Mechanisms of (p)ppGpp Action and Functional Consequences in *Staphylococcus aureus*

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Andrea Salzer

aus Heilbronn

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1. Berichterstatter/-in:	Apl. Prof. Dr. Christiane Wolz
2. Berichterstatter/-in:	Prof. Dr. Karl Forchhammer

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Tübingen, den

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Meiner Familie

Table of Contents

Abbreviations

%	percentage
(p)ppGpp	guanosine pentaphosphate and tetraphosphate
ATP	adenosine triphosphate
B. subtilis	*Bacillus subtilis*
BM2	basal medium 2
C. difficile	*Clostridioides difficile*
c-di-AMP	cyclic di-adenosine monophosphate
CDM	chemically defined medium
CFU	colony forming unit
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
e.g.	*exempli gratia*, for example
eDNA	extracellular DNA
ETC	electron transport chain
GDP	guanine diphosphate
GMP	guanine monophosphate
GTP	guanine triphosphate
IMP	inosine monophosphate
MIC	minimal inhibitory concentration
MRSA	methicillin-resistant *S. aureus*
MSSA	methicillin-sensitive *S. aureus*
OD_{600}	optical density at 600 nm
OPP	O-propargyl puromycin
PIA	polysaccharide intercellular adhesin
PMF	proton motive force
PNAG	poly-N-acetylglucosamine
PRPP	phosphoribosyl pyrophosphate
PSM	phenol-soluble modulin
RIF	region of increased fluidity
ROS	reactive oxygen species
RSG™	Redox Sensor Green™
RSH	RelA/SpoT homolog
S. aureus	*Staphylococcus aureus*
SAS	small alarmone synthetase
ssRNA	single strand RNA
TCA	tricarboxylic acid

tRNA	transfer RNA
TSB	tryptic soy broth
TSS	transcriptional start site
VBNC	viable but non-culturable
VRE	vancomycin-resistant *Enterococcus faecium*
WT	wildtype

1 Summary

The opportunistic human pathogen *Staphylococcus aureus* can cause a wide range of infections and is a growing threat to the healthcare systems due to the development of antibiotic resistance. To survive and multiply under unfavourable conditions, *S. aureus* has evolved strategies to sense and respond to various environmental stresses, such as nutrient limitation or antibiotic-induced cell wall stress. The stringent response is a bacterial stress response characterised by the synthesis of the alarmones (p)ppGpp, which enables bacterial survival under nutrient starvation, antibiotic exposure or other external stresses by transcriptional reprogramming and growth arrest. The functional consequences of the stringent response in *S. aureus* have been studied in previous work under various conditions, including transcriptionally or chemically induced amino acid limitation, and during infection in human macrophages and animal models. In this thesis, the role of the stringent response in biofilm formation and under nutrient limitation during the stationary growth phase was investigated. Additionally, the influence of the stringent response and (p)ppGpp on antibiotic tolerance was examined under these conditions. In the first part of this thesis, it was demonstrated that biofilm formation during relaxed conditions is independent of the stringent response. However, under nutrient-limited conditions or antibiotic exposure, (p)ppGpp synthesis by the small alarmone synthetases RelP and RelQ was found to be necessary for biofilm formation and maintenance. In addition, it was shown that an anti-biofilm peptide, originally proposed to inhibit (p)ppGpp function, acts independent of (p)ppGpp but inhibits biofilm formation and synergistically eradicates biofilms in combination with the antibiotic vancomycin. In the second part of this thesis, it could be demonstrated that (p)ppGpp synthesis is required to avoid excessive GTP accumulation during stationary growth phase. Maintaining GTP homeostasis ensures the culturability of *S. aureus* under nutrient starvation by preserving the proton motive force (PMF) and cell membrane architecture. (p)ppGpp-deficient *S. aureus* enters a division-incompetent, dormant state characterized by low levels of ATP, reduced metabolic activity, and alterations in membrane potential and architecture. It was demonstrated that control of GTP levels in nucleotide-sensitive promoters leads to the downregulation of genes involved in the TCA cycle and electron transport chain. To partially restore the culturability of the (p)ppGpp-deficient mutant, the transcription of *qoxABCD*, a terminal oxidase of the respiratory chain, was increased through mutation of the transcriptional start site. In addition, it was shown that the loss of the PMF renders the stationary phase bacteria vulnerable to various classes of antibiotics. These findings suggest that targeting the stringent response could be an effective strategy to combat bacterial infections. In particular, by targeting the functional consequences mediated by stringent response in *S. aureus*, e.g. by the application of biofilm, PMF or (p)ppGpp inhibitors, clinical applications could also be found.

2 Zusammenfassung

Der opportunistische Krankheitserreger *Staphylococcus aureus* kann ein breites Spektrum von Infektionen verursachen und stellt aufgrund der Entwicklung von Antibiotikaresistenzen eine wachsende Bedrohung für die Gesundheitssysteme dar. Um unter ungünstigen Bedingungen zu überleben und sich zu vermehren, hat *S. aureus* Strategien entwickelt, um verschiedene negative Umwelteinflüsse, wie z. B. Nährstoffmangel oder durch Antibiotika ausgelösten Zellwandstress, zu erkennen und darauf zu reagieren. Die stringente Kontrolle ist eine bakterielle Stressantwort, die durch die Synthese der Alarmone (p)ppGpp gekennzeichnet ist und das bakterielle Überleben bei Nährstoffmangel, Antibiotikaexposition oder anderen Umweltstressen durch transkriptionelle Umprogrammierung und Wachstumsstillstand ermöglicht. Die funktionellen Konsequenzen der stringenten Kontrolle in *S. aureus* wurden in vorangegangen Arbeiten unter verschiedenen Bedingungen untersucht, einschließlich transkriptionell oder chemisch induzierter Aminosäure-Limitierung und während der Infektion in humanen Makrophagen und Tiermodellen. In dieser Arbeit wurde die Rolle der stringenten Kontrolle bei der Biofilmbildung und unter Nährstofflimitierung während der stationären Wachstumsphase untersucht. Außerdem wurde der Einfluss der stringenten Kontrolle und von (p)ppGpp auf die Antibiotikatoleranz unter diesen Bedingungen untersucht. Im ersten Teil dieser Arbeit wurde gezeigt, dass die Biofilmbildung unter nährstoffreichen Bedingungen unabhängig von der stringenten Kontrolle ist. Unter nährstofflimitierten Bedingungen oder bei Antibiotikaexposition wurde jedoch festgestellt, dass die (p)ppGpp-Synthese durch die kleinen Alarmon-Synthetasen RelP und RelQ für die Bildung und Aufrechterhaltung des Biofilms notwendig ist. Darüber hinaus wurde gezeigt, dass ein Anti-Biofilm-Peptid, bei dem ursprünglich eine Hemmung der (p)ppGpp-Funktion gezeigt wurde, unabhängig von (p)ppGpp wirkt, aber dennoch die Biofilmbildung hemmt und in Kombination mit dem Antibiotikum Vancomycin Biofilme synergistisch eradiziert. Im zweiten Teil dieser Arbeit konnte gezeigt werden, dass die (p)ppGpp-Synthese erforderlich ist, um eine übermäßige GTP-Akkumulation während der stationären Wachstumsphase zu verhindern. Die Aufrechterhaltung der GTP-Homöostase gewährleistet die Kultivierbarkeit von *S. aureus* unter Nährstoffmangel, indem die Protonenmotorische Kraft (PMF) und die Zellmembranstruktur erhalten bleiben. (p)ppGpp-defizienter *S. aureus* tritt in einen teilungsunfähigen, dormanten Zustand ein, der durch niedrige ATP-Konzentrationen, reduzierte Stoffwechselaktivität und Veränderungen des Membranpotenzials und der Zellmembranstruktur gekennzeichnet ist. Es konnte gezeigt werden, dass die Kontrolle des GTP-Spiegels in nukleotid-sensitiven Promotoren zur Herunterregulierung von Genen führt, die am TCA-Zyklus und an der Elektronentransportkette beteiligt sind. Um die Kultivierbarkeit der (p)ppGpp-defizienten Mutante teilweise wiederherzustellen, wurde die Transkription von *qoxABCD*, einer terminalen Oxidase der

Atmungskette, durch Mutation der Transkriptionsstartstelle erhöht. Darüber hinaus wurde gezeigt, dass der Verlust der PMF die Bakterien in der stationären Phase die Toleranz gegenüber verschiedener Antibiotikaklassen verringert. Diese Ergebnisse legen nahe, dass die gezielte Beeinflussung der stringenten Kontrolle eine wirksame Strategie zur Bekämpfung bakterieller Infektionen sein könnte. Insbesondere durch die gezielte Beeinflussung der funktionellen Konsequenzen, die durch die stringente Kontrolle bei *S. aureus* vermittelt werden, z. B. durch die Applikation von Biofilm-, PMF- oder (p)ppGpp-Inhibitoren, könnten auch klinische Anwendungen gefunden werden.

3 List of publications and personal contributions

3.1 Accepted manuscripts

1. **Small alarmone synthetases RelP and RelQ of *Staphylococcus aureus* are involved in biofilm formation and maintenance under cell wall stress conditions**

   Andrea Salzer, Daniela Keinhörster, Christina Kästle, Benjamin Kästle and Christiane Wolz

   Front Microbiol. 2020 Sep 18;11:575882. doi: 10.3389/fmicb.2020.575882.

