding protein) TagH (BSU35700) and Udg (*ywqF*, BSU36230), a UDP-glucose dehydrogenase producing therefore one of the precursors of teichuronic acid, were upregulated in the proteome and transcriptome. Furthermore, DltE was one of the upregulated hits in both the proteome and transcriptome in S1 and S2. The *dlt* operon is responsible for the D-alanine esterification of LTA and wall teichoic acid (WTA), although the role of *dltE* has long been unclear. It is clear that DltE performs alanylation of teichoic acid, which can even lead to resistance to positively charged antimicrobial peptides. It is also thought that excessively long LTAs promote cell lysis (as seen in *Staphylococcus aureus*). In combination with the attempt of the cell to compensate for the problems in the cell wall by inhibiting PGN synthesis through LTA synthesis, the cell paradoxically ensures that it lyses more easily. Interestingly, this can be shown by independent growth experiments with LtaS mutants. In the absence of LtaS, and therefore with shorter LTA chains, FOS-treated cultures lyse less. This is not the case for YfnI, which probably takes over the LtaS function in this case. [50, 59, 65, 78, 93, 94, 95]

FOS leads to metabolic adaptation in the form of upregulation of fatty acid synthesis (fatR and fapR)

In addition to teichoic acids, *B. subtilis* showed an adaptation to an upregulation of fatty acid synthesis under the influence of FOS. The NADPH cytochrome P450 reductase YrhJ (BSU27160, SigM, W, X regulated) and its repressor FatR (BSU27170) were prominent in the proteome and transcriptome in S2. In addition, the methyltransferase YrhH (BSU27180, regulated by SigM, W, X and possibly also SigV), which is located in the same operon, was also prominent in both setups. The *fatR* regulon is considered to be a detoxification system for harmful fatty acids. In particular, in the proteome S2, more than half of the eleven fatty acid synthesis genes under FapR control were upregulated. This pathway was further highlighted by the fact that the beta-ketoacyl-acyl carrier protein synthase III FabHB (BSU10170) and the enoyl-acyl carrier protein reductase FabL (BSU08650) were also upregulated in the adaptive stress-independent S1 proteome and transcriptome analysis. The upregulation of these pathways is plausibly due to an imbalance in the energy metabolism of the cell. Numerous cofactors (NADPH, CoA) are involved, which play an important role in many metabolic pathways. An energy imbalance in the cell favours cell lysis. In addition, an imbalance in fatty acid synthesis leads to suboptimal membrane fluidity, which also favours lysis.[55, 66, 78, 96, 97, 98]

FOS interacts with iron homeostasis

Two enzymes involved in the biosynthesis of the siderophore bacillibactin (*dhbA* BSU32000, *dhbC* BSU31990) were prominent overlapping hits in the cellular stress response (S2). The cell attempts either to restore the iron balance or to stabilise the energy balance via NADH, which is involved in the *DhbA* reaction. [99, 100, 101]

The pyrimidine biosynthesis is downregulated by FOS.

PyrR regulates ten genes that together form an operon for pyrimidine biosynthesis. Nine of these genes were downregulated in the transcriptome under stress conditions (S2). Not worth mentioning compared to the other summary results, but the *pyr* operon was the only relevant group that was differentially regulated in wt and in the $\Delta fosB$ mutant. An interaction between a pyrimidine-like active site inhibitor and FosB has been described in the literature, suggesting that FosB levels, rather than the presence of FOS itself, may be critical for the *pyr* operon. The genes were upregulated in wt in the presence of FosB. It is possible that this effect is similar to that seen when little FosB is present at high FOS levels. Surprisingly, S1 of both omics studies, in which the genes of the operon were significantly overrepresented and upregulated, adds another facet to the consideration. In wt, the *pyr* operon was upregulated compared to the $\Delta fosB$ mutant. This was also the case at low levels of FOS in the intra strain comparison; at high levels of FOS this is reversed, and the operon is downregulated. [77, 102]

FOS downregulates numerous catabolic cellular processes

FOS has been shown to cause an imbalance in metabolic pathways at specific points in the metabolism. Several catabolic processes, such as the utilisation of sugars, peptides and amino acids, were downregulated. Interestingly, this occurs even at low concentrations of FOS. Much of this overlaps in the proteome and transcriptome and was specific within the setups. The cause-and-effect relationship is not trivial, but it appears that even at low concentrations (S1), FOS can induce changes in metabolic pathways that appear to be independent of general stress responses (S2). An example of an enzyme that was specific across omics methods and setups is the *N*-carbamoyl-L-ornithine hydrolase *RocB*, which is involved in the utilisation of the amino acids arginine, citrulline and ornithine to provide C, N and energy. In all analyses, although not always perfectly overlapping, the pathways for the utilisation of the amino acids arginine, citrulline and ornithine (*roc*...) were downregulated. Similarly, the expression of pathways for the utilisation of alanine/serine (*ald* BSU31930), sorbitol/glucitol (*gut*...), inositol (*iol*...), proline (*put*...), histidine (*his*...) acetoin (*aco*...), dipeptide (*app*...), maltodextrin (*mal*...) was reduced. All of these pathways play a role in central carbon metabolism, producing or consuming key metabolic compounds such as coenzyme A, NADH, 2-oxoglutarate or dihydroxyacetone phosphate.

By reducing the number of pathways, the cell is presumably attempting to downregulate the overall metabolism and be less susceptible to imbalances through fewer processes. Only in the proteome, but in both setups, genes of the *fnr* regulon were found to be downregulated. This large regulon controls the expression of proteins required for anaerobiosis, fermentation and overflow metabolism. It is currently unclear whether the lack of evidence for *fnr* regulation in the transcriptome data is due to exclusive regulation at the protein level.

There were hardly any pathways that were upregulated under FOS. On the one hand, glycerol utilisation (*glp...*) was upregulated under stress (S2) in both omics studies, which is also directly related to dihydroxyacetone phosphate or glycerol-3-phosphate. Surprisingly, *glpT* (glycerol-3-phosphate permease and FOS transporter, BSU02140) was not up- or downregulated in this pathway under any conditions, but is the key transporter for FOS, which has already been shown and independently tested (tested in preliminary experiments, results not shown). The synthesis of methionine (*mtn...*) in combination with cysteine (*tcy*, *snd...*, *mcc...*, *cys...*) was also upregulated. Of these, *mtnE* (BSU13580) was upregulated as a glutamine transaminase in both setups and omics studies and this is again a central point in metabolism. However, the methionine pathway is linked to cysteine metabolism via homocysteine and the upregulation makes sense as cysteine is required for FOS detoxification via FosB and bacillithiol for its synthesis. [23, 72, 73, 74, 75, 76, 79, 80, 81, 103, 104]

PadC is potentially involved in the detoxification of FOS.

The phenolic acid decarboxylase, PadC, was upregulated during FOS treatment, independent of a stress response (S1 and S2). PadC confers resistance to salicylic acid and other toxic compounds (nitric oxide, phenolic acids, flavonoids, oxalate). It may therefore play a role in the detoxification of FOS. The upregulation of bacillibactin synthesis genes mentioned above could generate salicylic acid-like substances that are toxic to the cell, which would then be eliminated by PadC. [105, 106, 107]

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AUTHOR CONTRIBUTIONS

SF developed the experiments and carried out all biological experiments and isolations, analysed the omics results and wrote the MS. MFW and BM performed the proteomics measurements and supported the analysis. TH and KN performed the transcriptome the analysis. SF and AW established the metabolomics workflow. AW support the work by providing bioinformatics tools for the analysis. CM provided scientific assistance and reviewed the MS.

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APPENDIX

A4: Transcriptome (samples1-3) and proteome (samples 1-4) sample overview.

A2: Metabolomics sample overview.

A3. *B. subtilis* growth curves of the omics experimental setup

A4. *B. subtilis* small scale growth curves.

A5: Principal component analysis (PCS) of the transcriptome analysis

A6: Principal component analysis (PCS) of the proteome analysis.

A7: Transcriptomics heatmaps gene clusters.

A8: Proteomics heatmaps gene clusters.

A9: Venn diagram of upregulated (A) and downregulated (B) differentially expressed genes (transcriptomics S1) between wt (left circle) and $\Delta fosB$ (right circle) under FOS treatment.

A10: Venn diagram of upregulated (A) and downregulated (B) differentially expressed genes (transcriptomics S2) between wt (left circle) and $\Delta fosB$ (right circle) under FOS treatment.

A11: Venn diagram of upregulated (A) and downregulated (B) proteins (proteomics S1) between wt (left circle) and $\Delta fosB$ (right circle) under FOS treatment

A12: Venn diagram of upregulated (A) and downregulated (B) proteins (proteomics S2) between wt (left circle) and $\Delta fosB$ (right circle) under FOS treatment.

A13: LC-MS analysis of the crucial PGN synthesis marker under FOS treatment of *B. subtilis* 168 wt

A14: Sankey diagram of the omics trend-comparing analysis of proteome and transcriptome data from S2.

A15: Analyses of the comparison between *B. subtilis* wt and *B. subtilis* $\Delta fosB$ in transcriptomics S1 and S2

A16: Analyses of the comparison between *B. subtilis* wt and *B. subtilis* $\Delta fosB$ in proteomics S1 and S2

A17: Sankey diagram of the omics trend-comparing analysis of proteome and transcriptome data from S1

A18: Sankey diagram of the omics trend-comparing analysis of proteome and transcriptome data from S1.

A19: Summary Zenodo digital appendix: Friz, S. Version v1, May 1, 2024, DOI: 10.5281/zenodo.11267447.[32]

APPENDIX:

Comprehending the responses of *Bacillus subtilis* to fosfomycin using multi-omics.

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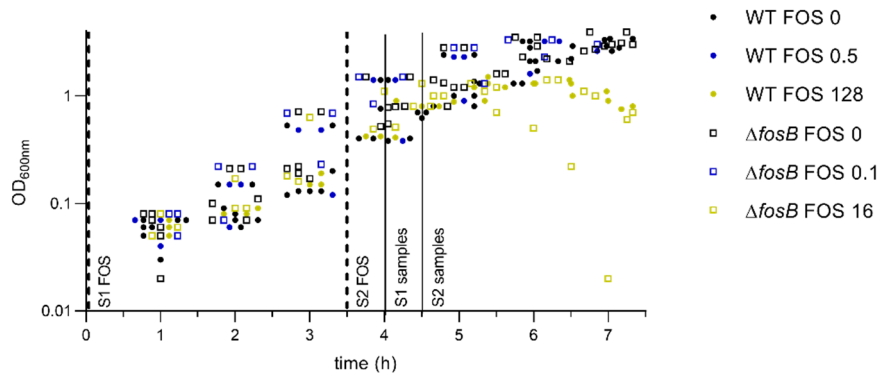
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A5: Transcriptome (samples 1-3) and proteome (samples 1-4) sample overview. All samples were inoculated to OD_{600nm} 0.05 and incubated at 37 °C and 120 rpm. The equivalent of OD_{600nm} 0.8 was harvested in 2 mL (transcriptomics) or 15 mL (proteomics) from the identical culture. S1: FOS from the beginning: wt: $0.5 \mu g mL^{-1}$, $\Delta fosB$: $0.1 \mu g mL^{-1}$, sampling at OD_{600nm} 0.8-1.0; S2: FOS in the exponential growth phase OD_{600nm} 0.5-0.8, wt: $128 \mu g mL^{-1}$, $\Delta fosB$: $16 \mu g mL^{-1}$, sampling 1 h after the treatment (OD_{600nm} 1.4-1.6).

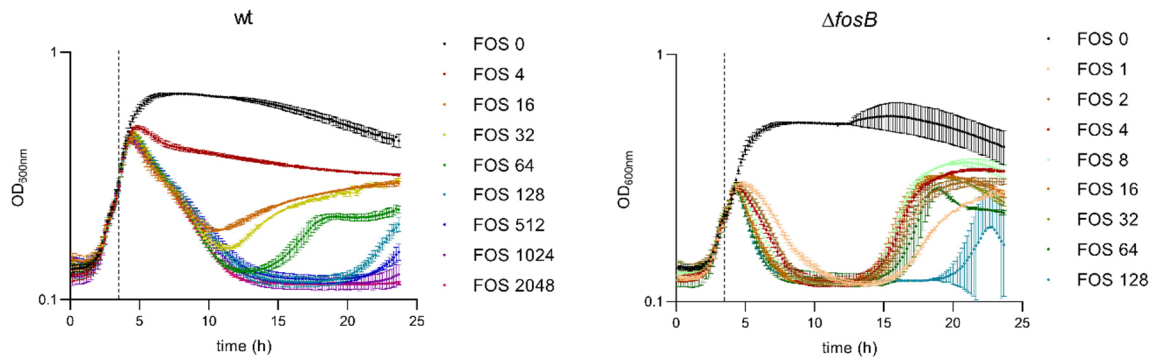
short	setup	strain	FOS	end time	end OD_{600nm}
wt+1	S1	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$0.5 \mu g mL^{-1}$	4.17 h	0.9
wt+2	S1	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$0.5 \mu g mL^{-1}$	4.17 h	0.9
wt+3	S1	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$0.5 \mu g mL^{-1}$	4.17 h	0.9
wt+4	S1	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$0.5 \mu g mL^{-1}$	4.17 h	1.0
wt-1	S1	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$0.0 \mu g mL^{-1}$	4.00 h	1.0
wt-2	S1	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$0.0 \mu g mL^{-1}$	4.00 h	1.1
wt-3	S1	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$0.0 \mu g mL^{-1}$	4.00 h	1.0
wt-4	S1	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$0.0 \mu g mL^{-1}$	4.00 h	1.2
$\Delta fosB$ +1	S1	<i>B. subtilis</i> subsp. <i>subtilis</i> , trpC2 $\Delta fosB$	$0.1 \mu g mL^{-1}$	3.67 h	0.9
$\Delta fosB$ +2	S1	<i>B. subtilis</i> subsp. <i>subtilis</i> , trpC2 $\Delta fosB$	$0.1 \mu g mL^{-1}$	3.67 h	1.0
$\Delta fosB$ +3	S1	<i>B. subtilis</i> subsp. <i>subtilis</i> , trpC2 $\Delta fosB$	$0.1 \mu g mL^{-1}$	3.67 h	1.0
$\Delta fosB$ +4	S1	<i>B. subtilis</i> subsp. <i>subtilis</i> , trpC2 $\Delta fosB$	$0.1 \mu g mL^{-1}$	3.67 h	1.1
$\Delta fosB$ -1	S1	<i>B. subtilis</i> subsp. <i>subtilis</i> , trpC2 $\Delta fosB$	$0.0 \mu g mL^{-1}$	3.50 h	0.8
$\Delta fosB$ -2	S1	<i>B. subtilis</i> subsp. <i>subtilis</i> , trpC2 $\Delta fosB$	$0.0 \mu g mL^{-1}$	3.50 h	0.8
$\Delta fosB$ -3	S1	<i>B. subtilis</i> subsp. <i>subtilis</i> , trpC2 $\Delta fosB$	$0.0 \mu g mL^{-1}$	3.50 h	0.8
$\Delta fosB$ -4	S1	<i>B. subtilis</i> subsp. <i>subtilis</i> , trpC2 $\Delta fosB$	$0.0 \mu g mL^{-1}$	3.50 h	0.8
wt+1	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$128 \mu g mL^{-1}$	4.5 h	1.6
wt+2	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$128 \mu g mL^{-1}$	4.5 h	1.6
wt+3	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$128 \mu g mL^{-1}$	4.5 h	1.6
wt+4	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$128 \mu g mL^{-1}$	4.5 h	1.8
wt-1	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$0.0 \mu g mL^{-1}$	4.5 h	1.5
wt-2	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$0.0 \mu g mL^{-1}$	4.5 h	1.6
wt-3	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$0.0 \mu g mL^{-1}$	4.5 h	1.7
wt-4	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$0.0 \mu g mL^{-1}$	4.5 h	2.0
$\Delta fosB$ +1	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> , trpC2 $\Delta fosB$	$16 \mu g mL^{-1}$	4.5 h	1.6
$\Delta fosB$ +2	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> , trpC2 $\Delta fosB$	$16 \mu g mL^{-1}$	4.5 h	1.5
$\Delta fosB$ +3	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> , trpC2 $\Delta fosB$	$16 \mu g mL^{-1}$	4.5 h	1.6
$\Delta fosB$ +4	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> , trpC2 $\Delta fosB$	$16 \mu g mL^{-1}$	4.5 h	1.4
$\Delta fosB$ -1	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> , trpC2 $\Delta fosB$	$0.0 \mu g mL^{-1}$	4.5 h	1.5
$\Delta fosB$ -2	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> , trpC2 $\Delta fosB$	$0.0 \mu g mL^{-1}$	4.5 h	1.5
$\Delta fosB$ -3	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> , trpC2 $\Delta fosB$	$0.0 \mu g mL^{-1}$	4.5 h	1.6
$\Delta fosB$ -4	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> , trpC2 $\Delta fosB$	$0.0 \mu g mL^{-1}$	4.5 h	1.4

A2: Metabolomics sample overview. All samples were inoculated to OD_{600nm} 0.05 and incubated at 37 °C and 120 rpm. The equivalent of OD_{600nm} 0.8 in 20 mL was harvested. S2: FOS in the exponential growth phase OD_{600nm} 0.5-0.8, wt: $128 \mu g mL^{-1}$, sampling 1 h after the treatment (OD_{600nm} 1.6-2.0).