   For this research article, I performed most biological experiments except analysis of biofilm composition (Fig. 3) and was mainly involved in data analysis and interpretation of all biological experiments. I designed all figures for the manuscript and contributed in large parts to writing the manuscript under the supervision of Christiane Wolz.

2. **Inducible expression of (pp)pGpp synthetases in *Staphylococcus aureus* is associated with activation of stress response genes**

   Petra Horvatek, Andrea Salzer, Andrew Magdy Fekry Hanna, Fabio Lino Gratani, Daniela Keinhörster, Natalya Korn, Marina Borisiova, Christoph Mayer, Dominik Rejman, Ulrike Mäder and Christiane Wolz

   PLoS Genet. 2020 Dec 30;16(12):e1009282. doi: 10.1371/journal.pgen.1009282.

   For this research article, I contributed by isolating RNA and performing quantitative real-time PCR (qRT-PCR) and Northern Blot analysis to determine gene expression levels of *psmα*, *rpsL*, *rsaD*, *dps*, *ftnA* and *agrA*, analysed and interpreted the data (Fig. 3, 4B and 6A). I was mainly involved in figure design and editing of the manuscript during revision.

3. **Role of (p)ppGpp in antibiotic resistance, tolerance, persistence and survival in Firmicutes**

   Andrea Salzer and Christiane Wolz

   Microlife. 2023 Mar 11;4:uqad009. doi: 10.1093/femsml/uqad009.

   For this review, I was involved in conception of the article and contributed by literature research and writing main parts of the manuscript (Abstract, Introduction, (p)ppGpp and antibiotic resistance, (p)ppGpp and antibiotic tolerance, (p)ppGpp and survival in persistent infections, Conclusion) under the supervision of Christiane Wolz. I designed Figure 1 and summarized literature in Table 1. In cooperation with Christiane Wolz, I was involved in manuscript editing to its final version.

3.2 Submitted manuscripts

1. **(p)ppGpp-mediated GTP homeostasis ensures survival and antibiotic tolerance of *Staphylococcus aureus* during starvation by maintaining the proton motive force**

   Andrea Salzer, Sophia Ingrassia, Lisa Sauer, Johanna Rapp, Ronja Dobritz, Jennifer Müller, Hannes Link and Christiane Wolz

   Preprint on biorxiv.org, https://doi.org/10.1101/2024.02.06.579068, (submitted to Communications Biology)

   For this research article, I made major contributions to the conception and design of the study. All experiments were performed or supervised and subsequently analysed by me. Metabolomics and RNAseq data were acquired and analysed in collaboration with co-authors. I designed all figures and wrote the manuscript under the supervision of Christiane Wolz.

2. **Triclosan alters biofilm structures and confers antibiotic tolerance in *Staphylococcus aureus* using multiple regulatory pathways**

   Dean Walsh, Andrea Salzer, Parvati Iyer, Christiane Wolz, Jonathan Aylott and Kim Rachel Hardie

   Preprint on biorxiv.org, doi: https://doi.org/10.1101/2023.02.01.525840 (submitted to npj Antimicrobials and Resistance)

   For this research article, I generated the HG001 *sigB* mutant and performed time-kill assays in planktonic cultures. I was involved in data analysis and interpretation.

4 Introduction

Parts of this introduction have been published in the following articles. The submitted manuscript has been uploaded to the preprint server bioRxiv and might differ from the final published article. All articles can be accessed under the following links:

- “Role of (p)ppGpp in antibiotic resistance, tolerance, persistence and survival in Firmicutes”, (review article), https://doi.org/10.1093/femsml/uqad009
- “Small alarmone synthetases RelP and RelQ of *Staphylococcus aureus* are involved in biofilm formation and maintenance under cell wall stress conditions”, (research article), https://doi.org/10.1101/2024.02.06.579068
- “(p)ppGpp-mediated GTP homeostasis ensures the survival and antibiotic tolerance of *Staphylococcus aureus* during starvation by preserving the proton motive force”, (submitted manuscript), https://doi.org/10.3389/fmicb.2020.575882

4.1 *Staphylococcus aureus* and its clinical relevance

Staphylococcus aureus is a gram-positive, coccus-shaped bacterium that belongs to the phylum Firmicutes. It is a commensal bacterium that asymptomatically colonizes the anterior nares of the nose and skin of approximately 30% of healthy individuals worldwide [1]. However, *S. aureus* is also an opportunistic pathogen that causes clinically significant infections, including skin and soft-tissue infections, life-threatening bacteraemia, endocarditis, necrotising sepsis, or device-related infections [2]. Nasal carriage of *S. aureus* has been identified as a major risk factor for acquiring invasive infections [1].

Although methicillin-resistant *S. aureus* (MRSA) is still low in prevalence in Northern Europe, the increasing resistance of *S. aureus* to various antibiotics worldwide complicates the treatment of these infections [3]. Therefore, it is essential to restrict MRSA spreading and prevent staphylococcal infections. To develop new antibiotics and alternative therapies in addition to antibiotic treatment, it is necessary to gain a better understanding of the regulation of virulence factors that contribute to the high pathogenicity of *S. aureus*, as well as the regulation of metabolism during infection.

4.2 The stringent response

The stringent response or stringent control is a bacterial response to different forms of stress including nutrient limitation and cell wall stress, and is characterised by the synthesis of the messenger molecules pppGpp and ppGpp [4-13]. For the first time (p)ppGpp was discovered in 1969 by Michael Cashel, where so-called magic spots could be observed by thin-layer

chromatography from amino acid starved *Escherichia coli*. These magic spots were later identified as pyrophosphorlyated GTP (pppGpp) or GDP (ppGpp) [14], collectively name (p)ppGpp hereafter. One main feature of the stringent control is to keep macromolecular synthesis tightly coupled to nutrient availability and growth rate. Accordingly, (p)ppGpp can specifically block replication, translation and transcription [9, 10, 12].

The stringent response is typically defined as the rapid and dramatic accumulation of (p)ppGpp in response to stress. However, even subtle fluctuations and steadily increasing basal (p)ppGpp levels, such as during entry into stationary growth phase, can modulate cellular processes and growth rate at (p)ppGpp concentrations that are far below the threshold required to activate the stringent response.

4.2.1 Synthesis and degradation of (p)ppGpp in Firmicutes

(p)pGpp is synthesized by RelA-SpoT-homologs (RSH) which catalyse the transfer of pyrophosphate originating from ATP to the 3´ OH group of GTP, GDP or GMP. Long RSH enzymes, characterised by an N-terminal enzymatic domain bearing distinct motifs for (p)ppGpp synthetase or hydrolase activity and a C-terminal regulatory domain, are present in nearly all bacteria and display both (p)ppGpp hydrolytic and biosynthetic activity [11, 15]. Firmicutes possess one long RSH enzyme, named Rel. Amino acid limitation through accumulation of uncharged tRNAs is so far the only common condition that has been shown to induce the (p)ppGpp synthetase activity of Rel [16, 17]. Rel can be allosterically activated by (p)ppGpp [18] and its activity can be modulated by the second messenger c-di-AMP [19-22]. In some Firmicutes, Rel directly interacts with the c-di-AMP-binding protein DarB thereby linking potassium deprivation with stringent response [21, 23]. In *Bacillus subtilis* lipid starvation [24], heat shock [25] or certain antibiotics [26] can also induce Rel dependent (p)ppGpp synthesis by so far ill-defined mechanisms. However, for *S. aureus* amino acid deprivation is the only known inducer for Rel dependent (p)ppGpp synthesis [16].

In addition to the long RSH Rel, Firmicutes possess one or two small alarmone synthetases (SAS), SasA/RelP and SasB/RelQ. The (p)ppGpp synthesis by SAS enzymes is induced through transcriptional activation of the encoding genes and/or posttranslational modifications. For example, RelP and RelQ from *S. aureus* are part of the VraRS cell-wall stress regulon and are induced after vancomycin treatment [27]. In *B. subtilis* phosphorylation of the response regulator WalR controls the heterogeneous expression of RelP [28], while in *Clostridioides difficile relQ* transcription is induced by sub-lethal concentrations of different antibiotics (clindamycin, metronidazole, vancomycin and fidaxomicin) [29, 30]. Post-translational activation or inhibition of SAS proteins is also important for function. RelQ from *B. subtilis* and *Enterococcus faecalis* form tetramers [31, 32]. RelQ activity is strongly inhibited through the

binding of single-stranded RNA (ssRNA). (p)ppGpp binding leads to disassociation of the RelQ:RNA complex and activation of RelQ [32]. RelP activity is inhibited by both pppGpp and ppGpp, activated by Zn^{2+} and insensitive to inhibition by RNA [33, 34]. Instead, in *S. aureus* RelQ seems to be insensitive to ssRNA, and RelP is not allosterically stimulated by the addition of pppGpp, ppGpp or pGpp [35]. In this bacterium, RelQ is mainly held in an inactive state when overexpressed [36]. Thus, the expression and activity of RelP and RelQ seem to differ considerably between species, although these two proteins are highly homologous. Post-translational regulatory mechanisms are likely important to fine-tune (p)ppGpp synthesis under different growth conditions.