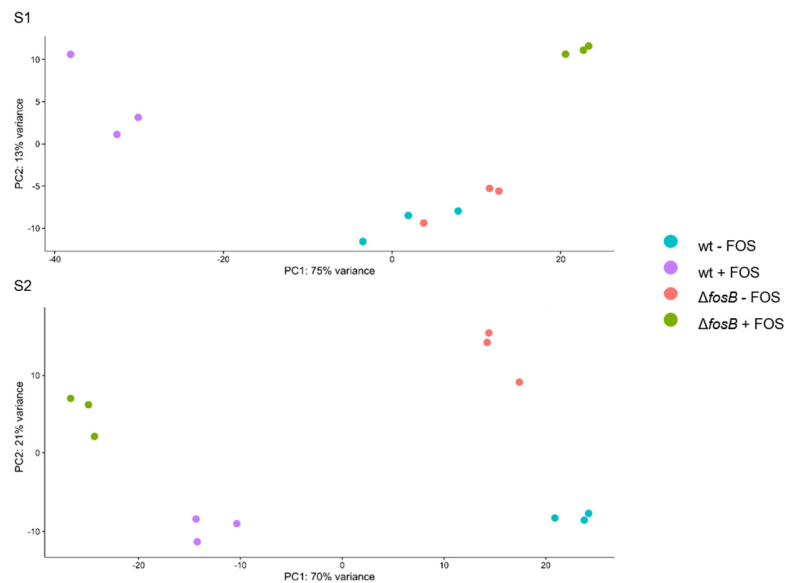
short	setup	strain	FOS	end time	end OD_{600nm}
wt+1	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$128 \mu g mL^{-1}$	5.25 h	1.6
wt+2	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$128 \mu g mL^{-1}$	5.25 h	1.7
wt+3	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$128 \mu g mL^{-1}$	5.25 h	1.7
wt-1	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$0.0 \mu g mL^{-1}$	5.50 h	2.0
wt-2	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$0.0 \mu g mL^{-1}$	5.50 h	1.8
wt-3	S2	<i>B. subtilis</i> subsp. <i>subtilis</i> str. 168 (trpC2)	$0.0 \mu g mL^{-1}$	5.50 h	1.8



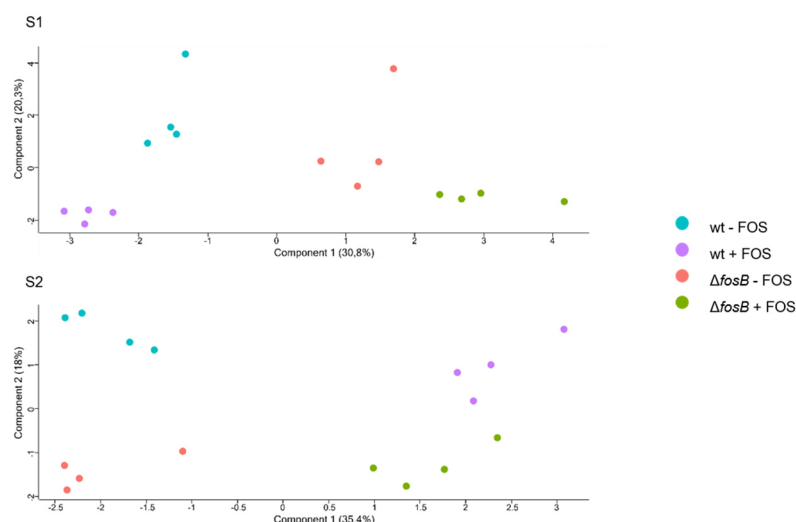
A3. *B. subtilis* growth curves of the omics experimental setup. Growth curves of the strains: *B. subtilis* 168 (*trpC2*) = wt, *B. subtilis* (*trpC2*) $\Delta fosB$ = $\Delta fosB$. FOS treatment with 0.5 $\mu\text{g mL}^{-1}$ (wt) and 0.1 $\mu\text{g mL}^{-1}$ ($\Delta fosB$) for S1 (from the beginning) and 128 $\mu\text{g mL}^{-1}$ (wt) and 16 $\mu\text{g mL}^{-1}$ ($\Delta fosB$) for S2 (after 3.5 h of incubation, in the exponential growth phase). Performed at least in triplicate in 30 mL cultures (100 mL flasks) at 37 °C under aerobic conditions. OD_{600nm} was measured with 1 mL cuvettes.



A4. *B. subtilis* small scale growth curves. Growth curves of the strains: *B. subtilis* 168 (*trpC2*) = wt, *B. subtilis* (*trpC2*) $\Delta fosB$ = $\Delta fosB$. FOS treatment with concentrations from 0-8192 $\mu\text{g mL}^{-1}$ after 3.5 h of incubation. Performed in triplicate or duplicate in 24-well plates covered with a semi-permeable sealing membrane (Breathe-Easy, Z380059, Sigma-Aldrich) shaking at 37 °C using the EPOCH2, BioTek Absorbance Microplate Reader, Agilent Technologies, by measuring OD_{600nm} every 10 min.



A5: Principal component analysis (PCA) of the transcriptome analysis. Shown is a PCA variance plot for S1 and S2 with *B. subtilis* wt and $\Delta fosB$, performed in triplicates.



A6: Principal component analysis (PCA) of the proteome analysis. Shown is a PCA variance plot for S1 and S2 with *Bacillus subtilis* wt and $\Delta fosB$, performed in quadruplicates.

A7: Transcriptomics heatmaps gene clusters. The clusters for S1 and S2 are listed with the BSU gene identifier (ordered locus names) and the common gene name (SubtiWiki status 2023-10-17, [45]).

S1 A: BSU34610 mdxE, BSU34580 yvdJ, BSU30560 pckA, BSU34620 yvdF, BSU34590 mdxG, BSU39350 hutH, BSU34600 mdxF

S1 B: BSU12930 dppB, BSU11382 appA/2, BSU31540 nupN, BSU11400 appC, BSU34570 yvdK, BSU29680 acsA, BSU26640 czcO, BSU34560 malL, BSU31550 nupO, BSU12950 dppD, BSU26650 czcD, BSU12920 dppA, BSU03010 amhX, BSU11390 appB, BSU34550 pgcM, BSU11381 appA/1, BSU12940 dppC, BSU14629 sr1

S1 C: BSU03200 putB, BSU29020 gapB

S1 D: BSU03210 putC, BSU11360 appD, BSU37790 rocG, BSU40340 rocD, BSU38590 licB, BSU37780 rocA, BSU38580 licC, BSU38560 licH, BSU37760 rocC, BSU08190 glvR, BSU40330 rocE, BSU11370 appF, BSU08180 malA, BSU40320 rocF, BSU37770 rocB, BSU08200 malP, BSU38570 licA

S1 E: BSU05480 mhqN, BSU05490 mhqO, BSU05500 mhqP

S1 F: BSU30280 melD, BSU04470 dctP, BSU30290 melC, BSU39580 yxeE, BSU17030 cotE, BSU11750 cotY, BSU11740 cotZ, BSU12440 phrA, BSU40530 cotF, BSU28900 pftB, BSU34490 yvdS

S1 G: BSU28250 leuD, BSU28270 leuB, BSU28280 leuA, BSU28300 ilvH, BSU28260 leuC, BSU28310 ilvB, BSU28290 ilvC

S1 H: BSU19030 yobO, BSU11460 oppD, BSU39750 iolB, BSU31560 nupP, BSU39710 iolF, BSU06310 gabP, BSU28760 araM, BSU29690 acuA, BSU17000 kbl, BSU35960 rbsB, BSU22550 qcrB, BSU39670 iolJ, BSU28810 abnA, BSU14900 ctaD, BSU09460 bcaP, BSU09880 yhaR, BSU39680 iolI, BSU11430 oppA, BSU11470 oppF, BSU38090 vpr, BSU06950 rhgK, BSU06230 iolT, BSU18010 yneN, BSU29140 citZ, BSU10570 yhjN, BSU33490 cadA, BSU08070 acoB, BSU27030 sacC, BSU29110 phoP, BSU17210 pksN, BSU34140 ganQ, BSU34290 epsI, BSU33800 opuCD, BSU39890 yxbB, BSU31570 nupQ, BSU11410 yjbA, BSU35860 pgdS, BSU28740 araP, BSU21870 ilvD, BSU39700 iolG, BSU32230 yuxL, BSU14910 ctaE, BSU39730 iolD, BSU39740 iolC, BSU35940 rbsA, BSU28730 araQ, BSU08060 acoA, BSU11440 oppB, BSU39370 hutI, BSU07610 citM, BSU28750 araN, BSU35920 rbsK, BSU39720 iolE, BSU29100 phoR, BSU27060 levE, BSU33810 opuCC, BSU29010 speD, BSU18320 ppsC, BSU34130 ganA, BSU11450 oppC, BSU28790 araB, BSU18000 citB, BSU34190 lutP, BSU18330 ppsB, BSU06940 yesL, BSU13940 ykwB, BSU39360 hutU, BSU10450 yhjB, BSU10460 yhjC, BSU35930 rbsD, BSU35950 rbsC, BSU27040 levG, BSU38800 yxkH, BSU03020 ycgA, BSU39380 hutG, BSU29700 acuB, BSU39690 iolH

S1 I: BSU17840 fosB

S1 J: BSU13890 ptsG

S1 K: BSU15560 pyrE, BSU28840 ysdA, BSU40050 gntR, BSU07810 treA, BSU15550 pyrF, BSU40060 gntK, BSU15480 pyrP, BSU07800 treP, BSU15530 pyrK, BSU15520 pyrAB, BSU15540 pyrD

S1 L: BSU01290 rpsN, BSU01100 rpsL, BSU01120 fusA, BSU01260 rplN, BSU40880 exoA, BSU01240 rpmC, BSU03990 mtID, BSU01200 rpsS, BSU03981 mtIA, BSU01040 rplJ, BSU01180 rplW, BSU01250 rpsQ, BSU29660 rpsD, BSU01270 rplX, BSU01110 rpsG, BSU01480 truA, BSU01190 rplB, BSU33950 cggR, BSU38050 sacP, BSU01230 rplP, BSU01050 rplL, BSU03982 mtIF, BSU16490 rpsB, BSU01210 rplV

S1 M: BSU22070 xpt, BSU13720 queC, BSU38330 ywbG, BSU06370 pbuG, BSU22060 pbuX, BSU15490 pyrB, BSU15500 pyrC, BSU15510 pyrAA

S1 N: BSU18380 iseA, BSU39180 yxiH, BSU16040 rplS, BSU12050 yjdH, BSU36510 nrgA, BSU28860 rpml, BSU28850 rplT, BSU35130 yvlA, BSU40070 gntP, BSU02850 znuA, BSU07820 treR, BSU28870 infC, BSU39200 yxzC

S2 A: BSU17840 fosB

S2 B: BSU38480 sasA, BSU27180 yrhH, BSU23830 yqjL, BSU23370 ypuA, BSU06380 yebC

S2 C: BSU22980 ypbG, BSU27170 fatR, BSU27160 yrhJ, BSU15230 murB, BSU03240 ycgQ, BSU27120 sigV, BSU27130 rsiV

S2 D: BSU31370 yugJ, BSU38540 dltE, BSU25070 ispG, BSU38110 nfrA, BSU40360 htrC, BSU40190 fbp, BSU22430 panB, BSU04690 rsbT, BSU39540 yxel, BSU38680 yxID, BSU37000 prmC, BSU31360 yugK, BSU35990 ywrO, BSU02930 yceG, BSU40930 yyaE

S2 E: BSU31990 dhbC, BSU04230 amj, BSU31970 dhbB, BSU02880 yceB, BSU34790 trxB, BSU33030 yuxN, BSU03610 tcyA, BSU08210 yfiB, BSU28040 radC, BSU27250 mcbB, BSU38260 efeB, BSU27370 mltG, BSU32000 dhbA, BSU09500 yhdK, BSU09510 yhdL, BSU09640 yhdY, BSU03250 ycgR, BSU14480 abh, BSU27270 mtnN, BSU11340 fabF, BSU29680 acsA, BSU11640 cpaA, BSU28020 mreC, BSU31310 yugP, BSU12900 htrA, BSU40410 walR, BSU38700 sigY, BSU02910 yceE, BSU31960 dhbF, BSU27280 yrrT, BSU24210 yqiG, BSU38100 ywcH, BSU31300 yugS, BSU36530 bcrC, BSU22320 ponA, BSU38120 rodA, BSU35920 rbsK, BSU39020 nupG, BSU07260 yfnI, BSU22970 ypbH, BSU38660 yxiF, BSU15220 murG, BSU38650 yxiG, BSU03040 amyE, BSU02900 yceD, BSU09410 phoA, BSU36540 ywnJ, BSU27140 oatA, BSU22310 recU, BSU39360 hutU, BSU35930 rbsD

S2 F: BSU03789 yczN, BSU19790 cgeB, BSU07290 yfnF, BSU17260 aprX, BSU07280 yfnG, BSU19780 cgeA, BSU07270 yfnH, BSU32650 yurS, BSU25710 cwiH, BSU36030 ywrK, BSU23350 ypzD

S2 G: BSU12170 forE1, BSU04430 ydbD, BSU06150 gutB, BSU03990 mtlD, BSU40450 yycD, BSU03981 mtlA, BSU10430 yhxD, BSU33830 opuCA, BSU04370 ydaS, BSU33410 yvgO, BSU18520 yoxB, BSU12150 yjgB, BSU09390 ygxB, BSU15960 sivC, BSU02585 BSU_02585, BSU15480 pyrP, BSU40420 purA, BSU15490 pyrB, BSU33890 yvbK, BSU29020 gapB, BSU40660 yybF, BSU15500 pyrC, BSU15520 pyrAB, BSU25010 yqgE, BSU28690 glcF, BSU15510 pyrAA, BSU09450 yhdF, BSU35678 BSU_35678, BSU03982 mtlF, BSU17230 pksS

S2 H: BSU20830 yopN, BSU38180 ywzA, BSU33760 sdpB, BSU21440 bdbB, BSU38130 ywcE, BSU09100 cspB, BSU06250 ydjM, BSU25880 yqxJ, BSU37460 rapF, BSU37250 narI, BSU28340 ysnF, BSU33750 sdpA, BSU20990 yonU, BSU22820 yphE, BSU21470 sunT, BSU20760 yopU, BSU09420 lytE, BSU19490 gerT, BSU18908 yozY, BSU29260 ytpI, BSU37260 narJ, BSU11920 yjcN, BSU37220 arfM, BSU37270 narH, BSU30660 ctaK, BSU24770 mgsR, BSU21450 sunS, BSU18960 yozM, BSU24800 yqgW, BSU15470 pyrR, BSU25890 yqxi, BSU18600 yozQ, BSU18978 bsrE, BSU33770 sdpC, BSU37360 sboX, BSU33820 opuCB, BSU18849 yozV, BSU26350 sknR, BSU00080 yaaC, BSU13310 tnrA, BSU18920 phrK, BSU11990 yjdB, BSU17710 tatAC, BSU20840 yopM, BSU37350 sboA, BSU14660 ykzI, BSU05690 ydhB, BSU36670 csbD, BSU20999 yoyJ, BSU29249 yzjN, BSU33222 rsoA, BSU03780 phrC, BSU01889 ybzH, BSU06390 yebD, BSU35980 ywsA, BSU18910 rapK, BSU31380 yuzA, BSU37380 albB, BSU18850 yobD, BSU30700 rpmEB, BSU21460 bdbA, BSU20820 yopO, BSU00490 spoVG, BSU26940 yraH, BSU07550 yfiT, BSU04039 yczO, BSU14629 srI, BSU14760 ylaF, BSU04400 gsiB, BSU20770 yopT

S2 I: BSU31140 cdoA, BSU11190 argC, BSU39580 yxeE, BSU17030 cotE, BSU23980 artP, BSU29450 argG, BSU11750 cotY, BSU11740 cotZ, BSU17310 ymaG, BSU40530 cotF

A8: Proteomics heatmaps gene clusters. The clusters for S1 and S2 are listed with the BSU gene identifier (ordered locus names) and the common gene name (SubtiWiki status 2023-10-17, [45]).

S1 A: BSU37790 rocG, BSU40340 rocD

S1 B: BSU31990 dhbC, BSU11250 argF, BSU31970 dhbB, BSU03000 opuAC, BSU34570 yvdK, BSU08070 acoB, BSU19310 dhaS, BSU09710 bmrC, BSU37780 rocA, BSU11220 argD, BSU23070 serA, BSU31980 dhbE, BSU40320 rocF

S1 C: BSU00160 yaaH, BSU08970 prkA, BSU27980 spoIVFA

S1 D: BSU11810 spoVIF, BSU15090 gerR, BSU10790 asnO, BSU25600 comER, BSU22800 spoIVA, BSU16940 recA, BSU15490 pyrB, BSU09300 glpD, BSU15530 pyrK, BSU27840 safA, BSU15520 pyrAB, BSU36760 murAA, BSU15540 pyrD, BSU15510 pyrAA

S1 E: BSU15500 pyrC, BSU09290 glpK

S1 F: BSU28410 gerE, BSU31060 gbsA, BSU12100 pdaC, BSU39580 yxeE, BSU27920 obg, BSU35240 ctpB, BSU23980 artP, BSU07930 yfkD, BSU00430 yabG, BSU07240 yetN, BSU31050 gbsB, BSU00310 holB, BSU34810 yvcD, BSU23480 dacF

S1 G: BSU37610 ywzC, BSU27500 trmU, BSU16060 rnhB

S1 H: BSU09780 yheC, BSU15560 pyrE, BSU30350 yttB, BSU14630 speA, BSU06620 ligA, BSU04650 ndoAI, BSU08020 yfjO, BSU32830 fadA, BSU11120 yitT, BSU20040 yosP, BSU01440 rpiQ, BSU22170 ypsC, BSU22150 ypvA, BSU38480 sasA, BSU27960 rplU, BSU35290 prfB, BSU19740 yodT, BSU01330 rpsE, BSU17440 ynbB, BSU13130 proA, BSU31780 yueH, BSU13720 queC, BSU03390 yckC, BSU16950 pbpX, BSU09000 yhbJ, BSU19690 kamA, BSU18210 yngE, BSU23090 rsiX, BSU24170 mmgA, BSU27720 queA, BSU25630 yqeK, BSU16870 ymfI, BSU15700 yloI, BSU10700 gerPC, BSU14770 ylaG, BSU36550 spoIIQ, BSU25260 glyS, BSU25510 lepA, BSU00510 prs, BSU27540 yrvM, BSU02780 ycdA, BSU09270 glpP, BSU39210 yxiF, BSU25120 yqfT, BSU25000 pbpA, BSU17380 nrdE, BSU15320 sigE, BSU29730 motP, BSU24140 mmgD, BSU31890 essB, BSU14470 mreBH, BSU11230 carA, BSU32000 dhbA, BSU38230 ywcB, BSU32840 fadN, BSU07420 yfmM, BSU09510 yhdL, BSU23560 mleN, BSU02600 cwiJ, BSU37820 spsK, BSU25410 rpsU, BSU03900 gabT, BSU00340 trmN6, BSU39960 yxal, BSU12010 manP, BSU33330 yvsh, BSU14480 abh, BSU12490 yjqC, BSU30890 ytxO, BSU00280 tmk, BSU15750 yloN, BSU15230 murB, BSU01180 rplW, BSU16910 rodZ, BSU23970 artQ, BSU17450 glnR, BSU15010 ylbH, BSU09440 citA, BSU28110 spoVID, BSU23960 artR, BSU25430 yqeV, BSU22240 ypqA, BSU19770 cgeC, BSU15470 pyrR, BSU36410 mbl, BSU24310 folD, BSU25680 yqeG, BSU06300 cotA, BSU01150 rpsJ, BSU12050 yjdH, BSU12180 yjha, BSU12980 ykfB, BSU29660 rpsD, BSU21920 ugtP, BSU31030 bstB, BSU40080 gntZ, BSU28850 rplT, BSU01110 rpsG, BSU02720 yccF, BSU17030 cotE, BSU14880 ctaB, BSU22830 gpsA, BSU14950 ylbB, BSU12960 dppE, BSU16600 nusA, BSU22420 panC, BSU38040 sacA, BSU36260 tkmA, BSU23220 scpA, BSU09570 yhdR, BSU01420 rpsK, BSU30410 ytrF, BSU36420 spoIIID, BSU10610 yhjR, BSU11730 cotO, BSU15690 yloH, BSU24260 yqxG, BSU17840 fosB, BSU03600 tcyB, BSU01190 rplB, BSU33240 oxdC, BSU13670 mhqR, BSU09400 spoVR, BSU17430 ynbA, BSU21820 thyB, BSU15480 pyrP, BSU29580 thil, BSU17050 mutL, BSU26740 cypA, BSU28380 gerM, BSU10850 iolX, BSU30480 ytgA, BSU33950 cggR, BSU06630 yerH, BSU04310 ydaN, BSU38050 sacP, BSU11820 yjcD, BSU31390 yugI, BSU16650 rbfA, BSU32820 fadE, BSU20400 yorF, BSU18260 yngJ, BSU27440 glnH, BSU36900 glyA, BSU36370 ywpB, BSU09550 mpfA, BSU19210 yocH, BSU40890 rpsR, BSU03830 fbpQ, BSU00220 yaaL, BSU06610 pcrA, BSU23460 spoIIAB, BSU04630 ssdC, BSU18240 yngH, BSU08950 yhbE, BSU17390 nrdF, BSU23310 sipS, BSU29010 speD, BSU00810 yacF, BSU25560 hola, BSU37150 pyrG, BSU27600 rel, BSU37000 prmC, BSU27120 sigV, BSU06760 dnmA, BSU27340 yrrO, BSU25460 dnaJ, BSU15220 murG, BSU32750 metN, BSU09830 yhaX, BSU31360 yugK, BSU28060 spoIIB, BSU29470 ackA, BSU40830 yyaK, BSU07820 treR, BSU01370 adk, BSU02270 pssA, BSU22610 tyrA, BSU19330 sodF, BSU04580 cshA, BSU12020 manA, BSU23370 ypuA, BSU40530 cotF, BSU01220 rpsC, BSU37490 speB, BSU39110 deaD, BSU04570 murF, BSU25480 grpE, BSU14170 ykuP, BSU02140 glpT, BSU27130 rsiV, BSU22410 panD, BSU16490 rpsB, BSU01280 rplE, BSU01210 rplV, BSU16610 rnpM, BSU06380 yebC, BSU28470 lysC, BSU28580 rqcU, BSU10170 fabHB, BSU25270 glyQ, BSU40930 yyaE, BSU00420 ksgA

S1 I: BSU15550 pyrF

S1 J: BSU36390 flhP, BSU24550 gcvPB, BSU16320 flhY, BSU16330 cheY, BSU35330 flhS, BSU16230 flhH, BSU14010 cheV, BSU07360 yfmS, BSU16430 cheA, BSU13950 mcpC, BSU16440 cheW, BSU16220 flhG, BSU21010 yonS, BSU35340 flhD, BSU24560 gcvPA, BSU16290 flgE, BSU16450 cheC, BSU35360 hag, BSU31260 mcpB, BSU16260 ylxF, BSU07350 yfmT, BSU13680 motB

S1 K: BSU39750 iolB, BSU35960 rbsB, BSU37710 bacD, BSU11430 oppA, BSU11520 mecA, BSU39270 bglP, BSU38200 galK, BSU04800 int, BSU29140 citZ, BSU38190 galT, BSU34350 epsC, BSU38590 licB, BSU29680 accA, BSU30560 pckA, BSU22880 rpsA, BSU38580 licC, BSU38560 licH, BSU39260 bglH, BSU39700 iolG, BSU27770 yrbE, BSU36080 ywrF, BSU24050 bkdAA, BSU24040 bkdAB, BSU38960 yxjG, BSU39730 iolD, BSU35790 pmi, BSU29540 ytdI, BSU29020 gapB, BSU04150 ycsN, BSU04410 ydbB, BSU38570 licA, BSU35930 rbsD, BSU39690 iolH

S1 L: BSU14071 fadG, BSU35440 yvyF, BSU11870 metI, BSU11460 oppD, BSU08560 yfhJ, BSU18380 iseA, BSU16070 ylgG, BSU28890 yscB, BSU28440 sdhA, BSU19410 cwIS, BSU04470 dctP, BSU34200 sigL, BSU23890 yidC2, BSU29710 acuC, BSU35970 ywsB, BSU13530 kinE, BSU03280 nasF, BSU29690 acuA, BSU20270 yorS, BSU22260 yppF, BSU20440 yorB, BSU17000 kbl, BSU11880 metC, BSU16180 flgB, BSU24090 ptb, BSU16310 flhM, BSU31250 tlpA, BSU39670 iolJ, BSU37460 rapF, BSU24060 lpdV, BSU14900 ctaD, BSU13180 metE, BSU22810 yphF, BSU21320 yomL, BSU39680 iolI, BSU08470 yfhB, BSU09760 yheE, BSU32240 thrB, BSU35780 lytD, BSU26060 yqbM, BSU38850 yxkC, BSU24220 spo0A, BSU21830 ypjQ, BSU16300 flhL, BSU10260 yhfK, BSU12650 xkdK, BSU00290 darA, BSU05870 gmuF, BSU06240 bdhA, BSU35310 hpf, BSU16980 spoVS, BSU09670 dat, BSU11470 oppF, BSU03420 nin, BSU23670 yqkA, BSU20880 yopI, BSU16240 flhI, BSU21940 degR, BSU11010 yitJ, BSU16270 flhK, BSU03440 tlpC, BSU38810 msmX, BSU19560 mhqD, BSU11300 mgtE, BSU31230 tlpB, BSU08390 bstA, BSU03990 mtlD, BSU35350 yvyC, BSU35320 flhT, BSU23270 ribE, BSU10360 yhfT, BSU12810 xlyA, BSU18980 yobJ, BSU29890 ytnP, BSU03790 thrD, BSU38950 yxjH, BSU17100 pksC, BSU05770 ydhI, BSU34610 mdx, BSU34040 lutB, BSU35680 ggaB, BSU30260 melR, BSU29120 mdh, BSU16100 sucD, BSU39250 yxiE, BSU35150 yvzB, BSU40970 parA, BSU33690 yvaQ, BSU23690 yqjY, BSU33490 cadA, BSU32940 yusV, BSU11850 yjcG, BSU02200 ybfG, BSU35230 swrA, BSU34030 lutC, BSU39770 iolR, BSU07560 pel, BSU31010 floT, BSU29110 phoP, BSU12600 xkdF, BSU12660 xkdM, BSU21330 yomK, BSU34560 malL, BSU16350 flhP, BSU28910 pftA, BSU35420 flgN, BSU39610 yxeB, BSU16470 sigD, BSU12870 mhqA, BSU27070 levD, BSU39760 iolA, BSU05830 gmuC, BSU35410 flgK, BSU16200 flhE, BSU08450 yfiZ, BSU03480 srFA, BSU16280 flgD, BSU35400 flgL, BSU34480 yvdT, BSU33320 thuD, BSU30510 ytpA, BSU02910 yceE, BSU00860 clpC, BSU13690 motA, BSU01540 salA, BSU13700 clpE, BSU21870 iolD, BSU24070 buk, BSU16990 tdh, BSU36400 flhO, BSU11860 yjcH, BSU39820 htpG, BSU16390 flhA, BSU37130 spo0F, BSU37690 bacF, BSU32230 yuxL, BSU21590 yokH, BSU14910 ctaE, BSU33840 opcR, BSU05480 mhqN, BSU03910 gabD, BSU40980 yyaB, BSU33430 cysL, BSU24080 bcd, BSU34620 yvdF, BSU10210 yhfF, BSU13340 ykoM, BSU20870 yopJ, BSU35940 rbsA, BSU05490 mhqO, BSU33120 liaH, BSU11440 oppB, BSU40330 rocE, BSU39540 yxel, BSU21630 yokD, BSU07450 yfmJ, BSU38920 pepT, BSU10380 hemAT, BSU27580 yrvJ, BSU16460 cheD, BSU02230 purT, BSU26230 yqaP, BSU35920 rbsK, BSU26210 yqaR, BSU39720 iolE, BSU03230 putR, BSU08180 malA, BSU13620 mtnD, BSU29100 phoR, BSU27060 levE, BSU34540 clpP, BSU29130 icd, BSU16190 flgC, BSU22580 reoY, BSU30300 melA, BSU19230 azoR1, BSU21770 ilvA, BSU00730 cysK, BSU21600 yokG, BSU20890 yopH, BSU39400 pdp, BSU34160 ganS, BSU16090 sucC, BSU23810 yqjN, BSU12210 yjiB, BSU05030 yddM, BSU34020 yvbX, BSU09190 yhcR, BSU34130 ganA, BSU11450 oppC, BSU37370 albA, BSU28790 araB, BSU36690 rapB, BSU38600 licR, BSU18000 citB, BSU29060 ytaG, BSU40300 rapG, BSU06560 year, BSU35690 ggaA, BSU09260 yhxA, BSU34190 lutP, BSU08200 malP, BSU26075 yqbK, BSU09370 lytF, BSU16210 flhF, BSU29940 ytlR, BSU39360 hutU, BSU10200 yhfE, BSU17230 pksS, BSU40960 parB, BSU27100 yrhP, BSU35950 rbsC, BSU38400 epr, BSU17480 ynzF, BSU38800 yxkH, BSU28900 pftB, BSU09990 scoC, BSU15100 ylbP, BSU06190 ydjG, BSU32780 yusF, BSU29700 acuB, BSU34440 pbpE, BSU38770 cimH, BSU34050 lutA

S1 M: BSU09750 sspB, BSU29570 sspA, BSU26970 adhB

S1 N: BSU00560 spoVT, BSU26980 yraE, BSU09180 yhcQ, BSU16880 ymfJ, BSU25540 gpr, BSU32100 yumB, BSU32720 yurZ, BSU34510 yvdQ, BSU09230 yhcV, BSU23390 spoVAF, BSU22920 ypeB

S1 O: BSU02070 csgA, BSU33540 azoR2, BSU29430 ytzD, BSU08640 yfhS, BSU29500 gerW, BSU13510 ykzE, BSU08660 sspE, BSU08490 yfhD, BSU26960 yraF, BSU00450 sspF, BSU01550 gerD, BSU14200 ykuS, BSU24480 yqhR, BSU08110 sspH, BSU08770 ygzA, BSU09140 yhcM, BSU30470 ytzC, BSU17240 ymzB, BSU37200 ywjD, BSU21950 ypzA, BSU07620 yfiN, BSU11260 yjzC, BSU29910 ytzH, BSU28660 sspl, BSU27830 coxA, BSU26950 yraG, BSU27680 yrbG, BSU22930 sleB, BSU23410 spoVAD, BSU32860 yusN, BSU19950 sspC, BSU18290 yngL, BSU13470 sspD, BSU03920 glcU, BSU14220 ahpA, BSU13930 splB, BSU09150 yhcN, BSU18200 nrmB, BSU29880 malS, BSU35980 ywsA, BSU03930 gdh, BSU31380 yuzA, BSU25010 yqgE, BSU38630 katX, BSU10280 yhfM, BSU34630 mdxR, BSU07140 yetF, BSU13390 ykoT, BSU17830 yndM

S2 A: BSU36760 murAA

S2 B: BSU15560 pyrE, BSU15550 pyrF, BSU00010 dnaA, BSU11240 carB, BSU03050 ldh, BSU16940 recA, BSU15490 pyrB, BSU15530 pyrK, BSU15520 pyrAB, BSU15540 pyrD, BSU15510 pyrAA, BSU09290 glpK

S2 C: BSU35960 rbsB, BSU14120 abbA, BSU33240 oxdC, BSU37280 narG, BSU39400 pdp, BSU23820 yqjM

S2 D: BSU00560 spoVT, BSU04470 dctP, BSU27760 csbX, BSU30580 ytmA, BSU37460 rapF, BSU14500 yqkA, BSU36810 atpD, BSU24460 papA, BSU39270 bglP, BSU26850 yrpG, BSU33880 yvbJ, BSU05790 ydhK, BSU19370 odhA, BSU30770 mntA, BSU29260 ytpI, BSU35330 flhS, BSU35350 yvyC, BSU34220 epsO, BSU11850 yjcG, BSU34570 yvdK, BSU29680 accA, BSU24770 mgsR, BSU21450 sunS, BSU26640 czcO, BSU34560 malL, BSU15650 zloB, BSU00100 dacA, BSU14880 ctaB, BSU35860 pgdS, BSU23980 artP, BSU34380 slrR, BSU18360 galM, BSU34620 yvdF, BSU07750 yfiA, BSU07240 yetN, BSU31290 yugT, BSU29270 ytol, BSU33890 yvbK, BSU26210 yqaR, BSU18910 rapK, BSU38150 qoxC, BSU31380 yuzA, BSU21600 yokG, BSU28150 hemC, BSU34130 ganA, BSU11450 oppC, BSU09260 yhxA, BSU18970 yobI, BSU18330 ppsB, BSU00490 spoV, BSU07200 yetJ, BSU17480 ynzF, BSU38800 yxkH

S2 E: BSU33870 yvbl, BSU28800 araA, BSU19030 yobO, BSU21750 sco, BSU32730 metQ, BSU32830 fadA, BSU10750 yisK, BSU39750 iolB, BSU32660 glxB, BSU07900 yfkH, BSU19410 cwIS, BSU35970 ywsB, BSU21440 bdbB, BSU39710 iolF, BSU13530 kinE, BSU07130 lpdD, BSU31540 nupN, BSU37390 albC, BSU17000 kbl, BSU37980 ywdF, BSU22550 qcrB, BSU39670 iolJ, BSU16950 pbpX, BSU19530 yodA, BSU37250 narI, BSU14900 ctaD, BSU18210 yngE, BSU13180 metE, BSU28530 etfB, BSU11400 appC, BSU09880 yhaR, BSU39680 iolI, BSU08470 yfhB, BSU14920 ctaF, BSU21650 yobK, BSU06770 yeeB, BSU11430 oppA, BSU09420 lytE, BSU38780 yxkI, BSU09730 yheG, BSU17200 pksM, BSU38810 msmX, BSU32840 fadN, BSU23140 resB, BSU06230 iolT, BSU01630 feuA, BSU37670 hemQ, BSU08440 yfiY, BSU37260 narJ, BSU21490 sunI, BSU17100 pksC, BSU37790 rocG, BSU22700 aroB, BSU30070 opuD, BSU04140 pbpC, BSU33490 cadA, BSU34030 lutC, BSU40340 rocD, BSU37270 narH, BSU29110 phoP, BSU08050 yfiL, BSU00110 pdxS, BSU38590 licB, BSU10860 bstC, BSU09710 bmrC, BSU37780 rocA, BSU39610 yxeB, BSU16470 sigD, BSU03380 yckB, BSU37310 fnr,

BSU14890 ctaC, BSU30560 pckA, BSU10140 hemY, BSU39760 iolA, BSU10120 hemE, BSU12180 yjH, BSU35910 rbsR, BSU05980 tatAY, BSU11600 sasB, BSU33320 fhuD, BSU36850 atpF, BSU22110 kdgK, BSU06030 groEL, BSU39700 iolG, BSU27770 yrbE, BSU00750 pabA, BSU37130 spoOF, BSU32230 yuxL, BSU14910 ctaE, BSU40980 yyaB, BSU37410 albE, BSU38740 cydC, BSU13340 ykoM, BSU03290 nasE, BSU39730 iolD, BSU10850 iolX, BSU00780 folB, BSU39740 iolC, BSU06160 gutP, BSU38270 efeO, BSU03220 putP, BSU25190 cccA, BSU22730 ndk, BSU07450 yfmJ, BSU11370 appF, BSU39370 hutI, BSU27580 yrvJ, BSU02230 purT, BSU38760 cydA, BSU29020 gapB, BSU03830 fpbQ, BSU39720 iolE, BSU32050 yuiE, BSU35340 fliD, BSU33180 yvrC, BSU29100 phoR, BSU34550 pgcM, BSU22580 reoY, BSU23310 sipS, BSU02850 znuA, BSU34160 ganS, BSU22560 qcrA, BSU13460 rsgI, BSU37770 rocB, BSU35530 tagO, BSU24560 gcvPA, BSU39350 hutH, BSU21460 bdbA, BSU10770 wprA, BSU00980 sigH, BSU28690 glcF, BSU37370 albA, BSU06560 year, BSU35520 tagV, BSU18340 ppsA, BSU34190 lutP, BSU38750 cydB, BSU08200 malP, BSU09370 lytF, BSU35270 cccB, BSU34600 mdxF, BSU23110 resE, BSU39360 hutU, BSU17230 pksS, BSU26940 yraH, BSU35950 rbsC, BSU37320 narK, BSU27660 comN, BSU17110 pksD, BSU28900 pftB, BSU24570 gcvT, BSU23150 resA, BSU03300 nasD, BSU39690 iolH

S2 F: BSU03200 putB, BSU03210 putC, BSU21610 yokF, BSU08090 acoL, BSU28680 glcD, BSU34610 mdxE, BSU08070 acoB, BSU30660 ctaK, BSU34140 ganQ, BSU33380 yvgL, BSU21010 yonS, BSU17620 yncB, BSU19620 ldcB, BSU08060 acoA, BSU40330 rocE, BSU06570 yerB, BSU08080 acoC, BSU40320 rocF, BSU10440 yjhA, BSU34590 mdx, BSU22540 qcrC

S2 G: BSU23410 spoVAD

S2 H: BSU09230 yhcV

S2 I: BSU35440 yvyF, BSU19750 cgeE, BSU16180 flgB, BSU16310 flmI, BSU08660 sspE, BSU08490 yfhD, BSU15600 cysC, BSU16230 flhI, BSU35680 ggaB, BSU10400 yhxC, BSU21330 yomK, BSU21660 sprA, BSU28660 sspI, BSU27830 coxA, BSU16350 flpP, BSU35420 flgN, BSU32100 yumB, BSU15130 mraZ, BSU12090 cotT, BSU20870 yopJ, BSU14220 ahpA, BSU31050 gbsB, BSU18200 nrmB, BSU06210 ydjI, BSU16450 cheC, BSU40300 rapG, BSU06190 ydjG, BSU22360 asnS

S2 J: BSU33540 azoR2, BSU31250 tlpA, BSU24550 gcvPB, BSU35780 lytD, BSU36050 cotB, BSU38850 yxkC, BSU16300 flil, BSU16320 flhY, BSU16330 cheY, BSU25080 yqfX, BSU21940 degR, BSU03440 tlpC, BSU36070 cotG, BSU08110 sspH, BSU31230 tlpB, BSU14010 cheV, BSU33690 yvaQ, BSU23970 artQ, BSU07360 yfmS, BSU16430 cheA, BSU15590 sat, BSU35410 flgK, BSU35400 flgL, BSU13950 mcpC, BSU27720 queA, BSU31830 yueE, BSU09750 sspB, BSU23250 ribH, BSU33120 liaH, BSU10380 hemAT, BSU16460 cheD, BSU03930 gdh, BSU19230 azoR1, BSU16290 flgE, BSU35360 hag, BSU31260 mcpB, BSU16260 ylxF, BSU16210 flhF, BSU10920 yitA, BSU07350 yfmT, BSU31240 mcpA, BSU13680 motB

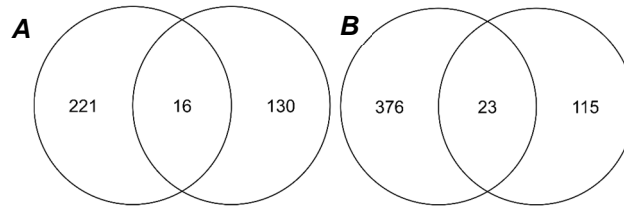
S2 K: BSU38480 sasA, BSU33030 yuxN, BSU11500 spx, BSU25000 pbpA, BSU07710 ltaS, BSU23160 rluB, BSU11340 fabF, BSU12100 pdaC, BSU17450 glnR, BSU27180 yrhH, BSU33360 yvgJ, BSU09300 glpD, BSU23830 yqjL, BSU28580 rqcU, BSU10170 fabHB

S2 L: BSU14150 ykuN, BSU10270 lcfB

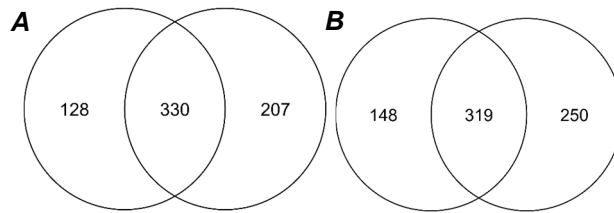
S2 M: BSU35290 prfB, BSU39520 yxeK, BSU00450 sspF, BSU29570 sspA, BSU24620 tasA, BSU39490 yxeN