Rel is so far the only described enzyme to degrade (p)ppGpp in Firmicutes and thus important to reset the system. Under non-inducing conditions, Rel is primarily in a hydrolase-On/synthetase-Off conformation [37], while starved ribosomes are sufficient to inhibit the hydrolytic activity [38]. The strong hydrolase activity of Rel from *S. aureus* makes the enzyme essential for the detoxification of (p)ppGpp produced by RelP or RelQ [16]. In *B. subtilis and E. faecalis rel* mutants are viable but severely impaired in growth due to (p)ppGpp overproduction [39-41]. The hydrolase NahA of *B. subtilis* efficiently hydrolyses (p)ppGpp to produce pGpp, thereby modulating alarmone composition and function [42]. These results indicate the existence and physiological relevance of pGpp as a third alarmone, with functions that can be distinct from those of (p)ppGpp.

The induction of the stringent response and the synthesis of (p)ppGpp in Firmicutes is summarized in Fig. 1.

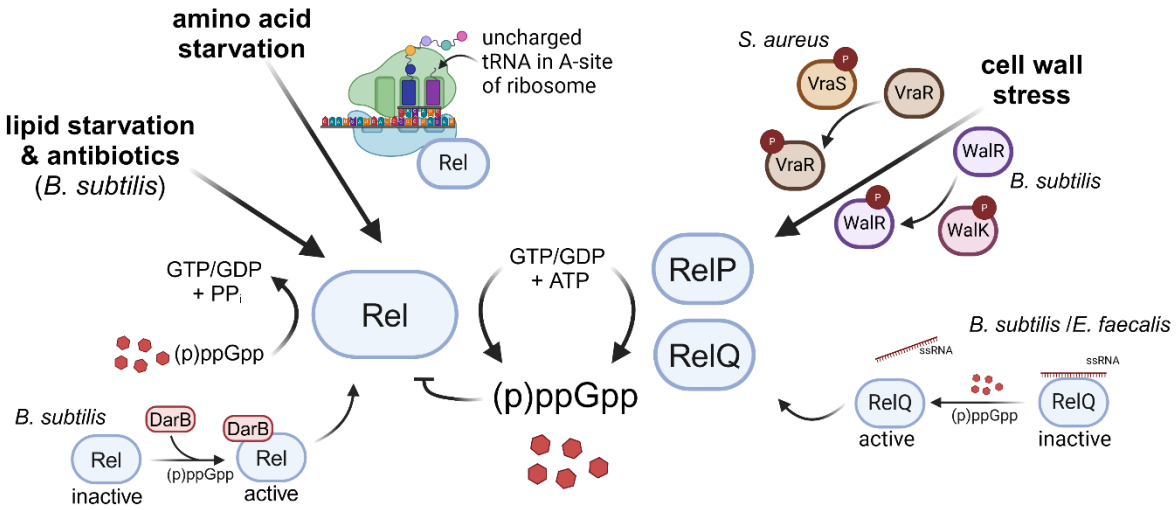

Fig. 1 Activation of the stringent response and synthesis of (p)ppGpp in *S. aureus* and other Firmicutes. Rel-dependent (p)ppGpp synthesis is induced during amino acid starvation (by sensing of stalled ribosomes by Rel), or in *B. subtilis* also during lipid starvation, heat shock, and antibiotic stress. RelP and RelQ are activated during cell wall stress by the two-component systems VraRS in *S. aureus* or WalKR in *B. subtilis*. RelQ is also

activated via the (p)ppGpp-induced release of an inactivating ssRNA. The substrates for synthesis are GTP or GDP and ATP, and the reaction is catalysed by the synthetase domain of Rel and SAS enzymes (RelP/RelQ). (p)ppGpp degradation occurs solely by the hydrolase domain of Rel. (Figure created with Biorender.com and adapted from [43]).

4.3 Targets and downstream effects of (p)ppGpp

In gram-positive pathogens, the stringent response plays an important role in virulence [44] as well as antibiotic tolerance. The global transcriptional effects of (p)ppGpp have been examined previously in several Firmicutes, such as *B. subtilis* [45], *Streptococcus pneumoniae* [46], *E. faecalis* [47], *Streptococcus mutans* [48] and *S. aureus* [36, 49]. Many of the observed downstream effects are mediated via specific binding of (p)ppGpp to target proteins resulting in inhibition of replication (DnaG), ribosome function (RsgA, RbgA, Der, Era, HflX, and ObgE), secretion (Ffh and FtsY) and GTP synthesis (GuaB, HprT, Gmk, GdpP, Pde, PgpH and PurR) [9, 10, 50]. Moreover, (p)ppGpp can control transcription via (p)ppGpp-specific riboswitches [51]. The main effects of (p)ppGpp on bacterial physiology in Firmicutes are summarized in Fig. 2.

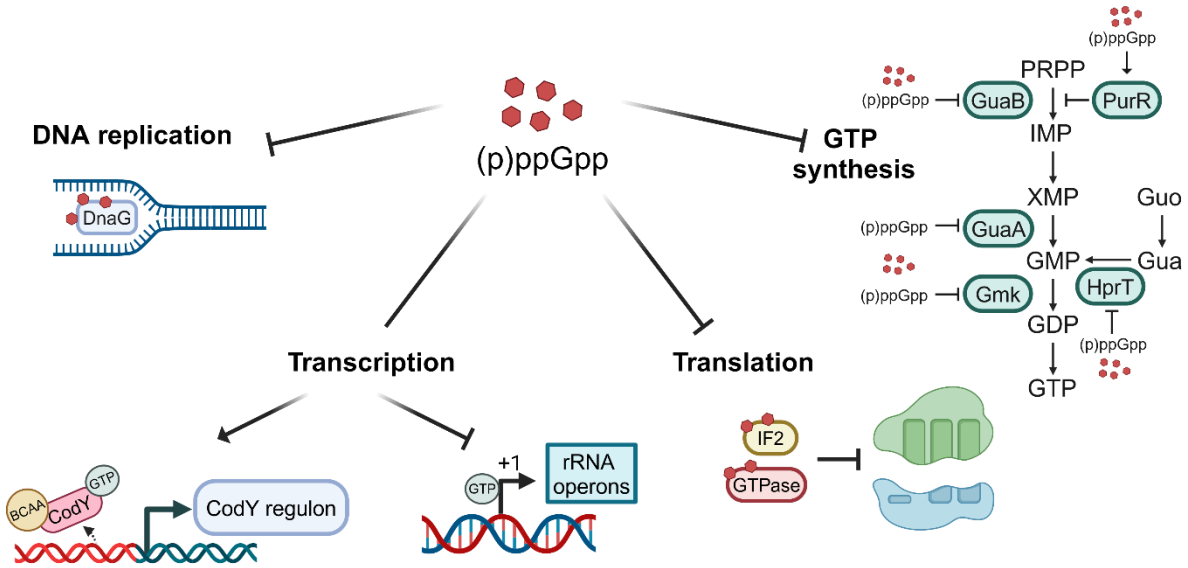

Fig. 2 Main effects of (p)ppGpp on bacterial physiology in *S. aureus* and other Firmicutes. Accumulation of (p)ppGpp modulates then bacterial physiology by directly binding to a variety of enzymes or via lowering of the GTP pools. (p)ppGpp regulates transcription indirectly by either de-repression of the transcriptional regulator CodY or by modulating transcription from nucleotide-sensitive promoters. Increasing (p)ppGpp levels also inhibit several steps in the GTP synthesis by direct binding and inhibition of enzymes involved in GTP synthesis. Furthermore, (p)ppGpp inhibits DNA replication (elongation) and translation (ribosome assembly) by binding to proteins involved in those processes. (Figure created with Biorender.com and adapted from [43]).

4.3.1 Transcriptional regulation by (p)ppGpp

In contrast to proteobacteria e.g. *E. coli* where (p)ppGpp directly binds to RNA polymerase and thereby regulates transcription, in Firmicutes (p)ppGpp does not interfere with RNA polymerase activity [52]. In *S. aureus* and other Firmicutes (p)ppGpp regulates transcription by controlling intracellular GTP levels [49, 53-55]. Upon (p)ppGpp synthesis, GTP levels drop due to its use as a substrate for (p)ppGpp synthesis and direct inhibition of enzymes involved in GTP synthesis. This reduction in cellular GTP levels allows the de-repression of genes under the control of the transcriptional repressor CodY. CodY is a global regulator, known to repress gene involved in amino acid synthesis and uptake but also virulence factors, when loaded with branch-chained amino acids and GTP [56-58].

Furthermore, a decrease in the GTP levels leads to the repression of nucleotide-sensitive, GTP-initiating promoters, e.g., those of rRNA genes [55, 59]. Some promoters are controlled by the concentration of the transcription-initiating nucleotide due to the requirement of high iNTP concentrations to stabilize the open complex formation in transcription initiation [60]. In *S. aureus*, the concentration of intracellular GTP levels could be correlated to the activity of rRNA promoters and linked to the GTP nucleotides in the initiation region of the promoters at the position between +1 and +4 [55]. This indicates that not only the transcriptional initiation but also the elongation requires high concentrations of the respective free nucleotides.