S2 N: BSU09780 yheC, BSU15090 gerR, BSU03390 yckC, BSU24600 sinI, BSU35310 hpf, BSU14470 mreBH, BSU39960 yxaI, BSU12010 manP, BSU28110 spoVID, BSU03060 lctP, BSU38040 sacA, BSU21900 bsaA, BSU38100 ywcH, BSU22800 spoIVA, BSU35920 rbsK, BSU15500 pyrC, BSU27840 safA, BSU05120 cspC, BSU28460 yslB, BSU00770 sul

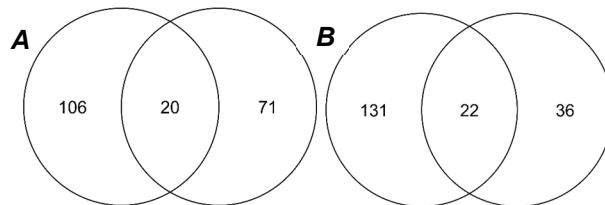
S2 O: BSU15640 rqcH, BSU03970 ycnL, BSU09210 yhcT, BSU32630 yurR, BSU27020 yraA, BSU17330 miaA, BSU22980 ypbG, BSU08560 yfhJ, BSU21140 yonC, BSU18380 iseA, BSU31990 dhbC, BSU39290 yxxD, BSU25740 yqeB, BSU33190 yvrD, BSU28270 leuB, BSU15120 bshC, BSU31970 dhbB, BSU02880 yceB, BSU08520 recX, BSU16970 ymdB, BSU13130 proA, BSU09100 cspB, BSU33510 copZ, BSU34790 trxB, BSU27720 queA, BSU31830 yueE, BSU09750 sspB, BSU23250 ribH, BSU34430 racX, BSU00880 disA, BSU10260 yhfK, BSU27500 trmU, BSU38540 dltE, BSU23510 xerD, BSU06280 ydjP, BSU00300 yaaR, BSU28390 racE, BSU16630 infB, BSU25120 yqfT, BSU25130 nfo, BSU15720 defA, BSU27170 fatR, BSU21050 yonN, BSU11330 fabHA, BSU16120 topA, BSU27370 mltG, BSU32000 dhbA, BSU36230 ugd, BSU26610 yrkA, BSU00670 tilS, BSU30010 ythP, BSU09510 yhdL, BSU05960 ydiG, BSU27640 yrvC, BSU27160 yrhJ, BSU35700 tagH, BSU23910 yqjE, BSU09980 yhaI, BSU02350 gamP, BSU23270 ribE, BSU28830 ysdB, BSU16220 trxA, BSU02450 glnL, BSU37050 maeA, BSU27390 yrrK, BSU15570 cysH, BSU00260 yaaN, BSU10890 yisX, BSU00580 yabN, BSU30550 metK, BSU04490 ydbJ, BSU06520 purH, BSU40990 noc, BSU31000 yuaI, BSU40500 rplI, BSU24290 xseB, BSU14480 abh, BSU33680 catR, BSU00410 mmV, BSU17880 ynzC, BSU40440 dnaC, BSU15230 murB, BSU32620 yurQ, BSU19450 rsbRC, BSU07560 pel, BSU29520 sppl, BSU31010 floT, BSU30960 glgD, BSU29640 ytsP, BSU15010 ylbH, BSU38940 yxjI, BSU09440 citA, BSU41020 thdF, BSU31530 malR, BSU25430 yqeV, BSU32010 besA, BSU29590 nifZ, BSU01150 rpsJ, BSU12900 htrA, BSU25650 yqel, BSU29660 rpsD, BSU19479 yoyC, BSU39580 yxeE, BSU38500 dltA, BSU35660 mnaA, BSU26950 yraG, BSU02890 yceC, BSU40410 walR, BSU38700 sigY, BSU01110 rpsG, BSU15790 rpeA, BSU33770 sdpC, BSU27920 obg, BSU02910 yceE, BSU16960 rny, BSU16140 codV, BSU26550 yrkD, BSU02360 gamA, BSU05660 ydgl, BSU40540 hypR, BSU35760 tagB, BSU06200 ydjH, BSU20490 yoqW, BSU31960 dhbF, BSU00200 yaaK, BSU00700 coaX, BSU24210 yqig, BSU06060 ydiO, BSU07990 yfjR, BSU28370 rph, BSU04550 ydbP, BSU34740 crh, BSU04560 ddl, BSU04690 rsbT, BSU13470 sspD, BSU24180 yqik, BSU31390 yugI, BSU00590 rqcP, BSU09280 glpF, BSU08030 yfjN, BSU22840 engA, BSU16650 rbfA, BSU03070 mdr, BSU03590 tcyC, BSU05700 ydhC, BSU05970 rex, BSU16130 trmFO, BSU15900 fabD, BSU19550 nox, BSU36370 ywpB, BSU08900 yhzB, BSU09550 mpfA, BSU21680 msrB, BSU38690 yxIC, BSU22270 yppE, BSU19300 yozC, BSU21620 yokE, BSU13620 mtnD, BSU01500 rpsI, BSU06070 ydiP, BSU37000 prmC, BSU38530 dltD, BSU33600 smpB, BSU08000 yfjQ, BSU06290 yeaA, BSU22380 tseB, BSU19340 yocR, BSU00140 dck, BSU12210 yjiB, BSU07660 yfiJ, BSU00310 holB, BSU28190 ysxC, BSU02900 yceD, BSU29550 ytcJ, BSU02330 ybfQ, BSU28870 infC, BSU12420 yjoB, BSU23370 ypuA, BSU40530 cotF, BSU07490 fecF, BSU01220 rpsC, BSU35990 ywrO, BSU36950 tsaC, BSU24270 dxs, BSU14170 ykuP, BSU08590 yfhM, BSU05420 ydfl, BSU01770 glmM, BSU16490 rpsB, BSU13810 ykvS, BSU00800 yazB, BSU16610 mpM, BSU10250 lplJ, BSU25440 yqeU, BSU32780 yusF, BSU15000 ylbG, BSU02970 yceK, BSU34440 pbpE



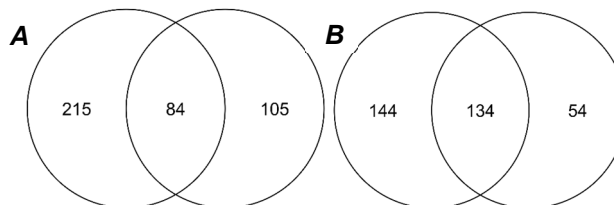
A9. Venn diagram of upregulated (A) and downregulated (B) differentially expressed genes (transcriptomics S1) between wt (left circle) and $\Delta fosB$ (right circle) under FOS treatment. Transcriptome analyses of the strains: *B. subtilis* 168 (*trpC2*) = wt, *B. subtilis* (*trpC2*) $\Delta fosB$ = $\Delta fosB$. FOS treatment with $0.5 \mu\text{g mL}^{-1}$ (wt) and $0.1 \mu\text{g mL}^{-1}$ ($\Delta fosB$) for S1 (from the beginning). Venn diagram was generated with the help of <https://molbiotools.com/listcompare.php>.



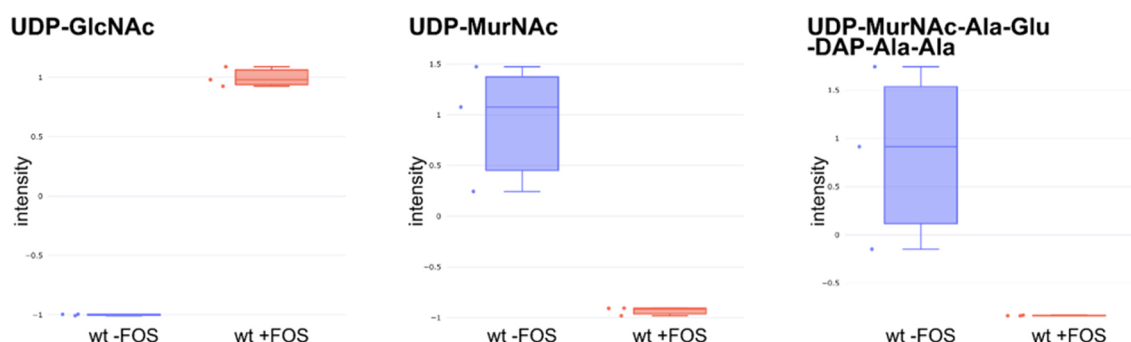
A10. Venn diagram of upregulated (A) and downregulated (B) differentially expressed genes (transcriptomics S2) between wt (left circle) and $\Delta fosB$ (right circle) under FOS treatment. Transcriptome analyses of the strains: *B. subtilis* 168 (*trpC2*) = wt, *B. subtilis* (*trpC2*) $\Delta fosB$ = $\Delta fosB$. FOS treatment with $128 \mu\text{g mL}^{-1}$ (wt) and $16 \mu\text{g mL}^{-1}$ ($\Delta fosB$) for S2 (after 3.5 h of incubation, in the exponential growth phase). Venn diagram was generated with the help of <https://molbiotools.com/listcompare.php>.



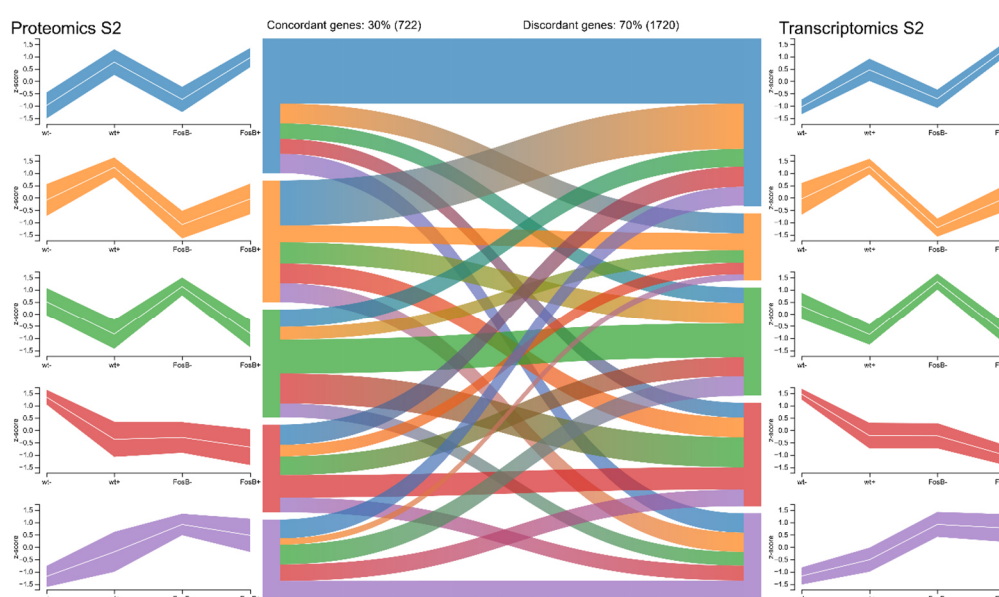
A11. Venn diagram of upregulated (A) and downregulated (B) proteins (proteomics S1) between wt (left circle) and $\Delta fosB$ (right circle) under FOS treatment. Transcriptome analyses of the strains: *B. subtilis* 168 (*trpC2*) = wt, *B. subtilis* (*trpC2*) $\Delta fosB$ = $\Delta fosB$. FOS treatment with $0.5 \mu\text{g mL}^{-1}$ (wt) and $0.1 \mu\text{g mL}^{-1}$ ($\Delta fosB$) for S1 (from the beginning). Venn diagram was generated with the help of <https://molbiotools.com/listcompare.php>.



A12. Venn diagram of upregulated (A) and downregulated (B) proteins (proteomics S2) between wt (left circle) and $\Delta fosB$ (right circle) under FOS treatment. Transcriptome analyses of the strains: *B. subtilis* 168 (*trpC2*) = wt, *B. subtilis* (*trpC2*) $\Delta fosB$ = $\Delta fosB$. FOS treatment with $128 \mu\text{g mL}^{-1}$ (wt) and $16 \mu\text{g mL}^{-1}$ ($\Delta fosB$) for S2 (after 3.5 h of incubation, in the exponential growth phase). Venn diagram was generated with the help of <https://molbiotools.com/listcompare.php>.



A13. LC-MS analysis of the crucial PGN synthesis marker under FOS treatment of *B. subtilis* 168 wt. Shown are the ANOVA test fold changes of the wt cultures ($n=3$) treated with FOS ($128 \mu\text{g mL}^{-1}$) with the corresponding control ($n=3$). P -values: UDP-GlcNAc: 2.0687, UDP-MurNAc: 0.0068, UDP-MurNAc-Ala-Glu-DAP-Ala-Ala: 0.0378.



A14. Sankey diagram of the omics trend-comparing analysis of proteome and transcriptome data from S2. Transcriptome and proteome analyses of the strains: *B. subtilis* 168 (*trpC2*) wt, *B. subtilis* (*trpC2*) ΔfosB . FOS treatment with $128 \mu\text{g mL}^{-1}$ (wt) and $16 \mu\text{g mL}^{-1}$ (ΔfosB) for S2 (after 3.5 h of incubation, in the exponential growth phase). Analysis of the intersecting hits with 5 clusters ($k=5$), shown as centroid profile plots in the individual conditions. Data: Z10, Z11 [32].

A15. Analyses of the comparison between *B. subtilis* wt and *B. subtilis* ΔfosB in transcriptomics S1 and S2. Transcriptome analyses of the strains: *B. subtilis* 168 (*trpC2*) = wt, *B. subtilis* (*trpC2*) ΔfosB = ΔfosB . FOS treatment with $0.5 \mu\text{g mL}^{-1}$ (wt) and $0.1 \mu\text{g mL}^{-1}$ (ΔfosB) for S1 (from the beginning) and $128 \mu\text{g mL}^{-1}$ (wt) and $16 \mu\text{g mL}^{-1}$ (ΔfosB) for S2 (after 3.5 h of incubation, in the exponential growth phase). The analyses carried out are not visualised or summarised in tables; all results and analyses can be found in the summary of all data (Z1).

The detailed investigation of the differences between *B. subtilis* wt and *B. subtilis* ΔfosB was mainly achieved by examining the fold changes of the comparable conditions (each set internally and with and without FOS), including the statistical analysis of specific groups using PANTHER (see methods section for details). In addition, the corresponding heatmaps (Fig.2) were also taken into account.

When comparing the transcriptomic results of S1 without FOS treatment, there are hardly any differences between ΔfosB and wt. In this comparison, 41 transcripts are overrepresented in wt ($\log_2$ fold change of at least 1 and adjusted p -value <0.05) and 20 in the ΔfosB mutant (Z1).

Based on the PANTHER analysis, there are no significantly overrepresented metabolic pathways in the hits of the transcripts upregulated in the wt. However, the UMP biosynthesis and pyrimidine biosynthesis pathways are the most abundant (not significant).

Among the transcripts upregulated by $\Delta fosB$, single hits related to sporulation, motility and flagella, as well as two genes of proline catabolism to glutamate (*putB*, BSU03200 and *putC*, BSU03210) are upregulated and their metabolic pathways are also statistically overrepresented (PANTHER, GO biological process complete). Although the number of differentially expressed transcripts is significantly higher under FOS treatment in S1 (474 overrepresented in wt, 573 overrepresented in $\Delta fosB$), no metabolic pathway of the hits is significantly overrepresented. The significantly higher number of differentially expressed genes can be clearly seen in Fig.2. As already described, wt with FOS behaves as an opponent to $\Delta fosB$ with FOS and the respective controls without FOS. Regardless of the reason, e.g. whether the lower concentration of FOS in the $\Delta fosB$ sample causes it to behave like an untreated sample, no meaningful comparison can be made between wt and $\Delta fosB$. The marker was the *fosB* gene, which logically showed an extreme fold change due to the knockout.

Comparison of the transcriptomic results of S2 (with and without FOS treatment) showed hardly any differences between $\Delta fosB$ and wt. The described heatmap (Fig.2) revealed in the totality of the results that only cluster F (and partly I) showed differences between the strains and therefore only a clear minority of the total transcripts. Looking at the fold changes between the strains with and without FOS, more hits were identified than in S1 (without FOS: 349 transcripts are overrepresented in wt and 263 in the $\Delta fosB$ mutant; with FOS: 139 transcripts are overrepresented in wt and 81 in the $\Delta fosB$ mutant; ($\log_2$ fold change of at least 1 and adjusted p-value <0.05)), but with the exception of the hits overrepresented in $\Delta fosB$ without FOS, the hits were distributed among different single hits, so that no metabolic pathway was significantly overrepresented. The metabolic pathways overrepresented in $\Delta fosB$ among the overrepresented hits were associated with sporulation, chemotaxis in general and specifically with flagella and cell motility (PANTHER, GO biological process complete). The marker $\Delta fosB$ gene was equally evident as in S1.

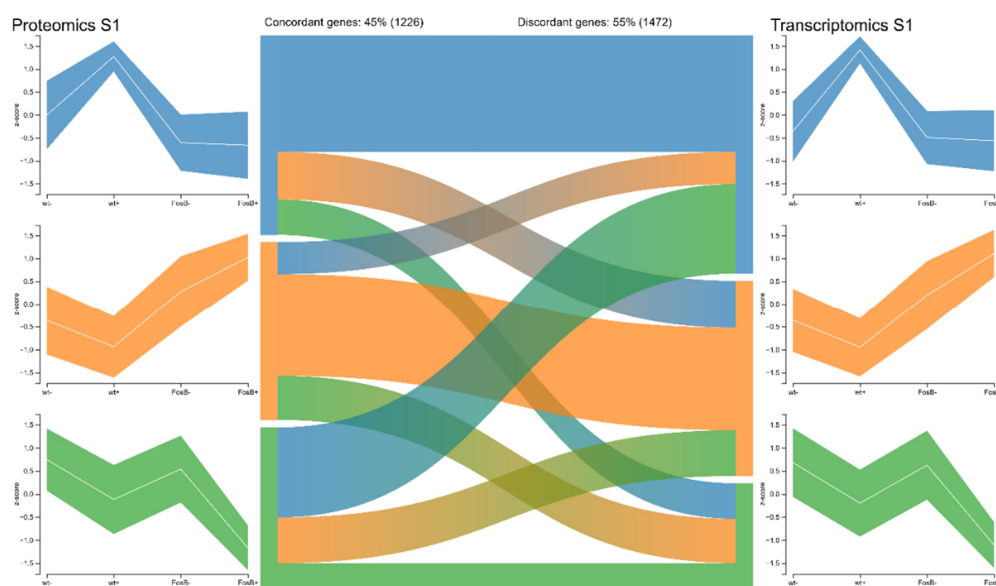
A16. Analyses of the comparison between *B. subtilis* wt and *B. subtilis* $\Delta fosB$ in proteomics S1 and S2. Proteome analyses of the strains: *B. subtilis* 168 (*trpC2*) = wt, *B. subtilis* (*trpC2*) $\Delta fosB$ = $\Delta fosB$. FOS treatment with $0.5 \mu\text{g mL}^{-1}$ (wt) and $0.1 \mu\text{g mL}^{-1}$ ($\Delta fosB$) for S1 (from the beginning) and $128 \mu\text{g mL}^{-1}$ (wt) and $16 \mu\text{g mL}^{-1}$ ($\Delta fosB$) for S2 (after 3.5 h of incubation, in the exponential growth phase). The analyses carried out are not visualised or summarised in tables; all results and analyses can be found in the summary of all data (Z1).