Beyond the indirect transcriptional regulation via the control of GTP levels, (p)ppGpp can also directly control transcription as an allosteric effector of a transcription regulator. Recently, it was shown that (p)ppGpp targets the transcriptional regulator PurR to downregulate purine biosynthesis gene expression upon amino acid starvation [61]. Hereby, (p)ppGpp directly binds to the effector pocket of transcriptional factor PurR to compete with phosphoribosyl pyrophosphate (PRPP) to alter PurR binding to the promoter DNA and therefore ensures downregulation of purine biosynthesis during nutrient downshift. PurR in turn regulates the transcription of several virulence genes and metabolism and thus is a critical regulator of staphylococcal pathogenesis [62].

4.3.2 (p)ppGpp-mediated regulation of GTP synthesis and homeostasis

In addition, (p)ppGpp has been implicated in regulation of GTP homeostasis by inhibiting enzymes involved in GTP synthesis including the guanylate kinase Gmk, the IMP dehydrogenase GuaB and the hypoxanthine-guanine phosphoribosyltransferase HrpT (Fig. 2) [39, 54, 63] or by binding to the transcriptional regulator PurR [64].

Elevated cellular GTP levels in (p)ppGpp-deficient *S. aureus* and *B. subtilis* during nutritional stress have been associated with cell death [24, 54, 65-67] making (p)ppGpp-mediated control of GTP homeostasis crucial for viability.

4.4 The role of (p)ppGpp in biofilm formation

Biofilms are sessile microbial communities attached to surfaces and embedded in an extracellular matrix. Biofilm-forming staphylococci cause many device-related or chronic infections [68-76]. Depending on the composition of the biofilm matrix, staphylococcal biofilms are classified as *ica*-dependent or *ica*-independent [73]. *Ica*-dependent biofilms are characterized by polysaccharide intercellular adhesin (PIA), also known as poly-N-acetyl glucosamine (PNAG), which is synthesized by enzymes encoded by the *icaADBC* operon [77]. In *ica*-independent biofilms, proteins [72] and extracellular nucleic acids (eDNA) [70] are the main matrix components. Biofilm formation is a three-step process that includes initial attachment to the surface, biofilm maturation due to intercellular aggregation and bacterial cell detachment. Initial attachment can happen to abiotic or biotic surfaces and is mostly driven by hydrophobic or electrostatic interactions and/or by the interaction of specific surface molecules [71]. When maturation starts and the biofilms grow by proliferation, a scaffolding extracellular matrix is produced. In a second maturation stage, disruptive processes form channels in the biofilm structure. These channels are important to guarantee nutrient supply in the deeper biofilm layers [71]. Furthermore, the disruptive forces are important since they also cause the detachment of cell clusters from a biofilm and therefore control biofilm expansion. Detachment and biofilm disruption is mediated by the enzymatic degradation of matrix components by proteases, nucleases or a group of small amphiphilic α-helical peptides, known as phenol-soluble modulins (PSMs) [71].

For several bacterial species, it has been demonstrated that the activation of the stringent response affects biofilm formation [78-91]. In *S. aureus*, the stringent response has been implicated in biofilm formation since the treatment with stringent response inducing agents, e. g mupirocin [92, 93] or serine hydroxamate [78], resulted in increased biofilm formation. Moreover, the anti-biofilm peptides IDR-1018 and DJK-5 have been suggested to directly interact with (p)ppGpp, preventing its signalling effects and, thus, biofilm formation [78, 94].

4.5 (p)ppGpp mediates growth control and survival *in vitro*

(p)ppGpp is a major regulator of growth rate control in *E. coli* [95, 96]. However, the effects of (p)ppGpp on global protein synthesis and survival to starvation seem to be highly dependent on the conditions (nitrogen- versus carbon-limitation) [97]. In *S. aureus* $pppGpp^0$ mutants are still able to inhibit rRNA synthesis in the later growth phase [55]. This indicates that, at least in *S. aureus*, (p)ppGpp independent mechanisms also account for growth rate control when nutrients become limited. (p)ppGpp seems to have different roles depending on its abundance in the cell [6]. The role of (p)ppGpp in bacterial survival under non-stressed conditions, when the levels of (p)ppGpp below those required for induction of the stringent response, is less

clear. It was proposed that basal levels of (p)ppGpp may have different effects than high level bursts during stressful situations [6]. Several binding partners of (p)ppGpp have been uncovered displaying dissociation constants from below one micromolar to more than one millimolar concentrations. Nucleotide metabolism enzymes are among the first targets to be affected by rising (p)ppGpp while more fundamental processes such as DNA replication are among the last [9].

(p)ppGpp^0 strains in Firmicutes usually show no growth defect under non-stressed conditions e.g., in rich medium [16, 54, 98]. However, (p)ppGpp^0 mutants have altered transcriptomes, even under non-stressed conditions [39] and basal levels of (p)ppGpp seem to be important to maintain GTP homeostasis in Firmicutes [99]. After shift to amino acid limiting conditions a sudden raise of (p)ppGpp is necessary to protect the bacteria from death [16, 54, 65, 100]. In *B. subtilis* it was shown that controlling GTP accumulation is required for survival during amino acid starvation [65]. Manipulating the GTP levels in pppGpp^0 mutants provided evidence that GTP regulates growth rate in a dose-dependent manner in *B. subtilis*. In the wildtype, (p)ppGpp restricts the upper limit of GTP to ensure optimal growth and survival. Additionally, the inhibition of fatty acid synthesis by cerulenin in *B. subtilis* was also found to be lethal only in the absence of (p)ppGpp synthesis [13]. Adaptation to fatty acid starvation in wildtype bacteria requires the activity of the Rel synthetase, although (p)ppGpp levels remain below detection limit. Thus, (p)ppGpp mediated balancing of the GTP/ATP ratio is essential for bacterial survival at least under fatty acid or amino acid starvation. Under such conditions the GTP/ATP ratio in pppGpp^0 strains remains high and probably keeps the cell growing, which will eventually perturb membrane function and lead to cell death [24].

In chemically defined medium the survival of the *S. aureus* (p)ppGpp^0 mutant is diminished in the stationary phase which has also been linked to uncontrolled GTP accumulation [67]. In stationary phase *S. aureus* acquires tolerance towards oxidative stress by maintenance of redox and iron homeostasis which is mediated by (p)ppGpp [36, 101]. Consistently, in the (p)ppGpp^0 mutant the antioxidant stress response genes were induced due to elevated reactive oxygen species (ROS) levels in stationary phase [101]. (p)ppGpp synthesis by the inducible synthetases resulted in the activation of ROS-inducing toxins and the simultaneous expression of detoxifying systems to protect the producer. Thereby, (p)ppGpp prepares the cells for survival to elevated ROS [36]. Thus, in S. *aureus* (p)ppGpp not only confers tolerance to antibiotics but also to ROS [36, 101], although oxidative stress *per se* does not seem to induce (p)ppGpp synthesis.

4.5.1 Dormancy and the VBNC state

The stringent response has been implicated not only in response to environmental stresses, such as amino acid and fatty acid starvation or antibiotics, but basal (p)ppGpp levels have been postulated to modulate and fine-tune general metabolism during normal growth in the absence of external stress factors [39, 52, 102]. However, these adaptations may result in slower growth rates and metabolic rearrangements [39, 47, 65] that can be advantageous for survival since reduced growth rates can promote entry into a dormant or quiescent state [103, 104].

The viable but non-culturable (VBNC) state is a unique survival strategy adopted by bacteria in response to adverse environmental conditions. It describes a non-growing, deep dormant state with reduced metabolic activity [105]. VBNC bacteria maintain membrane function and integrity, carry out respiration and continue transcription and translation, but have lost culturability in nutrient media which normally support their growth [106, 107]. In contrast, dead cells have damaged membranes and are metabolically inactive. Another bacterial non-growing state is dormancy. In contrast to VBNC bacterial cells, dormant bacteria do not display any measurable metabolic activity, but are able to resuscitate under more favourable environmental conditions [105, 106]. Although VBNC cells share many general characteristics with viable and cultivatable cells, they exhibits physiological differences including altered metabolism and gene expression as well as alterations in cell morphology, cell wall and membrane including e.g. fatty acid composition or peptidoglycan cross-linking [106, 108]. VBNC cells of *E. faecalis* exhibit an increase in peptidoglycan-crosslinking [108], ultimately altering cell membrane properties.

4.6 The role of (p)ppGpp in antibiotic tolerance

Antibiotic tolerance is an important contributor to antibiotic treatment failure, but it also acts as a precursor of antibiotic resistance [109, 110]. As most antibiotics target active metabolic processes, a decreased growth rate and reduced metabolic activity contribute to the development of multidrug tolerance. (p)ppGpp leads to an immediate shut-down of replication and translation [8, 50], therefore high (p)ppGpp levels likely contribute to antibiotic tolerance.