The detailed investigation of the differences between *B. subtilis* wt and *B. subtilis* $\Delta fosB$ was mainly achieved by examining the fold changes of the comparable conditions (each set internally and with and without FOS), including the statistical analysis of specific groups using PANTHER (see methods section for details). In addition, the corresponding heatmaps (Fig.3) were also taken into account. When comparing the proteomic results of S1 without FOS treatment, 62 proteins are overrepresented in wt (p-value <0.05) and 113 in the $\Delta fosB$ mutant (Z1). The hits upregulated by $\Delta fosB$ are mainly proteins associated with flagella, sporulation and chemotaxis. In contrast, proteins and their pathways for UMP biosynthesis and thus pyrimidine biosynthesis are more abundant and significantly overrepresented in the wt (PANTHER, GO biological process complete).

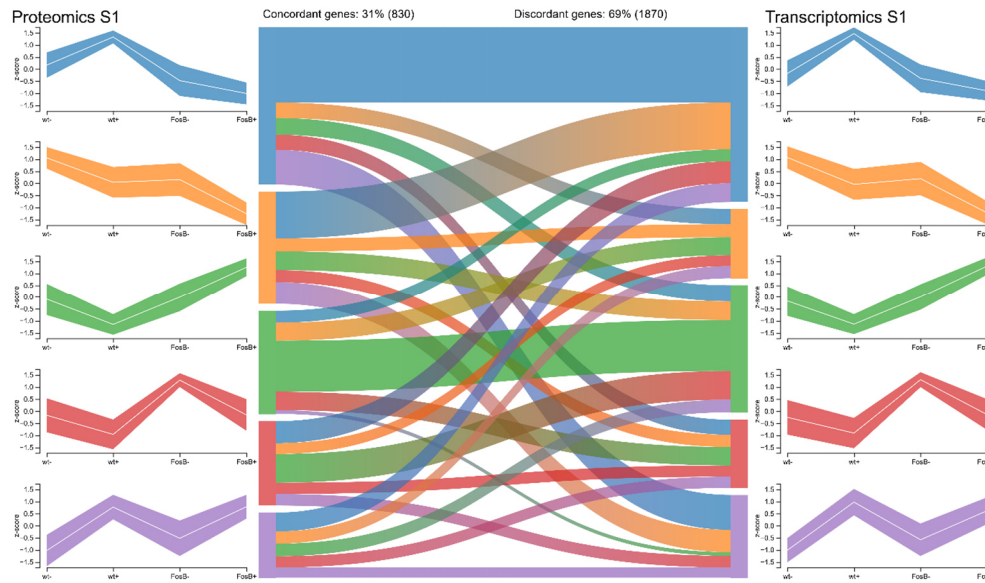
Under FOS treatment, the difference between wt and $\Delta fosB$ in S1 is significantly higher in terms of the number of differentially abundant proteins: 598 proteins are overrepresented in wt and 455 in the $\Delta fosB$ mutant. For the proteins upregulated in $\Delta fosB$, the picture was similar to that of the hits without FOS. The most statistically overrepresented pathways were those associated with flagella, sporulation and chemotaxis (PANTHER, GO biological process complete). Compared to the conditions without FOS, the overrepresented pathways are those of proteins belonging to the category of tricarboxylic acid cycle and carbohydrate metabolic processes or amino acid metabolic processes (alpha-amino acids). Conversely, many ribosomal proteins are overrepresented in the wt hits, as is UMP biosynthesis, which was also present without FOS.

The comparison between wt and $\Delta fosB$ mutant in S2 is fundamentally different from S1. When comparing the proteome results of S2 without FOS treatment, 39 proteins are overrepresented in wt (p-value <0.05) and 66 in the $\Delta fosB$ mutant (Z1). For the proteins upregulated in $\Delta fosB$, as in S1, the hits were statistically overrepresented in pathways associated with flagella, sporulation and chemotaxis (PANTHER, GO biological process complete). Among the hits upregulated in wt, the metabolic pathways of UMP biosynthesis and pyrimidine biosynthesis were overrepresented, as in S1. The situation with FOS treatment was identical not only with regard to the differentially occurring proteins (31 overrepresented in wt, 76 overrepresented in $\Delta fosB$), but also with regard to the statistically overrepresented pathways and significantly occurring proteins.

Although the direct comparison of wt and $\Delta fosB$ mutants reveals differences that vary significantly depending on the setup, a look at the heat map results described (Fig.3) clearly shows that I also clearly differentiate the entire set of samples between S1 and S2. In S2, only clusters H, I, J differ between wt and $\Delta fosB$, whereas in S1, not even the same FOS conditions of the strains cluster together (Fig.3, clustering at top).



A17. Sankey diagram of the omics trend-comparing analysis of proteome and transcriptome data from S1. Transcriptome and proteome analyses of the strains: *B. subtilis* 168 (*trpC2*) wt, *B. subtilis* (*trpC2*) $\Delta fosB$. FOS treatment with $0.5 \mu\text{g mL}^{-1}$ (wt) and $0.1 \mu\text{g mL}^{-1}$ ($\Delta fosB$) for S1 (from the beginning). Analysis of the intersecting hits with 3 clusters ($k=3$), shown as centroid profile plots in the individual conditions. Data: Z26, Z27 [32].



A18. Sankey diagram of the omics trend-comparing analysis of proteome and transcriptome data from S1. Transcriptome and proteome analyses of the strains: *B. subtilis* 168 (*trpC2*) wt, *B. subtilis* (*trpC2*) Δ *fosB*. FOS treatment with $0.5 \mu\text{g mL}^{-1}$ (wt) and $0.1 \mu\text{g mL}^{-1}$ (Δ *fosB*) for S1 (from the beginning). Analysis of the intersecting hits with 5 clusters ($k=5$), shown as centroid profile plots in the individual conditions. Data: Z26, Z27 [32].

A19: Zenodo digital appendix: Friz, S. Version v1, May 1, 2024, DOI: 10.5281/zenodo.11267447. [32].
<https://zenodo.org/records/11267447>. For access to the data, please contact Prof. Dr. Christoph Mayer: christoph.mayer@uni-tuebingen.de.

- Z1: Summary of all transcriptome and proteome data mapped to the entire *B. subtilis* genome (Basis: SubtiWiki)
- Z2: GO-biological process enrichment with panther.de for upregulated differentially expressed genes (Transcriptomics S2) of wt under FOS treatment
- Z3: GO-biological process enrichment with panther.de for upregulated differentially expressed genes (Transcriptomics S2) of Δ *fosB* under FOS treatment
- Z4: GO-biological process enrichment with panther.de for downregulated differentially expressed genes (Transcriptomics S2) of wt under FOS treatment
- Z5: GO-biological process enrichment with panther.de for downregulated differentially expressed genes (Transcriptomics S2) of Δ *fosB* under FOS treatment
- Z6: GO-biological process enrichment with panther.de for upregulated proteins (Proteome S2) of wt under FOS treatment
- Z7: GO-biological process enrichment with panther.de for upregulated proteins (Proteome S2) of Δ *fosB* under FOS treatment
- Z8: GO-biological process enrichment with panther.de for downregulated proteins (Proteome S2) of wt under FOS treatment
- Z9: GO-biological process enrichment with panther.de for downregulated proteins (Proteome S2) of Δ *fosB* under FOS treatment
- Z10: OmicsTIDE Input for S2: Proteomics (<https://omicstide-tuevis.cs.uni-tuebingen.de/>)
- Z11: OmicsTIDE Input for S2: Transcriptomics (<https://omicstide-tuevis.cs.uni-tuebingen.de/>)
- Z12: OmicsTIDE ($k=3$) Fig.7 cluster blue-blue BSU identifier
- Z13: OmicsTIDE ($k=3$) Fig.7 cluster orange-orange BSU identifier
- Z14: OmicsTIDE ($k=3$) Fig.7 cluster green-green BSU identifier
- Z15: GO-biological process enrichment (no correction) with panther.de for upregulated S2 hits (proteome and transcriptome) generated with OmicsTIDE and/or Venn comparisons
- Z16: OmicsTIDE ($k=5$) A14 cluster green-green BSU identifier
- Z17: GO-biological process enrichment (no correction) with panther.de for downregulated S2 hits (proteome and transcriptome) generated with OmicsTIDE
- Z18: GO-biological process enrichment with panther.de for upregulated differentially expressed genes (Transcriptomics S1) of wt under FOS treatment
- Z19: GO-biological process enrichment with panther.de for upregulated differentially expressed genes (Transcriptomics S1) of Δ *fosB* under FOS treatment
- Z20: GO-biological process enrichment with panther.de for downregulated differentially expressed genes (Transcriptomics S1) of wt under FOS treatment
- Z21: GO-biological process enrichment with panther.de for downregulated differentially expressed genes (Transcriptomics S1) of Δ *fosB* under FOS treatment
- Z22: GO-biological process enrichment with panther.de for upregulated proteins (Proteome S1) of wt under FOS treatment
- Z23: GO-biological process enrichment with panther.de for upregulated proteins (Proteome S1) of Δ *fosB* under FOS treatment
- Z24: GO-biological process enrichment with panther.de for downregulated proteins (Proteome S1) of wt under FOS treatment
- Z25: GO-biological process enrichment with panther.de for downregulated proteins (Proteome S1) of Δ *fosB* under FOS treatment
- Z26: OmicsTIDE Input for S1: Proteomics (<https://omicstide-tuevis.cs.uni-tuebingen.de/>)
- Z27: OmicsTIDE Input for S1: Transcriptomics (<https://omicstide-tuevis.cs.uni-tuebingen.de/>)
- Z28: OmicsTIDE ($k=5$) A18 cluster red-red BSU identifier
- Z29: OmicsTIDE ($k=5$) A18 cluster violet-violet BSU identifier
- Z30: GO-biological process enrichment (no correction) with panther.de for upregulated S1 and S2 hits (proteome and transcriptome) generated with OmicsTIDE or Venn comparisons
- T_raw: Transcriptome raw data including explanation
- P_raw: Proteome raw data including explanation

4.8 Manuscript 8

Friz S, Walter A, Mayer C. A cell wall metabolomics workflow to determine cytosolic peptidoglycan precursor levels in bacteria

Ready for submission. Manuscript and appendix are attached.

A cell wall metabolomics workflow to determine cytosolic peptidoglycan precursor levels in bacteria

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Key words: metabolomics, targeted metabolomics, cell wall metabolites, peptidoglycan biosynthesis, cell wall precursors, UDP-GlcNAc, UDP-MurNAc, fosfomycin, CRISPRi, LC-MS, mass spectrometry, UmetaFlow, OpenMS

ABSTRACT

The peptidoglycan biosynthesis pathway is essential for bacteria to build a stabilising cell wall that protects against environmental insults and osmotic lysis. This pathway is generally well conserved in bacteria and is tightly regulated at multiple levels to modulate the formation of a stable cell wall under constantly changing growth conditions and throughout the cell cycle or cell differentiation processes. Interference with peptidoglycan biosynthesis is a major target for antibiotics, with each individual step within the pathway being targeted by an antibiotic. In addition, environmental factors antibiotic compounds may indirectly cause metabolic perturbations that affect the cell wall integrity and peptidoglycan biosynthesis. Therefore, a comprehensive assessment of the peptidoglycan precursor levels is important to better understand the bacterial cellular responses to antibiotics and other factors. Here, we provide a rapid and simple workflow for liquid chromatography-mass spectrometry (LC-MS)-based analysis of cell wall metabolites, allowing detailed views on relative changes in cell wall precursor pool levels in bacteria in response to challenging conditions. This workflow covers all steps from sample preparation and measurement to publication-ready figures for the analysis of soluble cell wall precursors. It is based on an expandable library that covers all cell wall related metabolites, including all precursors of peptidoglycan *de novo* synthesis, and uniquely identifies them by retention time and exact mass. The workflow has been established to study the cell wall metabolome of the Gram-positive model bacterium *Bacillus subtilis* but can be easily adapted and extended to study the specific cell wall metabolites of the Gram-negative model organism *Escherichia coli* and other bacteria.

INTRODUCTION

The rapid emergence of multidrug-resistant (MDR) bacterial pathogens, combined with the slow pace of new antibiotic development and the lack of discovery of new antibiotic targets, poses a massive threat to healthcare systems worldwide. In order to optimise the use of established antibiotics, it is therefore essential to better understand the mechanisms of antibiotic action and bacterial metabolic responses. One of the most important targets for antibiotics is the synthesis of peptidoglycan (PGN), the major component of the bacterial cell wall, which is responsible for the shape and integrity of the bacterial cell. The paramount importance of the PGN cell wall in infection biology is demonstrated not only by its physical importance to bacterial cells and its role as a target for antibiotics, but also by its crucial role in pathogenicity and immune recognition. It is therefore not surprising that PGN composition analysis is a widely used and well-established method. It involves the quantitative and qualitative measurement of complex mixtures of muropeptides produced by the enzymatic degradation of peptidoglycan using liquid chromatography (LC) or LC coupled to mass spectrometry (LC-MS). [1, 2, 3, 4]

In contrast, the complete analysis of all precursors of *de novo* PGN synthesis is rarely performed. However, such analyses allow us to estimate the impact on the overall metabolism. Furthermore, by considering the intercellular links of PGN *de novo* synthesis, the critical importance of PGN *de novo* synthesis can be understood and the specific target of an antibiotic can be directly identified by measuring intermediate metabolites with LC-MS.

There is currently no standardised workflow for the accurate identification and quantification of soluble precursors of PGN biosynthesis (appendix: A1, A2). Firstly, PGN precursors are rapidly metabolised in living cells in order to be incorporated into PGN, or are rapidly modified or degraded upon cell disruption. Therefore, rapid cell disruption and sample preparation are crucial. In addition, the availability of standards is limited and a specific LC method is required. For this reason, we present an in-house library of all soluble cell wall precursors based on MS1 level retention time and mass-to-charge (m/z) for unambiguous identification. This in-house library was generated by a combination of commercial standards, accumulation products in mutants, antibiotic treated cultures, the use of the CRISPRi *Bacillus subtilis* library and the medium and growth dependent natural accumulation of cell wall precursors in *Escherichia coli* and *B. subtilis* [6]. Thus, we demonstrate a variety of valid methods to obtain metabolite-specific LC-MS features when commercial standards are not available. The entire workflow, as shown in Fig.1, includes sample preparation, optimised LC separation, MS measurement and bioinformatic evaluation. The importance of rapid harvesting and sample preparation is achieved using a modified rapid filtration approach adapted from Meyer *et al.* 2013 [7].

The samples are pre-cooled, filtered under vacuum, washed with saline and then flash frozen in 60 % ethanol solvent. After extraction with this solvent and water, the samples are dried and analysed by LC-MS. A 7 min gradient on a Gemini C18 column has been developed for the analysis, which separates all cell wall dependent metabolites. The analysis is therefore suitable for any analysis of PGN precursors. The workflow is completed by the LC-MS data analysis pipeline for metabolomics UmetaFlow, based on OpenMS [8]. Parts of the graphical user interface version of UmetaFlow were developed in close cooperation with this project.

Statistical data analysis was conducted based on the *Hitchhiker's guide to statistical analysis of non-targeted LC-MS/MS data* [5]. To validate the entire cell wall metabolomics workflow and to test all steps in combination, two types of basic experiments were performed: i) treatment of bacterial cells with an antibiotic targeting cell wall metabolism and ii) down-regulation of the expression of an essential enzyme of PGN biosynthesis by mRNA interference: *B. subtilis* cultures treated with fosfomycin (FOS) and *B. subtilis* CRISPRi MurC cytosolic fractions were extracted and analysed by LC-MS for peptidoglycan precursor metabolites. In both cases, the expected accumulation products, UDP-GlcNAc and UDP-MurNAc, were detected at high levels compared to the control.

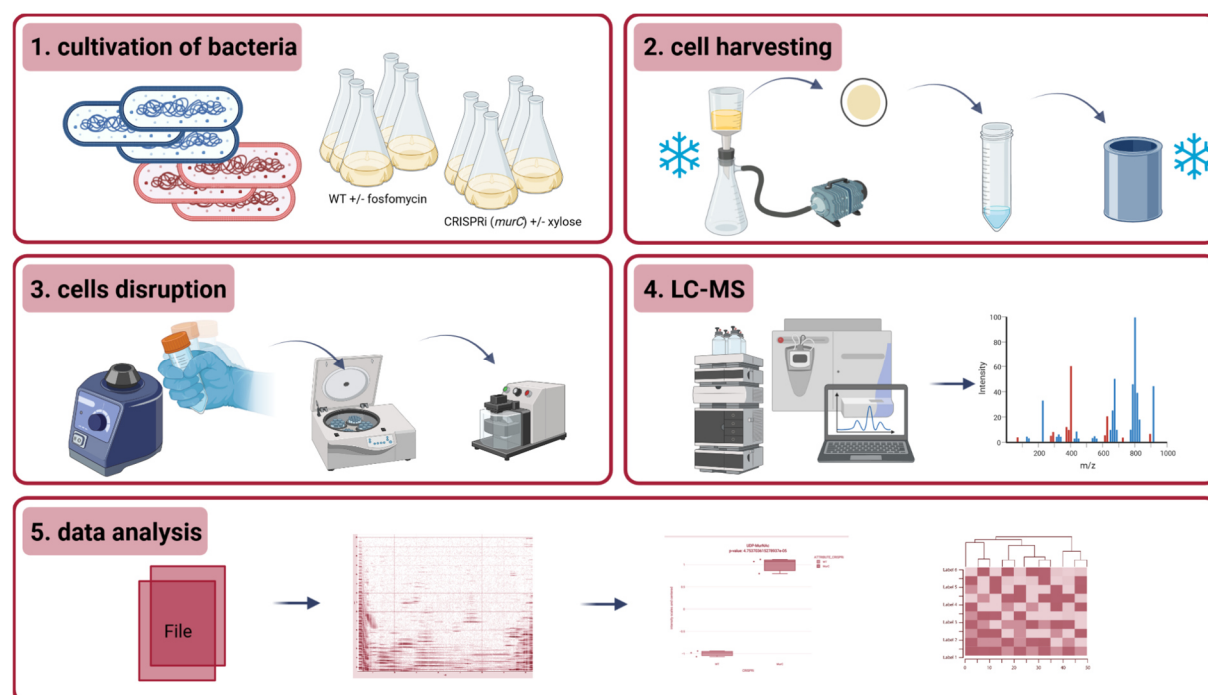


Figure 1: Overview of the presented workflow in 5 steps. 1: cultivation of different bacterial strains and different treatments, 2: fast cooled vacuum fast filtration to harvest the cells and freezing in 60 % ethanol with liquid nitrogen, 3: cells disruption (thawing, shaking, vortexing) centrifugation and speed vac drying, 4: LC separation and MS measurement, 5: bioinformatics evaluation via targeted and untargeted mass search, metabolite identification, statistics and visualisation. Created with BioRender.com.

MATERIAL AND METHODS

Bacterial strains

Bacillus subtilis subsp. *subtilis* str. 168 strain 168; (trpC2). (BGSC, 1A1, [9])

Bacillus subtilis CRISPRi: *murC* BEC29790, Essential Gene Knockdown Library [6]

Escherichia coli BW25113. F- Δ (*araD-araB*)567, Δ *lacZ*4787(::*rrnB*-3), λ -, *rph*-1, Δ (*rhaD-rhaB*) 568, *hsdR*514

Experimental setup and preparation of standards

Experiments to verify the presented method were performed in biologically independent quadruplicates (control, treatment and filter control). The standards for creating the MS 1 library were generated in several ways.