In *S. aureus*, *relP* and *relQ* expression are induced by cell wall stress with the cell wall active antibiotics ampicillin and vancomycin [27]. The elevated (p)ppGpp levels in turn protect from antibiotic killing, since a *relPQ* double mutant showed reduced survival rate upon treatment with these antibiotics, but no difference in MIC between wild-type and *relPQ* mutants could be observed [27]. Likewise, mutation of *ileS* encoding the isoleucyl-tRNA synthetase leads to Rel dependent (p)ppGpp synthesis and again increased vancomycin tolerance [111]. Similar

observations were reported for *E. faecalis.* In growth curve experiments with sub-inhibitory concentrations of vancomycin enhanced tolerance was observed in a *relA* mutant strain that exhibited higher (p)ppGpp levels due to missing hydrolase activity [98]. By contrast, a *relQ* mutant strain grew slower than its parental strain and was more rapidly killed in time-kill experiments using 5x MIC of vancomyin. In *B. subtilis* chloramphenicol treatment resulted in Rel dependent (p)ppGpp synthesis and thereby increased tolerance to vancomycin [26].

The impact of (p)ppGpp on tolerance towards other antibiotics is less clear. In *E. faecalis* a (p)ppGpp^0 (*rel, relQ*) strain is more sensitive to killing by norfloxacin and ampicillin [39]. In methicillin-sensitive *S. aureus* (MSSA) mutations of *rel* that likely lead to higher (p)ppGpp accumulation and an overall stringent-response like transcription pattern have been linked to ciprofloxacin tolerance [112]. However, Geiger et al. did not observe ciprofloxacin tolerance in *S. aureus* mutants with defective (p)ppGpp synthetases [27]. During prolonged daptomycin exposure, *S. aureus* acquired several mutations, one of which within *rel* (L61F). A *de novo* generated strain carrying the sole mutation L61F in Rel was less susceptible to daptomycin in time-kill assays [113]. Of note, it is not clear whether the mutation made the bacteria produce more or less (p)ppGpp. Chemical inhibition of r*el* by the (p)ppGpp analog relacin or transcriptional repression by antisense *rel* RNA reduced tolerance to clindamycin and metronidazole [29]. Very recently, Yang et al. showed that *B. subtilis* and *E. faecalis* produce (p)ppGpp in response to chloramphenicol treatment through *rel*, which protected the bacteria from antibiotic killing [26]. The protective effect could be linked to the decreased intracellular GTP pool. Elevated GTP levels in a (p)ppGpp^0 strain promoted the bactericidal effect of chloramphenicol.

4.6.1 Biofilm-related antibiotic tolerance

Biofilms are normally more tolerant to high concentrations of antibiotics than planktonic cultures. In gram-negative bacteria e.g. *Pseudomonas aeruginosa*, the stringent response was shown to play a crucial role for the antibiotic tolerance of nutrient-limited and biofilm bacteria [114]. In (p)ppGpp-deficient *P. aeruginosa*, the antibiotic tolerance was severely reduced allowing enhanced efficacy of antibiotic treatment in an animal infection model [114]. The widely used antimicrobial triclosan is a bacteriostatic inhibitor of fatty acid synthesis and was shown to increase tolerance to bactericidal antibiotics in *E. coli* but also MRSA biofilms [115]. A persistent vancomycin-resistant *Enterococcus faecium* (VRE) isolate harbored a *rel* mutation resulting in elevated (p)ppGpp levels [116]. The strain showed tolerance towards linezolid and daptomycin when growing in biofilm but remained susceptible in MIC testing and during planktonic growth.

In summary, several studies indicate that elevated (p)ppGpp either through mutations of the Rel hydrolase or via activation of synthetases can contribute to antibiotic tolerance without necessarily conferring resistance. However, (p)ppGpp is likely not the only or even main trigger for bacterial tolerance.

5 Aim of this thesis

The stringent response is a conserved bacterial stress response that is characterised by the synthesis of the alarmones (p)ppGpp by three synthetases, Rel, RelP and RelQ, present in *Staphylococcus aureus*. Upon nutrient limitation or cell wall stress, the synthesis of (p)ppGpp mediates physiological changes including a reduction in growth and metabolic activity as well as transcriptional reprogramming. Additional, basal (p)ppGpp levels below those levels required for stringent response induction have been implicated in maintenance of cell physiology. The aim of this work was to elucidate the physiological adaptations that ensure survival under adverse environmental conditions, as well as antibiotic tolerance, particularly in biofilms and the stationary growth phase.

The first part of this thesis focuses on the role of the stringent response in biofilm formation in *S. aureus*, as an influence of (p)ppGpp on biofilm formation had been demonstrated in other bacterial species. Biofilms are primarily responsible for infections that are difficult to treat, such as device-related infections, infections on body surfaces (such as skin and soft tissue, lungs, bladder, or endocarditis), and chronic infections. I aimed to clarify the role of the small alarmone synthase RelP and RelQ in biofilm formation and maintenance during antibiotic treatment. Additionally, the effect of an anti-biofilm peptide, which was proposed to inhibit (p)ppGpp action, and its synergistic effect with antibiotics on biofilm formation and eradication were investigated.

The second part of this thesis investigates the molecular mechanisms behind (p)ppGpp-mediated GTP homeostasis and its significance for the viability of *S. aureus* in nutrient-restricted conditions, such as in stationary growth phase. (p)ppGpp-dependent restriction of the intracellular GTP pool was shown to be crucial for the survival and culturability of starved cells and antibiotic tolerance in other gram-positive bacteria. This thesis aimed to investigate whether the maintenance of a balanced GTP pool during nutrient starvation is necessary for the survival of *S. aureus*. At first, viability assays were applied to characterize the metabolic state of the staved *S. aureus* cells. Furthermore, transcriptomic analysis was used to elucidate the molecular mechanism that leads to reduced culturability in a (p)ppGpp-deficient *S. aureus* mutant that displays highly elevated guanine nucleotide levels during starvation. The stringent response is often associated with antibiotic tolerance. In particular, non-growing bacteria, display a higher tolerance towards antibiotic treatment. Therefore, I investigated the requirement of (p)ppGpp during stationary phase starvation for tolerance towards different classes of antibiotics.

6 Results

6.1 The role of (p)ppGpp in biofilm formation and antibiotic tolerance in *Staphylococcus aureus*

The results presented here are part of the accepted research article “Small alarmone synthetases RelP and RelQ of *Staphylococcus aureus* are involved in biofilm formation and maintenance under cell wall stress conditions” which can accessed und the following doi: https://doi.org/10.3389/fmicb.2020.575882.

6.1.1 (p)ppGpp synthetases show no significant effect on biofilm formation under non-stress conditions

It was recently proposed that the stringent response facilitates biofilm formation in several pathogens, including *S. aureus* [78]. However, the basal medium 2 (BM2) used in this study allowed only for very limited *S. aureus* growth, resulting in a final OD_{600} below 1 after overnight growth. Therefore, we first analysed the impact of (p)ppGpp on biofilm formation using different media. These media included tryptic soy broth (TSB) with the addition of 0.5% glucose and 3% NaCl, which is widely used for *S. aureus* biofilm analyses [117], and chemically defined medium (CDM) [56], used to define the stringent response phenotype in *S. aureus*. To distinguish between the Rel- and RelP/RelQ-mediated stringent response, we included a rel_{syn} mutant (mutation in the synthetase domain, leaving hydrolase activity unaltered), a *relP, relQ* double mutant and a (p)ppGpp^0 mutant in which all three synthetases were non-functional in the analysis. Independent of the (p)ppGpp synthetases, all strains showed the strongest biofilm formation in CDM. Interestingly, in the prototypic biofilm medium TSB, biofilm formation was lower than in CDM (Fig. 3A). However, there was no significant difference in biofilm formation between the different strains in either medium. In BM2, a trend towards a slightly higher biofilm-forming capacity in the wildtype compared to the mutant strains was observed. This suggests that under these nutrient-limited conditions, the stringent response might be slightly activated. Thus, under non-stressed conditions, (p)ppGpp synthetases have little to no influence on the ability to form biofilms.

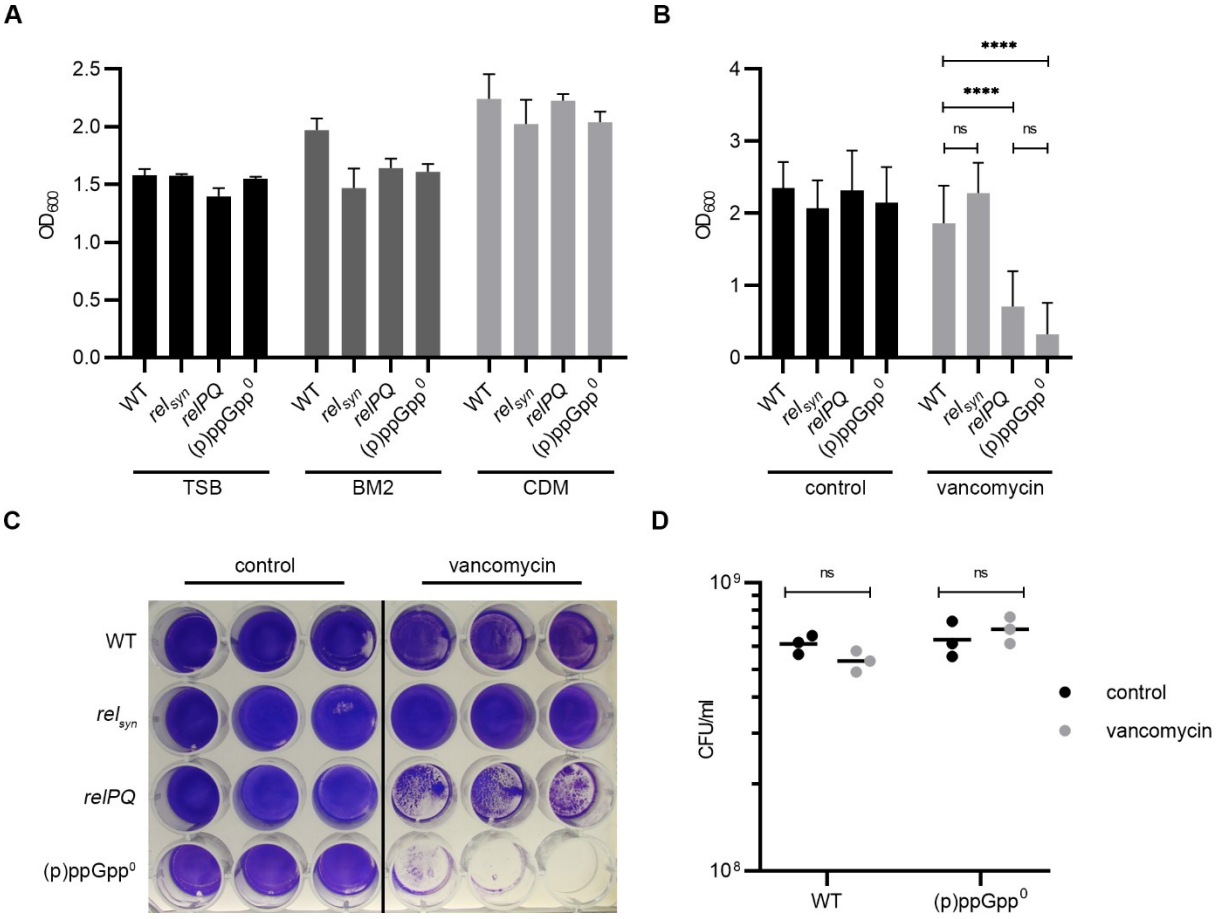

Fig. 3 (p)ppGpp synthases affect biofilm formation under cell wall stress conditions. (A) Biofilm formation in TSB (+3% NaCl, +0.5% glucose), BM2 and CDM after 24 h. (B) Biofilm formation under uninduced and vancomycin-stress (subinhibitory vancomycin 0.78 µg/ml) conditions in CDM after 24 h. Three separate experiments were performed with biological triplicates each. Error bars represent the standard deviation, statistical significance based on ordinary one-way ANOVA (ns: not significant, ****: $P < 0.0001$). (C) Representative plate stained with crystal violet. (D) CFU was determined from resolved biofilm and planktonic bacteria after 24 h of static incubation. (Figure adapted from [118].)

6.1.2 RelP and RelQ regulate biofilm formation under cell wall stress

It was demonstrated that sub-inhibitory concentrations of vancomycin transcriptionally activate relP and relQ [27]. Therefore, we speculated that the stringent response induced by vancomycin may interfere with biofilm formation. The minimal inhibitory concentration (MIC) (1 µg/ml) of vancomycin in planktonic grown bacteria did not differ between the analysed strains. At a sub-inhibitory concentration of vancomycin (0.78 µg/ml), the relPQ double mutant and the (p)ppGpp0 mutant exhibited significantly reduced biofilm formation compared to the wildtype and the relsyn mutant (Fig. 3B - C). When treated with vancomycin, the wildtype formed an almost uniform thick layer of biofilm. In contrast, the relPQ mutant and the (p)ppGpp0 strain showed significantly decreased biofilm formation, with some cell aggregates remaining after the washing procedure (Fig. 3B - C). The sub-inhibitory concentrations of vancomycin did not impair the bacterial survival of the tested strains (Fig. 3D). Therefore,

growth inhibition or bacterial killing did not cause the RelP- and RelQ-dependent changes in biofilm formation.

6.1.3 RelP and RelQ synergistically affect biofilm formation

To determine the impact of each of the synthetases on biofilm formation under vancomycin-induced conditions, we analysed single relP and relQ mutants. Both relP and relQ were found to contribute to biofilm formation in the presence of vancomycin. They act synergistically, as evidenced by the relPQ double mutant showing the lowest biofilm formation (Fig. 4). The relPQ double mutant phenotype was complemented by integration of either relP or relQ into the chromosome (Fig. 4). Thus, the induction of either relP or relQ by vancomycin is sufficient to maintain S. aureus biofilm formation under cell wall stress conditions.

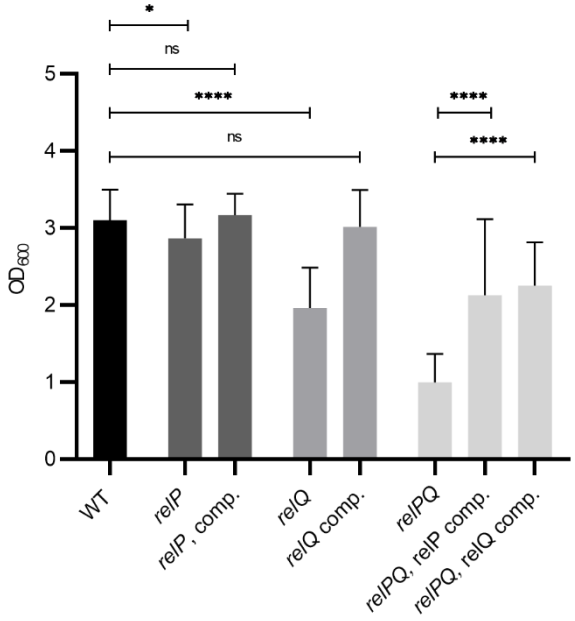

Fig. 4 RelP and RelQ affect biofilm formation synergistically. Biofilm formation under vancomycin-induced cell wall stress (0.78 µg/ml vancomycin) in CDM. Strains: HG001 wildtype, *relP* and *relQ* single mutants, *relPQ* double mutant and complemented strains. Three separate experiments were performed with biological triplicates each. Error bars represent the standard deviation, statistical significance based on ordinary one-way ANOVA (ns: not significant, *: $P < 0.1$, ****: $P < 0.0001$). (Figure adapted from [118].)

6.1.4 The biofilm composition is not affected by the stringent response

The biofilm matrix consists of PIA, proteins or eDNA [73, 75]. We analysed which matrix components were involved in the observed RelP/Q-dependent biofilm alterations under vancomycin treatment. Preformed biofilms were treated with sodium periodate, proteinase K or DNase to selectively degrade PIA, proteins or eDNA matrix components, respectively [119]. The biofilms formed by the wildtype or (p)ppGpp0 stain were almost completely degraded by proteinase K and DNase treatment in the absence of vancomycin, whereas sodium periodate

had no effect on the biofilm matrix (Fig. 5A - B). To confirm that vancomycin does not affect the biofilm composition, we also analysed the matrix components after vancomycin treatment. Once again, the biofilms consisted almost exclusively of proteins, and the biofilm matrix was not degraded by eDNA or sodium periodate treatment. Under the applied conditions, HG001 forms Ica-independent biofilms, and the composition of the biofilm remains unaltered by the stringent response.

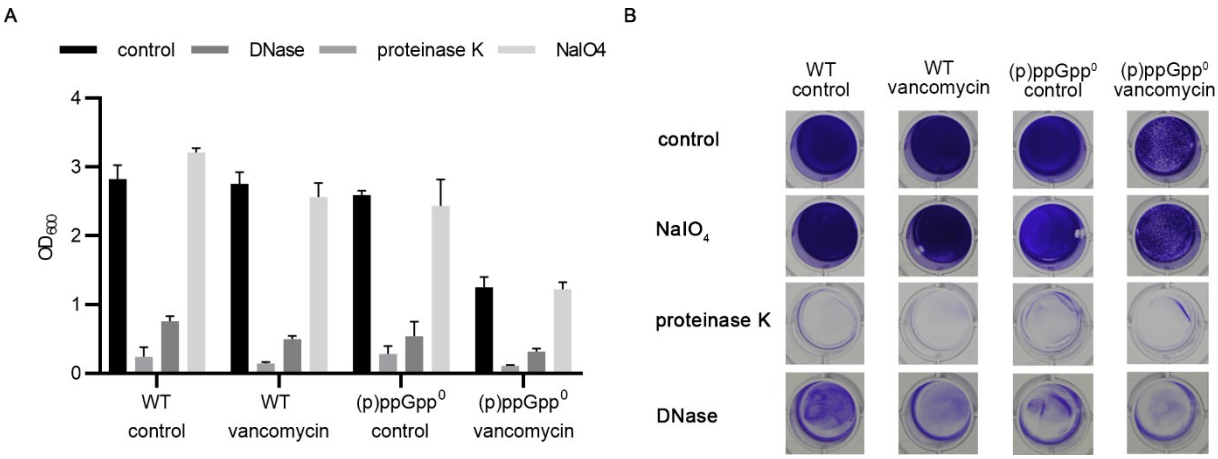

Fig. 5 Biofilm composition is not affected by (p)ppGpp. (A) Preformed biofilms of the wildtype and the isogenic (p)ppGpp^0 strain were treated with DNase or proteinase K for 4 h at 37°C or with sodium periodate for 24 h at 4°C. After treatment, the remaining biofilm was stained with crystal violet and quantified by OD_{600} measurement. Three separate experiments were performed, each with biological triplicates. Error bars represent the standard deviation. (B) Representative plate with wildtype, *rel*$_{syn}$, *relPQ* and the (p)ppGpp^0 strain stained with crystal violet. (Figure adapted from [118].)