All commercially available standards were purchased (Merck, Sigma Aldrich, Bachem and Carl Roth) and the 1 mM stock solution was measured. For the non-commercially available metabolites, the respective proFolin kits (MurC, D, E, F) were used and, if required, the reaction was carried out to obtain the corresponding product. An overview including manufacturer and catalogue number is given in appendix A5.

Growth conditions and treatment

Cells were cultured in lysogeny broth (LB broth Lennox, Carl Roth) at 37 °C with continuous shaking at 120 rpm. Cells were treated according to the experiment. For antibiotic inhibition, the *B. subtilis* culture was treated with 128 µg mL⁻¹ FOS at an OD_{600nm} of approximately 0.7 and incubated for 60 min before the cells were harvested immediately. The *B. subtilis* CRISPRi MurC knockdown mutant was treated with 1 % xylose at an OD_{600nm} of approximately 0.5 and incubated for 180 min before harvesting.

An overview of the experiments performed is shown in the table below.

Table 1: Conducted experiments.

experiment	strain	treatment	time of treatment/harvesting (OD _{600nm} min)
filter control	no	no	-
Gram-negative Test	<i>E. coli</i> BW25113	no	-
FOS control	<i>B. subtilis subsp. subtilis</i> str. 168 strain 168	no	0.7 60
FOS treatment	<i>B. subtilis subsp. subtilis</i> str. 168 strain 168	128 µg mL ⁻¹ FOS	0.7 60
CRISPRi control	<i>B. subtilis</i> CRISPRi: <i>murC</i> BEC29790	no	0.5 180
CRISPRi treatment	<i>B. subtilis</i> CRISPRi: <i>murC</i> BEC29790	1 % xylose	0.5 180

Sample preparation by fast filtration

The method shown is based on the development of Meyer *et al.* 2013 [7]. All biological samples were adjusted to a sample size of 20 mL with an OD_{600nm} of 0.8. The appropriate amount was cooled with liquid nitrogen (3x immersion for 3-5 s each, without freezing). The sample then has an estimated temperature of 5 °C. The cooled sample was passed through a cooled filter system (XX1014700, Millipore Classic Glass Filter Holder-Kit 47 mm, glass frit membrane support, 300 mL funnel, S-Pak filter, 0.45 µm, 47 mm, MERCK everything chilled at 4 °C for at least 30 min) in less than 5 s. The filter was washed with 5 mL of cooled sterile isotonic saline. The filters were immediately placed in 5 mL of 60 % ethanol (50 mL Falcon), frozen in liquid nitrogen and stored at -80 °C for at least overnight. The cells were disrupted by thawing (room temperature), shaking and vortexing 10-15 times each.

After centrifugation at 4 °C for 5 min at 10000 g, 4 mL of supernatant was removed and the extraction was repeated with 2 mL of cooled water. This resulted in a 6 mL sample, which was dried in a SpeedVac system at 30 °C and then dissolved in H₂O_{dd}.

Pre-experiments with different sample preparation

In preliminary experiments, sample preparation methods were varied and tested. In addition to filtration studies, several sample preparation methods were tested. The sample preparation methods described below were combined. Either fast filtration or centrifugation was used to harvest the cells, followed by cell suspension using either glass beads or 60 % ethanol solution, and finally optional protein precipitation. These steps were tested in all technically feasible combinations. For example, the combination of centrifugation and rapid filtration means that harvesting is done by *centrifugation* followed by *freeze and thaw cell disruption*. Standard for centrifugation: *centrifugation*, *glass bead cell disruption* and *protein precipitation with acetone*; for filtration: *fast filtration* and *freeze and thaw cell disruption*, in both cases followed by speed vacuum drying.

fast filtration

To investigate whether the saline wash step had an effect on extraction, the filters were not washed after filtration according to the protocol.

centrifugation

The cultures were centrifuged at 4000 g for 10 min at 4 °C and then washed in 20 mL of cooled water (repeat centrifugation).

glass bead cell disruption

Samples were thawed from storage at -80°C on ice and 800 µL were transferred to cryotubes containing 0.35 g glass beads (0.25-0.5 mm). Cell disruption was performed in the cell homogeniser for 3x 30 s (60 s break) followed by centrifugation (10,000 g) to recover the supernatant.

freeze and thaw cell disruption

The samples were mixed with the extraction solution of 60 % ethanol and frozen with liquid nitrogen at -80 °C. The cells were disrupted by thawing (room temperature), shaking and vortexing, 10-15 times each. After centrifugation at 4 °C and 10000 g for 5 min, 4 mL of the supernatant was removed and the extraction was repeated with 2 mL of cooled water.

The volume of extraction solution (60 % ethanol, 3-5 mL and water 2-4 mL) was tested as well as various extraction solutions: 80 % methanol, 40:40:20 acetonitrile:methanol:water, 40:40:20 acetonitrile:methanol:water + 0.1 M formic acid.

protein precipitation with acetone

The cell supernatants were incubated with ice-cooled acetone in a ratio of 1:4 for 1 h at -20 °C. The cell supernatants were then centrifuged at -20 °C. After centrifugation at 10000 g, the supernatant was saved and the protein pellet was discarded.

LC-MS measurement

Measurements were performed using an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific™) connected to an ACQUITY ultra-performance liquid chromatography (UPLC) system (Waters Corporation). Separation in the UPLC was carried out using a Phenomenex C18-Gemini® column (150 × 2 mm, 3 µm, 110 Å, Phenomenex) at 37 °C with 0.1 % formic acid and 0.05 % HCO₂NH₄ (A) and CH₃CN (B) buffer system. A single run (injection volume of 5 µL) was performed with a flow rate of 0.2 mL min⁻¹ and a two-step gradient: after 2.5 min of equilibration with 100 % A, a 1 min gradient up to 5 % B is followed by a 4 min gradient up to 70 % B. After 2 min at 70 % B, a re-equilibration step of 2.5 min follows at a flow rate of 4 mL min⁻¹. Analysis of the mass to charge ratios was carried out in the positive ion mode in the range of 50-200 m z⁻¹ and 150-1200 m z⁻¹. The MS settings were as follows: heater temperature 300°C, sheath gas/aux gas/sweep gas flow rate 30/10/0 arb, I spray voltage 4 kV, capillary temperature 275 °C, S-lens RF level 50%, multipole 00 offset -2.28 V, lens 0 voltage -2.78 V, multipole 0 offset -12.00 V, lens 1 voltage -7.00 V, multipole 1 offset -15.56 V, multipole RF amplitude 800.00 V PP, front lens -12.00 V.

Data processing

Raw data MS files were converted to mzML format using msConvert [10]. MS data processing was done with UmetaFlow GUI [11, 12]. For the untargeted metabolomics workflow, default settings were used except for a noise intensity threshold of 1000 in the feature detection step. Four replicates of blank samples were used to filter out blank features. For the quantification of PGN precursor metabolites, ion chromatograms were extracted based on the library (A2, A4) with known retention time and m z⁻¹ values. A retention time range of 40 s around the peak apex (± 20 s) and a mass tolerance of 10 ppm were used. The metadata template was filled with meaningful attributes for statistical analysis (A5). Statistical analysis was performed using the application for statistical analysis of metabolomics data [13]. Blank metabolites were removed with a threshold of 0.3 (mean metabolite intensities in samples must be greater than 30 % of the mean blank intensity), missing values were imputed with random values between zero and the lowest measured intensity (limit of detection), and data were scaled and centred. Parametric assumptions were tested with the Shapiro test for normal distribution and the Levene test for equal variance between the treatment and control groups. Significantly differentially regulated metabolites were detected by one-way ANOVA and confirmed by Tukey's post hoc test.

RESULTS AND DISCUSSION

Optimisation of the metabolite extraction workflow

In our attempt to develop a standardised workflow to enable accurate identification and quantification of soluble PGN biosynthesis precursors, we first optimised the metabolite extraction. Critical steps in all metabolomics approaches are (i) the rapid and mild but thorough cell disruption, (ii) the fast metabolism quenching to avoid metabolite modification or degradation, and finally, (iii) the efficient extraction of metabolites. We optimised these sample preparation steps for the analysis of the cell wall precursor metabolites based on the previously described metabolomics workflow for *B. subtilis* [6]. Numerous pre-experiments were carried out to evaluate influences and to adjust parameters by comparing different sample preparations based on the number of detected features in an untargeted metabolomics workflow [11]. The fast, cooled filtration method appeared to be superior to other methods based on the number of features detected, as shown in an experiment with FOS-treated cells (Fig.2). The filtration method involves fast filtration, cell quenching with liquid nitrogen, washing, cell disruption by freezing and thawing and extraction with 60 % ethanol and water. Obviously, the selection of an optimised cell extraction protocol must also take into account the technical implementation. For example, glass bead disruption is more elaborate and time consuming than other methods of cell disruption.

In addition, this method is usually very time-consuming and the volumes are very small and difficult to adapt; on the other hand, metabolites, especially nucleotides (not shown), are eliminated in this step. In conclusion, our investigations have allowed us to establish an optimal procedure for cell harvesting (fast filtration advantageous over centrifugation), cell disruption (freeze-thaw advantageous over glass beads and various solvents) and protein precipitation/metabolite extraction (good recovery with ethanol-water mixtures). Although the focus of the metabolite extraction method and optimisations described here is on *B. subtilis*, it was possible to perform cell wall metabolomics with *Escherichia coli* in a similar way. For the transfer of the workflow to other bacteria, the methodology and the solvents used for extraction may need to be individually reviewed, depending on the downstream analysis goal, in order to achieve optimal results. [7, 14, 15, 16]

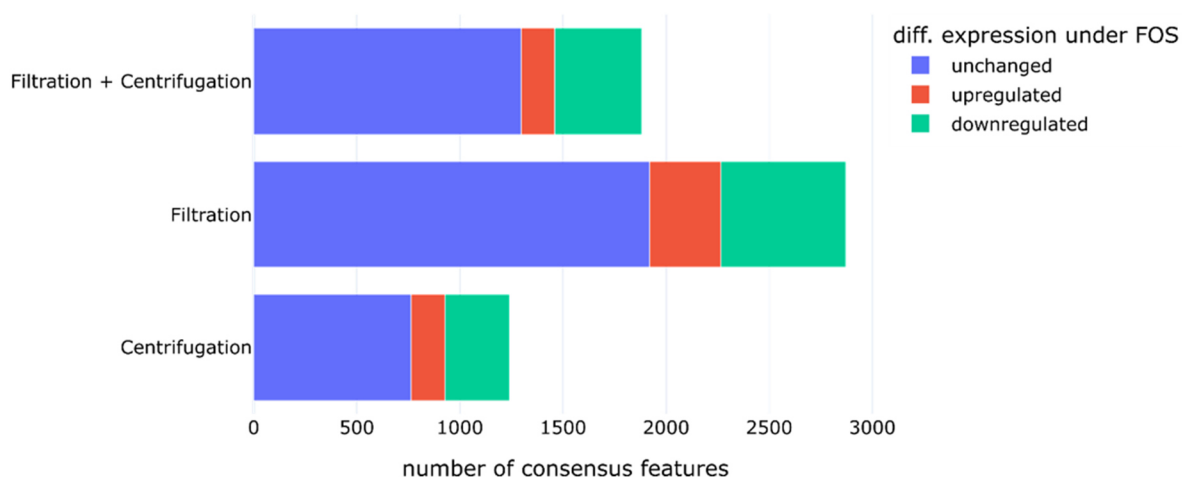


Figure 2: Analysis of the pre-experiments using the consensus feature and the benchmark metabolite UDP-GlcNAc. Comparison of different methods for sample preparation with the main focus on centrifugation or filtration, evaluated on the basis of the consensus features (for explanation see method section).

LC-MS analysis workflow

The next step in the metabolomics workflow is the analysis via LC-MS. Here, we investigated different modes of chromatography (normal phase, HILIC and reverse-phase chromatography). In the end, we chose a specific RP-C18 column for our analyses (Phenomenex C18-Gemini®; column (150 × 2 mm, 3 μm, 110 Å) because the Gemini C18 showed better separation and performance for cell wall related precursors compared to classical C18 columns and other LC modes. Moreover, we were able to achieve a successful separation without the need for reagents or chemical modification of the sample.

We further optimised the LC eluent gradient to be shorter in time but still offering a suitable separation as well as a sensitive detection via MS. Our explorations resulted in a 7 min gradient (with 2.5 min equilibration and re-equilibration, i.e. 10 min LC running time in total), which allowed the separation of all common cell wall metabolites and related primary metabolism compounds. The separation is divided into 2 parts. In the first minute the eluent is increased to 5 % to allow a good separation of the polar metabolites. Subsequently, the eluent is increased to 70 % in 4 min and finally, the gradient is held for 2 min to detach the most hydrophobic metabolites from the column. To test for the optimal gradient and elution conditions, we applied mixture of commercially available metabolites (tryptophan, FOS, GlcNAc, GlcNAc-6P, MurNAc, UDP-GlcNAc) and also included nucleotide standards of the PGN biosynthetic pathway, e.g.UDP-MurNAc-5 (sample)). These compounds reflect the different properties relevant for the separation of all soluble cell wall precursors. The final separation of the soluble cell wall precursors is shown in Fig.3. To ensure high accuracy and intensity of detection of each metabolite, the required mass range of 50-1200 m z⁻¹ was divided into two segments: 50-200 m z⁻¹ and 150-1200 m z⁻¹.

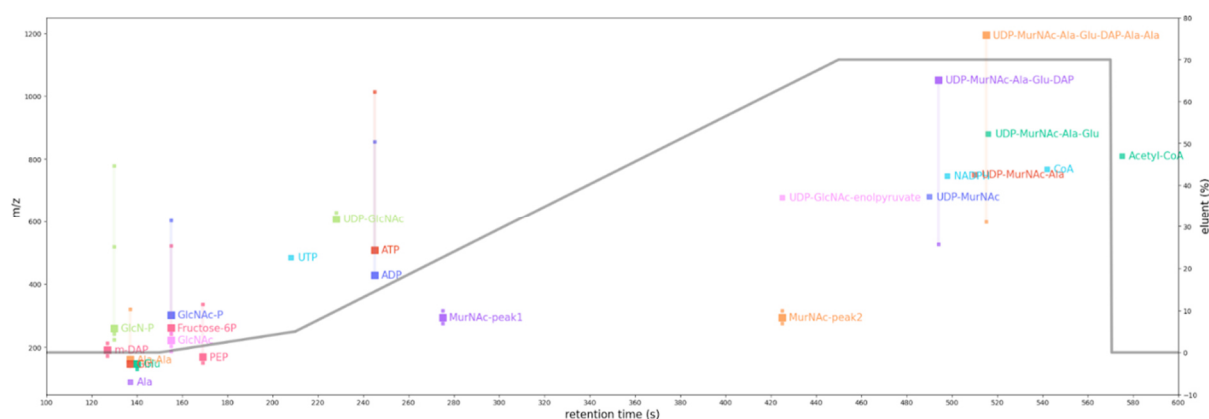


Figure 3: Result of the developed LC gradient. Illustration of the described gradient with the retention times of the relevant metabolites.

Data processing and analysis

Our aim was to provide an understandable semi-automated workflow for the detection and identification of cell wall metabolites in bacterial samples. The process should readily allow automated feature detection and graphical data evaluation; however, it was not designed to be fully automated by purpose to allow data exploration along the metabolite identification process. Since all soluble PGN precursors were present as standards, identification could be achieved by retention time, exact mass detection (i.e. m/z^{-1} on MS1 level) and quantification by extracted ion chromatograms (XICs) within strict RT and m/z^{-1} boundaries. This approach, together with our specially optimised LC-MS method, provided accurate and reproducible results. In parallel, we applied an untargeted metabolomics approach to demonstrate differences in metabolite expression between experiments and to check the reproducibility within our replicates via principal component analysis and hierarchical clustering (Fig.4, Fig.5). This allows us to expand the range of metabolites analysed on demand.

The graphical user interface implementation of UmetaFlow was chosen for the MS data analysis part because of its accessibility and ease of use [11]. It is based on OpenMS and provides the standard untargeted metabolomics data pre-processing workflow as well as the rapid extraction of ion chromatograms from multiple samples [8].

The results of both workflows are two files, a consensus feature matrix with intensities per metabolite and sample, and a template for a metadata table. These files can be used directly as input for a convenient collection of statistical functions for metabolomics data. In collaboration with the Virtual Multi Omics Labs (VMOL), a graphical user interface has been developed which allows data clean-up, exploration and statistical analysis resulting in publication-ready figures [5, 12, 13]. The untargeted metabolomics data were used for hierarchical clustering and heatmap generation as well as principle component analysis (PCA) to visualize general differences between the experimental datasets and to validate reproducibility across replicates. The targeted metabolomics data, based on XICs with our curated standard library, was used to investigate the peptidoglycan biosynthesis metabolites in more detail. Since the data met parametric assumptions, significantly differentially expressed metabolites were identified using one-way ANOVA with followed by Tukey's post-hoc test (Fig.4, Fig.5, Fig.6, Fig.7).

Demonstration of cell wall precursor detection

To demonstrate that the developed workflow works correctly, two independent experiments were performed. First, metabolite samples from *B. subtilis* 168 wt treated with the antibiotic FOS were analysed, and second, an extract from a *B. subtilis* 168 CRISPRi MurC knockdown that was induced with 1 % xylose was analysed. We processed these two data sets in two ways: We analysed the data in an untargeted manner using PCA, clustering and heatmaps, and in a targeted manner using our in-house PGN precursor library. To demonstrate the possibilities of extending this workflow and to verify and analyse the samples as a whole set, a holistic untargeted analysis was chosen as the first step. Using a PCA analysis (Fig.4), a clustering and the corresponding heatmap (Fig.5), we showed with an untargeted metabolomics approach that the samples clustered as expected and that clear differences between the conditions and strains could be detected with this method.

In this analysis, in addition to the FOS and CRISPRi experiments described above, an *E. coli* set (untreated) and a negative background control were used. Fig.5 shows a heatmap combined with a clustering of each sample, highlighting the differences and similarities of each sample in relation to the total sample set. By enlarging the region where UDP-GlcNAc (accumulation during FOS treatment) is present in *B. subtilis* wt samples treated with FOS, an interesting cluster of previously unknown metabolites that accumulate under FOS treatment becomes visible. The reproducibility of the replicates was given and is confirmed by the figures. It was shown that the method works in principle for both Gram-positive and Gram-negative samples.

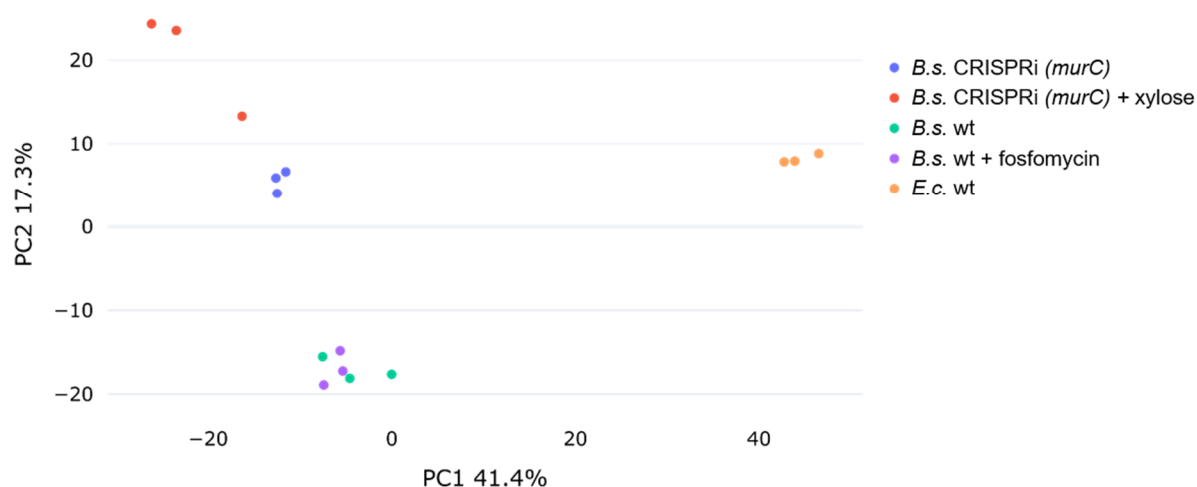


Figure 4: Untargeted principal component analysis (PCA) of all generated results. Samples: A5.