6.1.5 The stringent response induces biofilm formation independent of the global regulators Agr and CodY

The quorum-sensing system Agr, particularly the target genes *psm*, [71] and the transcriptional regulator CodY [120] have been identified as key controllers of biofilm structure and detachment. The synthesis of (p)ppGpp results in the de-repression of the CodY regulon and upregulation of Agr-dependent *psm* genes [49]. Therefore, we hypothesised that Agr and/or CodY activity could interfere with the observed (p)ppGpp-dependent biofilm. However, the mutation of *agr* or *codY* did not impact biofilm formation (Fig. 6). Thus, under our assay conditions, biofilm formation occurs independently of the global regulators CodY and Agr.

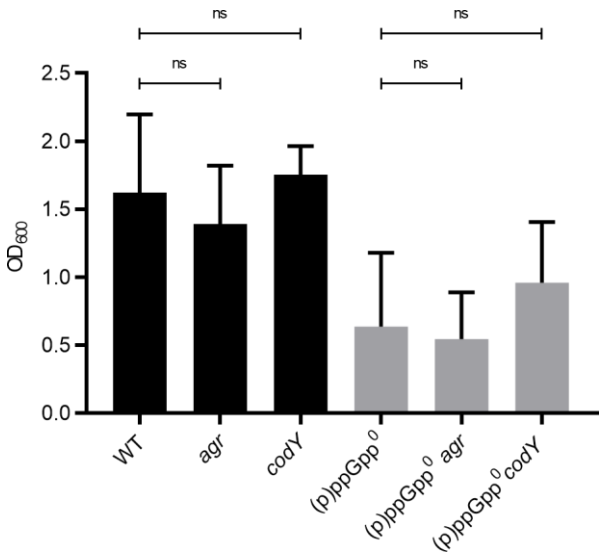

Fig. 6 Biofilm formation under stringent conditions is independent of CodY and Agr. Biofilm formation under non-stressed and vancomycin-induced cell wall stress (0.78 µg/ml vancomycin) in CDM after 24 h. Three separate experiments were performed, each with biological triplicates. Error bars represent the standard deviation, statistical significance based on ordinary one-way ANOVA (ns: $P > 0.05$). (Figure adapted from [118].)

6.1.6 (p)ppGpp contributes to biofilm antibiotic tolerance

Biofilms are typically more tolerant to high concentrations of antibiotics than planktonic cultures. We hypothesized that the stringent response contributes to biofilm antibiotic tolerance in *S. aureus*. To test this hypothesis, we compared biofilm antibiotic tolerance between the wildtype and the isogenic (p)ppGpp^0 mutant. Preformed biofilms were exposed to increasing concentrations of vancomycin for 16 hours. At the MIC (1 µg/ml for planktonic grown bacteria), vancomycin did not cause biofilm dispersal. However, when concentrations were 10- and 100-fold higher than the MIC, the biofilm produced by the (p)ppGpp^0 strain was significantly reduced, while the biofilm produced by the wildtype was more resistant to vancomycin treatment (Fig. 7A - B). Therefore, (p)ppGpp synthesis contributes to biofilm-related antibiotic tolerance.

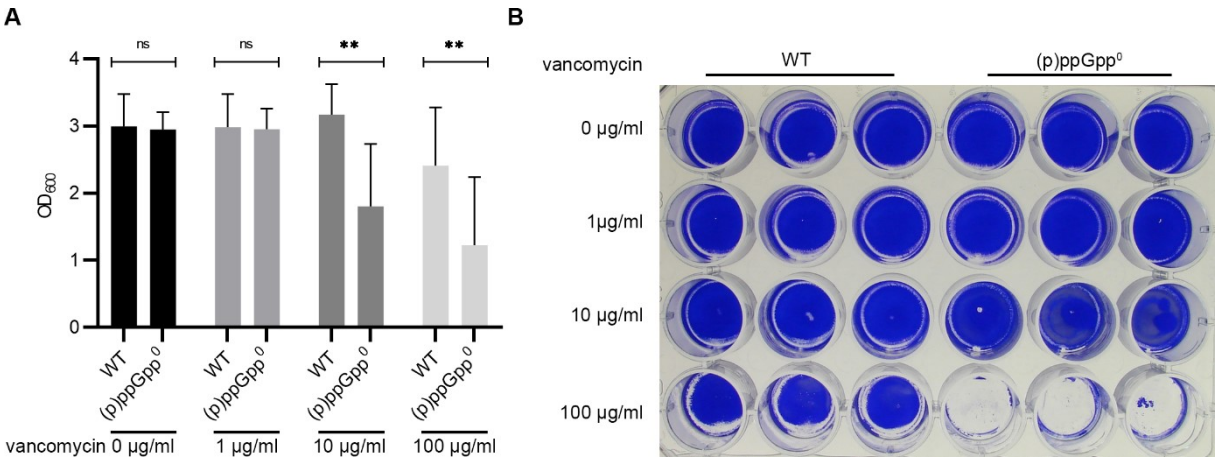

Fig. 7 (p)ppGpp contributes to biofilm related antibiotic tolerance. (A) Preformed biofilms (8 h) were exposed to increasing concentrations of vancomycin for 16 h. Three separate experiments were performed, each with biological triplicates. Error bars represent the standard deviation, statistical significance is based on ordinary one-way ANOVA (ns: not significant, **: $P < 0.01$). (B) Representative plate stained with crystal violet. (Figure adapted from [118].)

6.1.7 The anti-biofilm peptide DJK-5 exerts its effects independent of (p)ppGpp

Recently, two peptides, IDR-1018 [78] and DJK-5 [94], were proposed to prevent biofilm formation by specifically targeting intracellular (p)ppGpp. If correct, these peptides are expected to inhibit biofilm formation under stringent conditions in the wildtype but not in the pppGpp^0 background. We confirmed that DJK-5 interferes with biofilm formation in *S. aureus* (Fig. 8A - B). However, biofilm formation by the wildtype and pppGpp^0 strains was equally affected by DJK-5, without vancomycin treatment, indicating that the effect was independent of (p)ppGpp. The combination of a sub-inhibitory concentration of vancomycin and DJK-5 resulted in the complete inhibition of biofilm formation in the pppGpp^0 strain. This can be explained by bacterial killing of the pppGpp^0 strain through the synergistic action of vancomycin and DJK-5 (Fig. 8C). Thus, it is evident that (p)ppGpp synthesis in the wildtype provides protection against cell death. These results contradict the proposal made by de la Fuente-Nunez et al. [78, 94] that the biofilm-inhibiting activity of DJK-5 is achieved through (p)ppGpp inhibition.