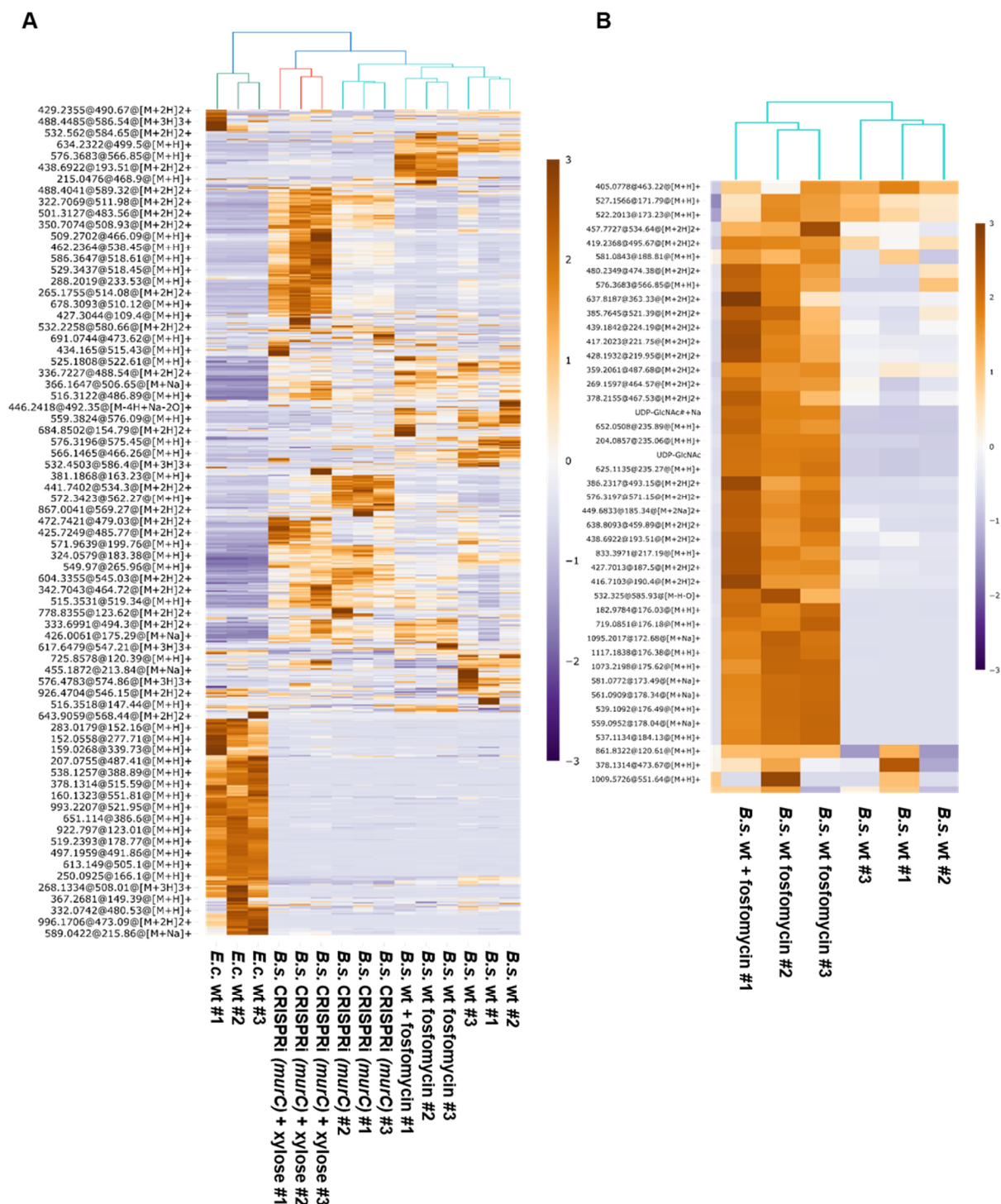


Figure 5: Untargeted metabolomics to qualify the experiments. Shown is a heatmap in connection with a clustering of all samples. A: heatmap with clustering of all metabolomics features; B: Zoom into *B. subtilis* wt FOS experiment with the interesting cluster around UDP-GlcNAc. A5.

To test the method on a specific biological question, *B. subtilis* was treated with FOS. The FOS-caused inhibition of MurA leads to an accumulation of the cell wall precursor and MurA substrate UDP-GlcNAc. Fig.4 shows a relevant selection of images that can be generated in the form shown as the final result of the statistical workflow. The ANOVA (Fig.6, top) test performed shows that the expected UDP-GlcNAc is among the significant results.

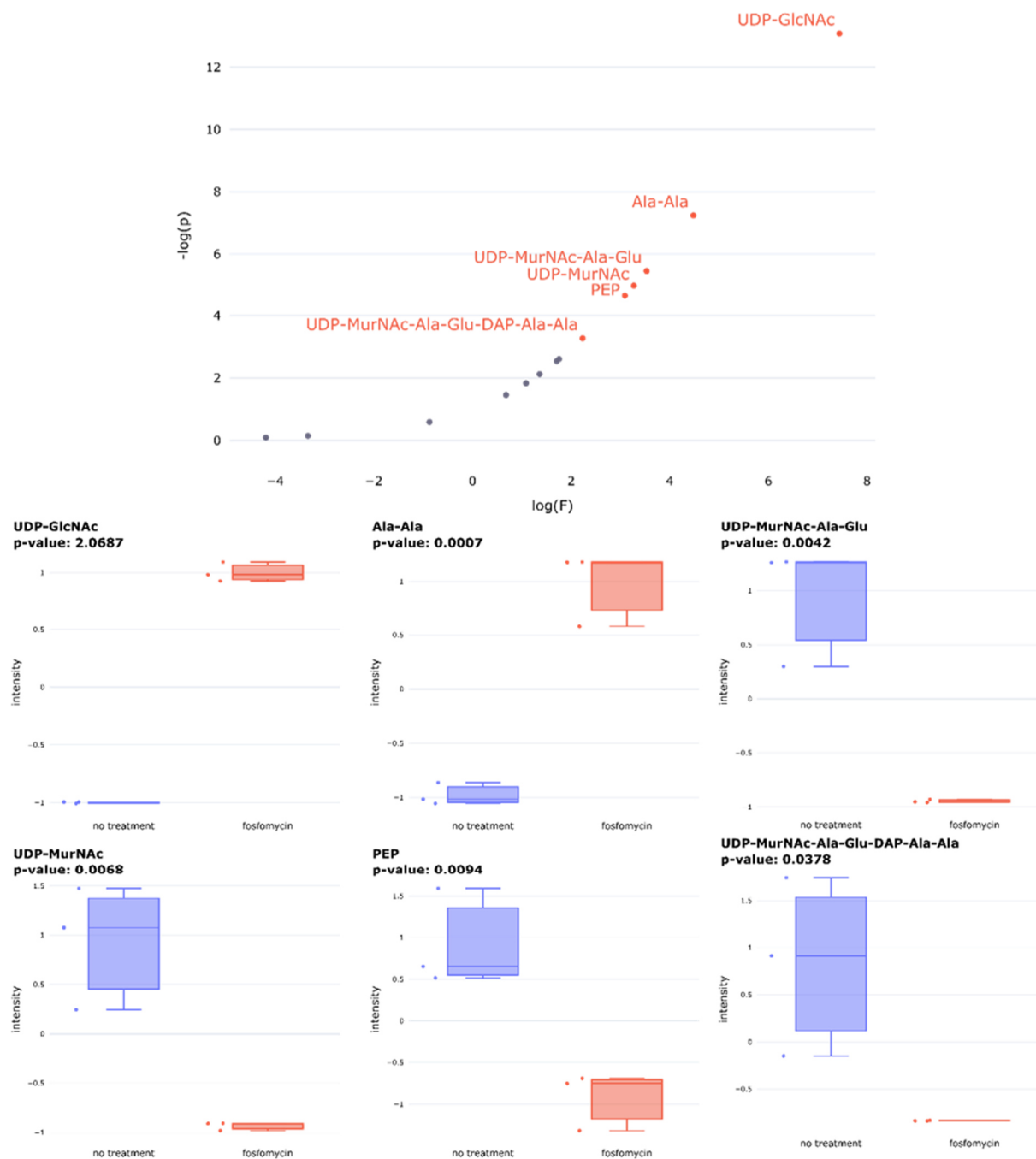


Figure 6: Fosfomycin treatment of *B. subtilis* 168 wt. A: ANOVA test; B: boxplots of all ANOVA significant features confirmed with Tukey's test. Samples: *B. subtilis* wt treated with $128 \mu\text{g mL}^{-1}$ FOS, A5.

For the second biological question, *B. subtilis* 168 CRISPRi MurC knockdown was induced with 1 % xylose. Activation of the CRISPRi system leads to inhibition of MurC, resulting in the accumulation of UDP-MurNAc. Also in this case, the ANOVA plot (Fig.6, top) shows the expected result and metabolite UDP-MurNAc among the significant results, supported by the boxplot.

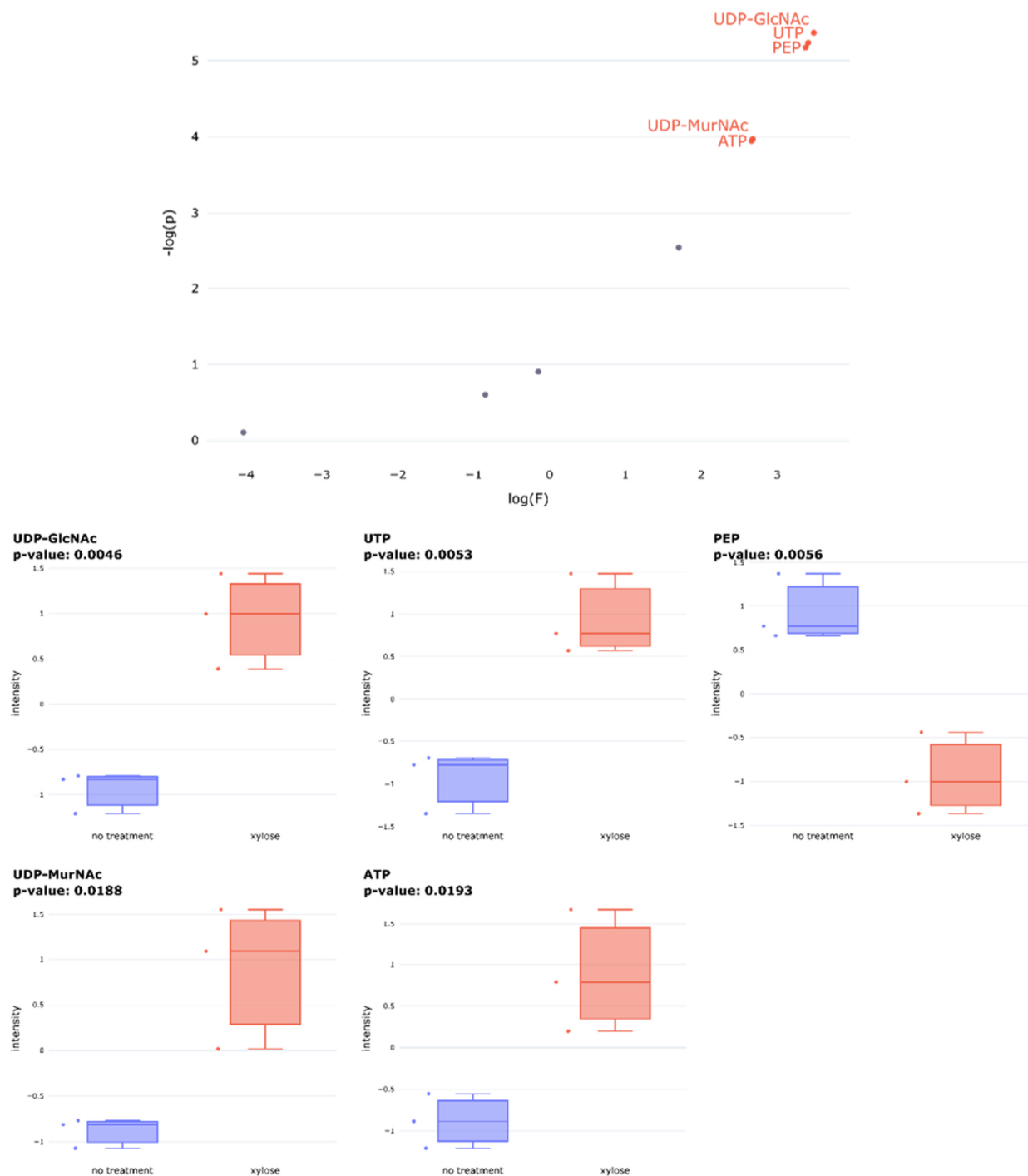


Figure 7: Induction of *B. subtilis* 168 CRISPRi MurC with 1 % xylose. A: ANOVA test; B: boxplots of all ANOVA significant features confirmed with Tukey's test. Samples: *B. subtilis* CRISPRi MurC treated with 1 % xylose, A5

In both examples shown, more significant metabolites are detected (Fig.5, Fig.6, ANOVA (top)). UDP-GlcNAc accumulates under FOS treatment and UDP-MurNAc accumulates under CRISPRi induction of the CRISPRi (*murC*) mutant. The levels of the subsequent metabolites decrease in both cases due to the lack of precursors. Under CPSPRi induction, UDP-GlcNAc accumulates as a result of "backlog" and UDP-MurNAc is at the same time a very competent inhibitor of MurA. In both cases, the accumulation of D-Ala-D-Ala as a substrate for the formation of the UDP-MurNAc pentapeptide is obvious, since the reaction partner in the form of UDP-MurNAc tripeptide is hardly present due to the inhibition of earlier essential steps.

To further analyse the possibilities and advantages, Fig.8 shows a graphical representation of the two experiments described. This has been done using the *de novo* cell wall biosynthesis pathway, which is central to this project. In addition to a helpful and easy to read table (A1) with all substrates, products and enzymes (*B. subtilis* and *E. coli*), Fig.8 shows a flow diagram of the PGN *de novo* synthesis with a reduced presentation of the results in relation to the entire metabolic pathway. The visual representation shows the effects on the metabolic pathways clearly and at a glance.

Conclusion

In conclusion, we present a workflow from sample preparation to publication-ready figures for the analysis of soluble cell wall precursors. In addition, we publish a standard library of all soluble cell wall precursors (including all non-commercially available metabolites to uniquely identify and accurately quantify them at MS1 level (retention time and exact mass)). Using both untargeted metabolomics and EIC-based evaluation approaches. Although the focus and optimisation is based on the Gram-positive example organism *B. subtilis*, the workflow has been shown to work for sample sets of both Gram-negative and Gram-positive origin. However, depending on the application, additional optimisation is required. Similarly, for other metabolites or pathways, the identification of soluble cell wall precursors is simple, reliable and reproducible, but a different focus may require different fine-tuning of the workflow.

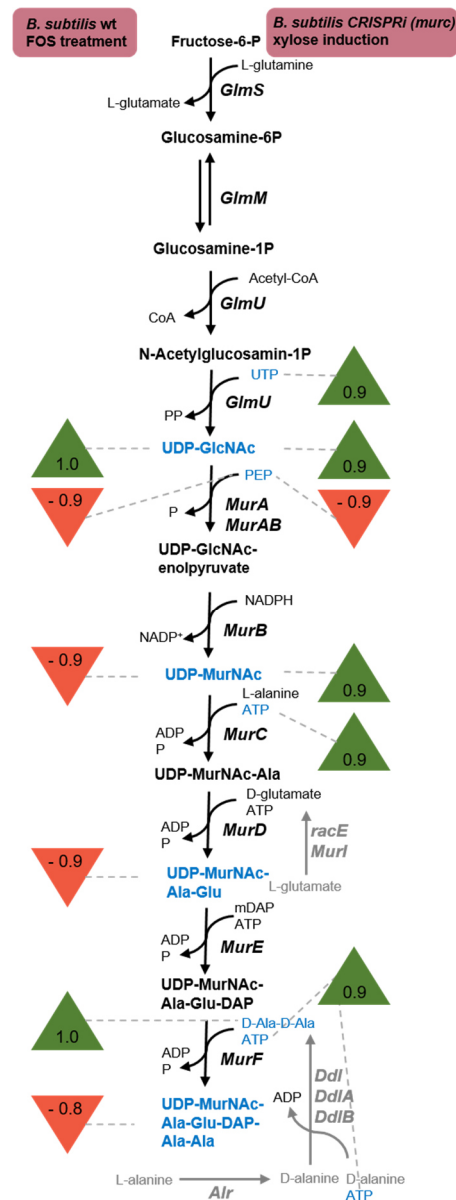


Figure 8: Illustration of total de novo PGN synthesis under different conditions. In addition to the presentation of all steps of the PGN synthesis with all substrates, products and enzymes (tabular presentation of this scheme in A1), some selected metabolites are presented across two different experiments. On the left side the inhibition of *B. subtilis* wt with fosfomycin is shown and on the right side the xylose induction of the *B. subtilis* CRISPRi MurC mutant. Upregulation of metabolites is marked with a green upwards triangle, accordingly, a less present metabolite is marked with a red triangle facing downwards. The number in the triangles corresponds to the normalised change mean (untreated-treated)/2. Shown are only ANOVA significant metabolites

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AUTHOR CONTRIBUTIONS

SF and AW developed the experiments. SF conducted the biological experiments, AW performed the bioinformatic analyses. SF and AW wrote the MS and visualised the results. CM provided scientific assistance and reviewed the MS.

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APPENDIX

A1: cytoplasmic steps of peptidoglycan biosynthesis in *Escherichia coli* and *Bacillus subtilis*.

A2: Metabolites of the cytoplasmic steps of cell wall de novo synthesis.

A3: Sources of the standard compounds used for cell wall metabolomics.

A4: Metabolite standards (UmetaFlow).

A5: Metadata Table.

Appendix

A cell wall metabolomics workflow to determine cytosolic peptidoglycan precursor levels in bacteria

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A1: cytoplasmic steps of PGN biosynthesis in *Escherichia coli* and *Bacillus subtilis*. *equilibrium reaction, #bifunctional enzyme

5,5					substrate 1		substrate 2		substrate 3		product 1		product 2		product 3		product 4	
B. subtilis (BSU)		E.coli (EG)		EC N.	name (PubChem ID)				name (PubChem ID)				name (PubChem ID)					
GlmS	BSU01780	GlmS	EG10382	2.6.1.16	D-fructose 6-phosphate	CHEBI:61527	L-glutamine	CHEBI:58359			D-glucosamine 6-phosphate	CHEBI:58725	L-glutamate	CHEBI:29985				
GlmM*	BSU1770	GlmM	EG11553	5.4.2.10	D-glucosamine 6-phosphate	CHEBI:58725					α-D-glucosamine 1-phosphate	CHEBI:58516						
GlmU#	BSU00500	GlmU	EG11198	2.3.1.157	α-D-glucosamine 1-phosphate	CHEBI:58516	acetyl-CoA	CHEBI:57288			N-acetyl-α-D-glucosamine 1-phosphate	CHEBI:57776	CoA	CHEBI:57287	H+	CHEBI:15378		
GlmU#	BSU00500	GlmU	EG11198	2.7.7.23	N-acetyl-α-D-glucosamine 1-phosphate	CHEBI:57776	UTP	CHEBI:46398	H+	CHEBI:15378	UDP-N-acetyl-α-D-glucosamine	CHEBI:57705	diphosphate	CHEBI:33019				
MurAA	BSU36760	MurA	EG11358	2.5.1.7	UDP-N-acetyl-α-D-glucosamine	CHEBI:57705	phosphoenolpyruvate	CHEBI:58702			UDP-N-acetyl-3-O-(1-carboxyvinyl)-α-D-glucosamine	CHEBI:68483	phosphate	CHEBI:43474				
MurAB	BSU37100	-	-	2.5.1.7	UDP-N-acetyl-α-D-glucosamine	CHEBI:57705	phosphoenolpyruvate	CHEBI:58702			UDP-N-acetyl-3-O-(1-carboxyvinyl)-α-D-glucosamine	CHEBI:68483	phosphate	CHEBI:43474				
MurB	BSU15230	MurB	EG11205	1.3.1.98	UDP-N-acetyl-3-O-(1-carboxyvinyl)-α-D-glucosamine	CHEBI:68483	NADPH	CHEBI:57783	H+	CHEBI:15378	UDP-N-acetyl-α-D-muramate	CHEBI:70757	NADP+	CHEBI:58349				
MurC	BSU29790	MurC	EG10619	6.3.2.8	UDP-N-acetyl-α-D-muramate	CHEBI:70757	L-alanine	CHEBI:57972	ATP	CHEBI:30616	UDP-N-acetyl-α-D-muramoyl-L-alanine	CHEBI:83898	ADP	CHEBI:456216	phosphate	CHEBI:43474		
RacE	BSU28390	MurI	EG11204	5.1.1.3	L-glutamate	CHEBI:29985					D-glutamate	CHEBI:29986						
MurD	BSU15200	MurD	EG10620	6.3.2.9	UDP-N-acetyl-α-D-muramoyl-L-alanine	CHEBI:83898	D-glutamate	CHEBI:29986	ATP	CHEBI:30616	UDP-N-acetyl-α-D-muramoyl-L-alanyl-D-glutamate	CHEBI:83900	ADP	CHEBI:456216	phosphate	CHEBI:43474		
MurE	BSU15180	MurE	EG10621	6.3.2.13	UDP-N-acetyl-α-D-muramoyl-L-alanyl-D-glutamate	CHEBI:83900	meso-2,6-diaminoheptanedioate	CHEBI:57791	ATP	CHEBI:30616	UDP-N-acetyl-α-D-muramoyl-L-alanyl-γ-D-glutamyl-meso-2,6-diaminoheptanedioate	CHEBI:83905	ADP	CHEBI:456216	phosphate	CHEBI:43474		
Alr/Dal/AlrA	BSU04640	Alr/DadX	EG10001/EG1408	5.1.1.1	L-alanine	CHEBI:57972					D-alanine	CHEBI:57416						
Ddl	BSU04560	DdlA/DdlB	EG10213/EG10214	6.3.2.4	D-alanine	CHEBI:57416	D-alanine	CHEBI:57416	ATP	CHEBI:30616	D-alanyl-D-alanine	CHEBI:57822	ADP	CHEBI:456216	phosphate	CHEBI:43474		
MurF	BSU04570	MurF	EG10622	6.3.2.10	UDP-N-acetyl-α-D-muramoyl-L-alanyl-γ-D-glutamyl-meso-2,6-diaminoheptanedioate	CHEBI:83905	D-alanyl-D-alanine	CHEBI:57822	ATP	CHEBI:30616	UDP-N-acetyl-α-D-muramoyl-L-alanyl-γ-D-glutamyl-meso-2,6-diaminopimeloyl-D-alanyl-D-alanine	CHEBI:61386	ADP	CHEBI:456216	phosphate	CHEBI:43474		

A2: Metabolites of the cytoplasmic steps of PGN de novo synthesis.

common name	name	compound	neutral	[M-H] 1+	[M+Na] 1+	[M+K] 1+	[M+2H] 2+
D-Ala	D-alanine	C3H7NO2	89.0477	90.055	112.0369	128.0108	45.5311
L-Ala	L-alanine	C3H7NO2	89.0477	90.055	112.0369	128.0108	45.5311
L-Gln	L-glutamine	C5H10N2O3	146.0691	147.0764	169.0584	185.0323	74.0418
L-Glu	L-glutamate	C5H9NO4	147.0532	148.0604	170.0424	186.0163	74.5339
D-Glu	D-glutamate	C5H9NO4	147.0532	148.0604	170.0424	186.0163	74.5339
Ala-Ala	D-alanyl-D-alanine	C6H12N2O3	160.0848	161.0921	183.074	199.048	81.0497
PEP	phosphoenolpyruvate	C3H5O6P	167.9824	168.9897	190.9716	206.9455	84.9985
mDAP	<i>meso</i> -2,6-diaminoheptanedioate	C7H14N2O4	190.0954	191.1026	213.0846	229.0585	96.055
GlcNAc	<i>N</i> -acetyl- α -D-glucosamine	C8H15NO6	221.0899	222.0972	244.0792	260.0531	111.5522
GlcN-6P	D-glucosamine 6-phosphate	C6H14NO8P	259.0457	260.053	282.0349	298.0089	130.5301
GlcN-1P	D-glucosamine 1-phosphate	C6H14NO8P	259.0457	260.053	282.0349	298.0089	130.5301
fructose-6P	D-fructose 6-phosphate	C6H13O9P	260.0297	261.037	283.0189	298.9929	131.0221
MurNAc	<i>N</i> -acetyl- α -D-muramic acid	C11H19NO8	293.1111	294.1183	316.1003	332.0742	147.5628
GlcNAc-1P	<i>N</i> -acetyl- α -D-glucosamine 1-phosphate	C8H16NO9P	301.0563	302.0635	324.0455	340.0194	151.5354
ADP	adenosine 5'-diphosphate	C10H15N5O10P2	427.0294	428.0367	450.0186	465.9926	214.522
UTP	uridine 5'-triphosphate	C9H15N2O15P3	483.9685	484.9758	506.9578	522.9317	242.9915
ATP	adenosine-5'-triphosphate	C10H16N5O13P3	506.9958	508.003	529.985	545.9589	254.5052
UDP-GlcNAc	UDP- <i>N</i> -acetyl- α -D-glucosamine	C17H27N3O17P2	607.0816	608.0889	630.0708	646.0447	304.5481
enolpyruvyl-UDP-GlcNAc	UDP- <i>N</i> -acetyl-3-O-(1-carboxyvinyl)- α -D-glucosamine	C20H29N3O19P2	677.0871	678.0943	700.0763	716.0502	339.5508
UDP-MurNAc	UDP- <i>N</i> -acetyl- α -D-muramate	C20H31N3O19P2	679.1027	680.11	702.0919	718.0659	340.5586
NADPH	Dihyronicotinamide-adenine dinucleotide phosphate	C21H30N7O17P3	745.0911	746.0984	768.0803	784.0543	373.5528
UDP-MurNAc-L-Ala	UDP- <i>N</i> -acetyl- α -D-muramoyl-L-alanine	C23H36N4O20P2	750.1398	751.1471	773.129	789.103	376.0772
CoA	coenzyme A	C21H36N7O16P3S	767.1152	768.1225	790.1044	806.0784	384.5649
acetyl-CoA	acetyl-coenzyme A	C23H38N7O17P3S	809.1258	810.1331	832.115	848.0889	405.5702
UDP-MurNAc-L-Ala-D-Glu	UDP- <i>N</i> -acetyl- α -D-muramoyl-L-alanyl-D-glutamate	C28H43N5O23P2	879.1824	880.1897	902.1716	918.1456	440.5985
UDP-MurNAc-tripeptide	UDP- <i>N</i> -acetyl- α -D-muramoyl-L-alanyl- γ -D-glutamyl- <i>meso</i> -2,6-diaminoheptanedioate	C35H55N7O26P2	1051.2672	1052.2745	1074.2564	1090.2304	526.6409
UDP-MurNAc-pentapeptide	UDP- <i>N</i> -acetyl- α -D-muramoyl-L-alanyl- γ -D-glutamyl- <i>meso</i> -2,6-diaminopimeloyl-D-alanyl-D-alanine	C41H65N9O28P2	1193.3414	1194.3487	1216.3307	1232.3046	597.678

A3: Sources of the standard compounds used for cell wall metabolomics.

common name	name	compound	neutral	standard origin	type	brand	catalogue number	final concentration
D-Ala	D-alanine	C3H7NO2	89.0477	weigh-in	chemical	Merck-Sigma Aldrich	A7377-5G	1 mM
L-Ala	L-alanine	C3H7NO2	89.0477	weigh-in	chemical	Merck	1007	1 mM
L-Gln	L-glutamine	C5H10N2O3	146.0691	weigh-in	chemical	Merck	289	1 mM
L-Glu	L-glutamate	C5H9NO4	147.0532	weigh-in	chemical	Merck	291	1 mM
D-Glu	D-glutamate	C5H9NO4	147.0532	weigh-in	chemical	Merck-Sigma Aldrich	G1001-1G	1 mM
Ala-Ala	D-alanyl-D-alanine	C6H12N2O3	160.0848	weigh-in	chemical	Bachem	4000279.0001	1 mM
PEP	phosphoenolpyruvate	C3H5O6P	167.9824	weigh-in	chemical	Merck-Sigma Aldrich	P7127-1G	1 mM
mDAP	<i>meso</i> -2,6-diaminoheptanedioate	C7H14N2O4	190.0954	weigh-in	chemical	Merck-Sigma Aldrich	07036-50MG	1 mM
GlcNAc	<i>N</i> -acetyl- α -D-glucosamine	C8H15NO6	221.0899	weigh-in	chemical	Fluka	01140	1 mM
GlcN-6P	D-glucosamine 6-phosphate	C6H14NO8P	259.0457	weigh-in	chemical	Merck-Sigma Aldrich	G5509-1G	1 mM
GlcN-1P	α -D-glucosamine 1-phosphate	C6H14NO8P	259.0457	weigh-in	chemical	Merck-Sigma Aldrich	G9753-5MG	1 mM
fructose-6P	D-fructose 6-phosphate	C6H13O9P	260.0297	dilution	KIT standard	Merck-Sigma Aldrich	ML0013-1KT	1 mM
MurNAc	<i>N</i> -acetyl- α -D-muramic acid	C11H19NO8	293.1111	weigh-in	chemical	Bachem	4011440.1000	1 mM
GlcNAc-1P	<i>N</i> -acetyl- α -D-glucosamine 1-phosphate	C8H16NO9P	301.0563	weigh-in	chemical	Merck-Sigma Aldrich	A2142	1 mM
ADP	adenosine 5'-diphosphate	C10H15N5O10P2	427.0294	weigh-in	chemical	Merck-Sigma Aldrich	A2754-1G	1 mM
UTP	uridine 5'-triphosphate	C9H15N2O15P3	483.9685	weigh-in	chemical	Merck	U6875-100MG	1 mM
ATP	adenosine-5'-triphosphate	C10H16N5O13P3	506.9958	weigh-in	chemical	Carl Roth	HN35.4	1 mM
UDP-GlcNAc	UDP- <i>N</i> -acetyl- α -D-glucosamine	C17H27N3O17P2	607.0816	dilution	KIT standard	proFoldin	MURA-100EC	1 mM
enolpyruvyl-UDP-GlcNAc	UDP- <i>N</i> -acetyl-3-O-(1-carboxyvinyl)- α -D-glucosamine	C20H29N3O19P2	677.0871	KIT reaction	KIT product	proFoldin	MURA-100EC	approx. 1 mM
UDP-MurNAc	UDP- <i>N</i> -acetyl- α -D-muramate	C20H31N3O19P2	679.1027	dilution	KIT standard	proFoldin	MURC-100EC	1 mM
NADPH	Dihydronicotinamide-adenine dinucleotide phosphate	C21H30N7O17P3	745.0911	weigh-in	chemical	Merck	10107824001	1 mM
UDP-MurNAc-L-Ala	UDP- <i>N</i> -acetyl- α -D-muramoyl-L-alanine	C23H36N4O20P2	750.1398	dilution	KIT standard	proFoldin	MURD-100EC	1 mM
CoA	coenzyme A	C21H36N7O16P3S	767.1152	weigh-in	chemical	Merck-Sigma Aldrich	C4282-10MG	1 mM
acetyl-CoA	acetyl-coenzyme A	C23H38N7O17P3S	809.1258	dilution	KIT standard	Merck-Sigma Aldrich	ML0013-1KT	1 mM
UDP-MurNAc-L-Ala-D-Glu	UDP- <i>N</i> -acetyl- α -D-muramoyl-L-alanyl-D-glutamate	C28H43N5O23P2	879.1824	dilution	KIT standard	proFoldin	MURE-100EC	1 mM
UDP-MurNAc-tripeptide	UDP- <i>N</i> -acetyl- α -D-muramoyl-L-alanyl- γ -D-glutamyl- <i>meso</i> -2,6-diaminoheptanedioate	C35H55N7O26P2	1051.2672	dilution	KIT standard	proFoldin	MURF-100EC	1 mM
UDP-MurNAc-pentapeptide	UDP- <i>N</i> -acetyl- α -D-muramoyl-L-alanyl- γ -D-glutamyl- <i>meso</i> -2,6-diaminopimeloyl-D-alanyl-D-alanine	C41H65N9O28P2	1193.3414	KIT reaction	KIT product	proFoldin	MURF-100EC	approx. 1 mM

Substrates of all cytoplasmic steps of peptidoglycan de novo synthesis

A4: Metabolite standards (UmetaFlow).

standard	mz	retention time
Acetyl-CoA	810.133	575
ADP	428.037	245
ADP#Dimer	855.066	245
Ala	90.055	137
Ala-Ala	161.092	137
Ala-Ala#Dimer	321.177	137
ATP	508.003	245
ATP#Dimer	1014.999	245
CoA	768.123	542
Fructose-6P	261.037	155
Fructose-6P#Dimer	521.067	155
GlcN-P	260.053	130
GlcN-P#-2H2O	224.032	130
GlcN-P#-H2O	242.042	130
GlcN-P#Dimer	519.099	130
GlcN-P#Trimer	778.144	130
GlcNAc	222.097	155
GlcNAc#+Na-H	244.079	155
GlcNAc#-2H2O	186.076	155
GlcNAc#-H2O	204.087	155
GlcNAc-1P	302.064	155
GlcNAc-1P#Dimer	603.120	155
Glu	148.060	140
Glu#-H2O	130.050	140
L-Gln	147.076	137
L-Gln#-NH3	130.050	137
m-DAP	191.103	127
m-DAP#+Na-H	213.085	127
m-DAP#-H2O	173.092	127
MurNAc#first	294.118	275
MurNAc#second	294.118	425
MurNAc#first+Na-H	316.100	275
MurNAc#second+Na-H	316.100	425
MurNAc#first-H2O	276.108	275
MurNAc#second-H2O	276.108	425
NADPH	746.098	498
PEP	168.990	169
PEP#-H2O	150.979	169
PEP#Dimer	336.972	169
UDP-GlcNAc	608.089	228
UDP-GlcNAc#+Na-H	630.071	228
UDP-GlcNAc-enolpyruvate	678.094	425
UDP-MurNAc	680.110	490
UDP-MurNAc-Ala	751.147	510
UDP-MurNAc-Ala-Glu	880.190	516
UDP-MurNAc-Ala-Glu-DAP	1052.275	494
UDP-MurNAc-Ala-Glu-DAP#2+	526.641	494
UDP-MurNAc-Ala-Glu-DAP-Ala-Ala	1194.349	515
UDP-MurNAc-Ala-Glu-DAP-Ala-Ala#2+	597.678	515
UTP	484.976	208

Ready to use for the metabolomics workflow.

A5: Metadata Table.

filename	ATTRIBUTE_Sample_Type	ATTRIBUTE_Organism	ATTRIBUTE_Strain	ATTRIBUTE_Experiment	ATTRIBUTE_Treatment
<i>B.s.</i> CRISPRi #1.mzML	Sample	<i>Bacillus subtilis</i>	CRISPRi MurC	<i>B.s.</i> CRISPRi	no treatment
<i>B.s.</i> CRISPRi #2.mzML	Sample	<i>Bacillus subtilis</i>	CRISPRi MurC	<i>B.s.</i> CRISPRi	no treatment
<i>B.s.</i> CRISPRi #3.mzML	Sample	<i>Bacillus subtilis</i>	CRISPRi MurC	<i>B.s.</i> CRISPRi	no treatment
<i>B.s.</i> CRISPRi +xylose #1.mzML	Sample	<i>Bacillus subtilis</i>	CRISPRi MurC	<i>B.s.</i> CRISPRi +xylose	xylose
<i>B.s.</i> CRISPRi +xylose #2.mzML	Sample	<i>Bacillus subtilis</i>	CRISPRi MurC	<i>B.s.</i> CRISPRi +xylose	xylose
<i>B.s.</i> CRISPRi +xylose #3.mzML	Sample	<i>Bacillus subtilis</i>	CRISPRi MurC	<i>B.s.</i> CRISPRi +xylose	xylose
<i>E.c.</i> WT #1.mzML	Sample	<i>Escherichia coli</i>	Wild Type	<i>E.c.</i> WT	no treatment
<i>E.c.</i> WT #2.mzML	Sample	<i>Escherichia coli</i>	Wild Type	<i>E.c.</i> WT	no treatment
<i>E.c.</i> WT #3.mzML	Sample	<i>Escherichia coli</i>	Wild Type	<i>E.c.</i> WT	no treatment
Blank #1.mzML	Blank			Blank	
Blank #2.mzML	Blank			Blank	
Blank #3.mzML	Blank			Blank	
<i>B.s.</i> WT #1.mzML	Sample	<i>Bacillus subtilis</i>	Wild Type	<i>B.s.</i> WT	no treatment
<i>B.s.</i> WT #2.mzML	Sample	<i>Bacillus subtilis</i>	Wild Type	<i>B.s.</i> WT	no treatment
<i>B.s.</i> WT #3.mzML	Sample	<i>Bacillus subtilis</i>	Wild Type	<i>B.s.</i> WT	no treatment
<i>B.s.</i> WT +fosfomycin #1.mzML	Sample	<i>Bacillus subtilis</i>	Wild Type	<i>B.s.</i> WT +fosfomycin	fosfomycin
<i>B.s.</i> WT +fosfomycin #2.mzML	Sample	<i>Bacillus subtilis</i>	Wild Type	<i>B.s.</i> WT +fosfomycin	fosfomycin
<i>B.s.</i> WT +fosfomycin #3.mzML	Sample	<i>Bacillus subtilis</i>	Wild Type	<i>B.s.</i> WT +fosfomycin	fosfomycin

Ready to use for the metabolomics workflow.

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