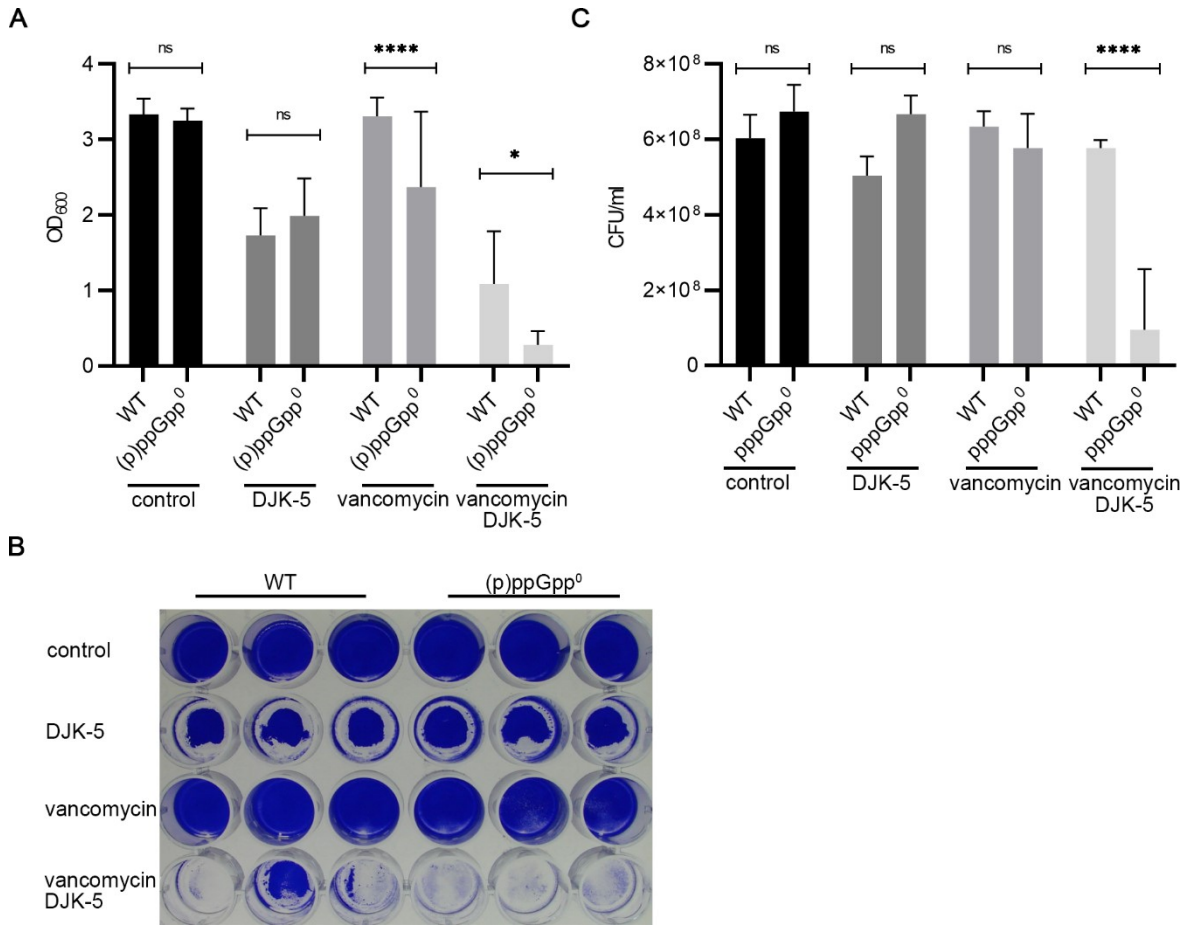

Fig. 8 DJK-5 interferes with biofilm formation under relaxed and stringent conditions. (A) Biofilms grown with or without vancomycin (0.78 µg/ml) and/or the anti-biofilm peptide DJK-5 (5 µg/ml) in CDM for 24 h. (B) Representative plate stained with crystal violet. (C) CFU of planktonic and biofilm bacteria determined in parallel after static growth for 24 h. Three separate experiments were performed with biological triplicates each. Error bars represent the standard deviation, statistical significance based on ordinary one-way ANOVA (ns: not significant, *: $P < 0.1$, ****: $P < 0.0001$). (Figure adapted and modified from [118].)

6.2 The role of (p)ppGpp-mediated GTP homeostasis during starvation for survival and antibiotic tolerance of *Staphylococcus aureus*

The results presented here are part of the manuscript "(p)ppGpp-mediated GTP homeostasis ensures the survival and antibiotic tolerance of *Staphylococcus aureus* during starvation by preserving the proton motive force" submitted to Communications Biology. The manuscript has been uploaded to the preprint server bioRxiv and can be accessed under the following doi: https://doi.org/10.1101/2024.02.06.579068. The manuscript might differ from the final published article.

6.2.1 (p)ppGpp contributes to culturability in the stationary phase

To study the requirement of (p)ppGpp synthesis for stationary phase survival, we monitored the growth and culturability of *S. aureus* wildtype strain HG001 and its isogenic (p)ppGpp^0 mutant in chemically defined medium (CDM) for 24 h. The (p)ppGpp^0 mutant is unable to synthesize (p)ppGpp due to mutations in all three (p)ppGpp synthetases (full deletion of *rel*, synthetase mutations in *relP* and *relQ)* [121]. There was no significant difference in growth measured by optical density (OD_{600}) between the two strains (Fig. 9A). However, after entry into the stationary phase, the culturability of the (p)ppGpp^0 mutant quickly started to decrease until an approximately one log reduction in CFU/ml was observed after 24 h compared to that of wildtype (Fig. 9B & Fig. 10A).

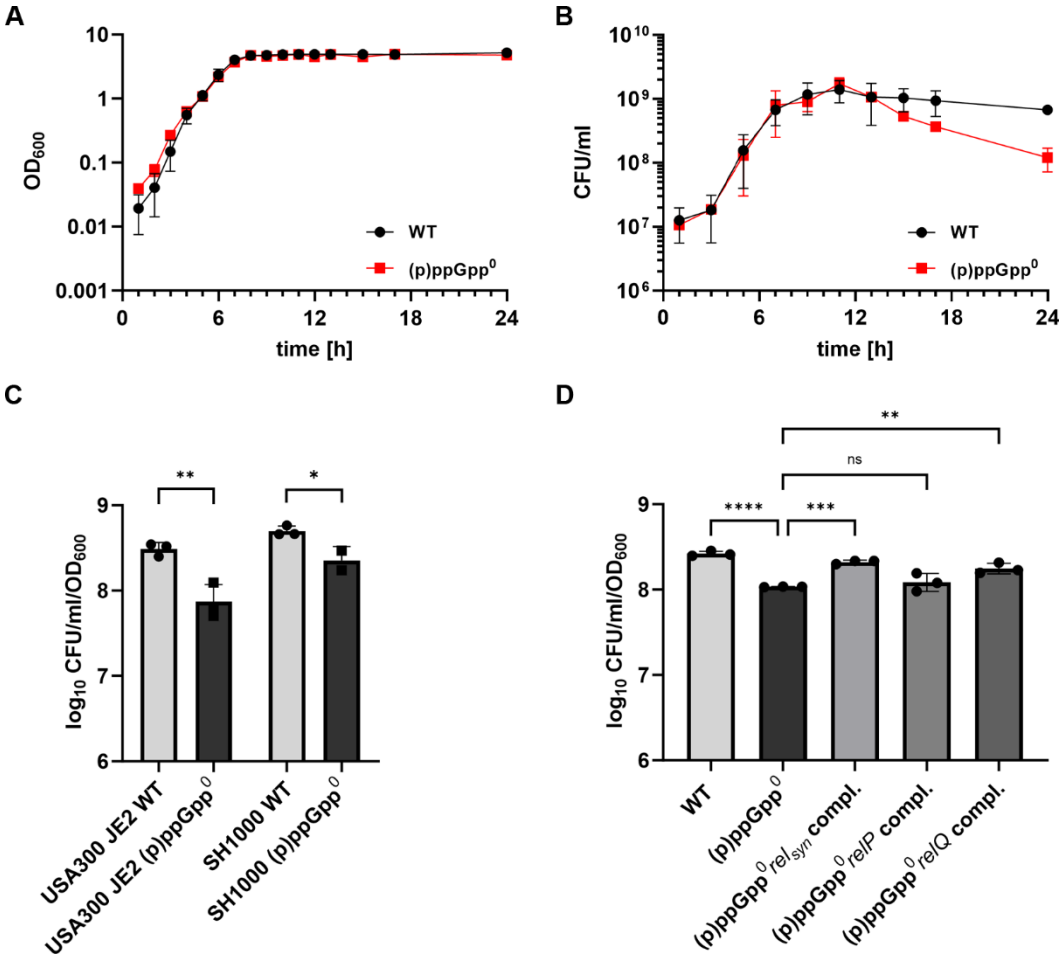

Fig. 9 Culturability of the (p)ppGpp^0 mutant decreases during the stationary growth phase. (A) *S. aureus* HG001 wildtype (WT) and the isogenic (p)ppGpp^0 strain were cultured in CDM [56] and growth was monitored by optical density OD_{600} and (B) CFU/ml determination. Data are shown as mean ± SD (n=3 biological replicates). (C) USA300 JE2 and SH1000 and their isogenic (p)ppGpp^0 mutants were grown in CDM for 24h and culturability was determined by CFU/ml enumeration normalized to OD_{600}. Data are shown as mean ± SD (n=3 biological replicates). Statistical significance was determined by two-tailed unpaired t-tests performed on log_{10} transformed data (**p-value <0.01, *p-value <0.05). (D) Strains were grown for 24h in CDM and culturability was determined by CFU/ml enumeration normalized to OD_{600}. Data shown are mean ± SD (n=3 biological replicates). Statistical significance

was determined by one-way analysis of variance (ANOVA) with a Šidák's post-test on log_{10} transformed data (****p-value <0.0001, ***p-value <0.001, **p-value <0.01, ns >0.05).

Reduced culturability of the (p)ppGpp^0 mutant was also observed in the background of the USA300 JE2 or SH1000 strains (the SigB-positive derivative and phage-cured derivative of HG001, respectively) (Fig. 9C). We next induced Rel-dependent (p)ppGpp synthesis in exponentially growing bacteria with a sub-inhibitory concentration of mupirocin, a tRNA synthetase inhibitor. These conditions mimicked amino acid starvation and resulted in significantly reduced culturability of the (p)ppGpp^0 mutant (Fig. 10B). Furthermore, we monitored expression levels of the known stringent response-activated genes *psmα* and *rsaD* [36, 122] during the mid- and late stationary phases (17 h and 24 h) in wildtype and (p)ppGpp^0. We observed a stringent response-like expression pattern for *psmα* and strong de-repression of *rsaD* in wildtype, indicating elevated (p)ppGpp levels (Fig. 10C). As expected, in the (p)ppGpp^0 mutant, expression of stringent response genes was not induced, but *rsaD* expression was strongly downregulated, suggesting elevated intracellular GTP levels (Fig. 10C). Individual complementation of (p)ppGpp^0 with *rel$_{syn}$*, *relP* or *relQ* (Fig. 9D) confirmed that Rel and RelQ contribute to basal (p)ppGpp levels to ensure survival during the stationary phase. Thus, (p)ppGpp is essential for bacterial culturability in the stationary phase as well as under induced stress conditions in exponentially growing bacteria.

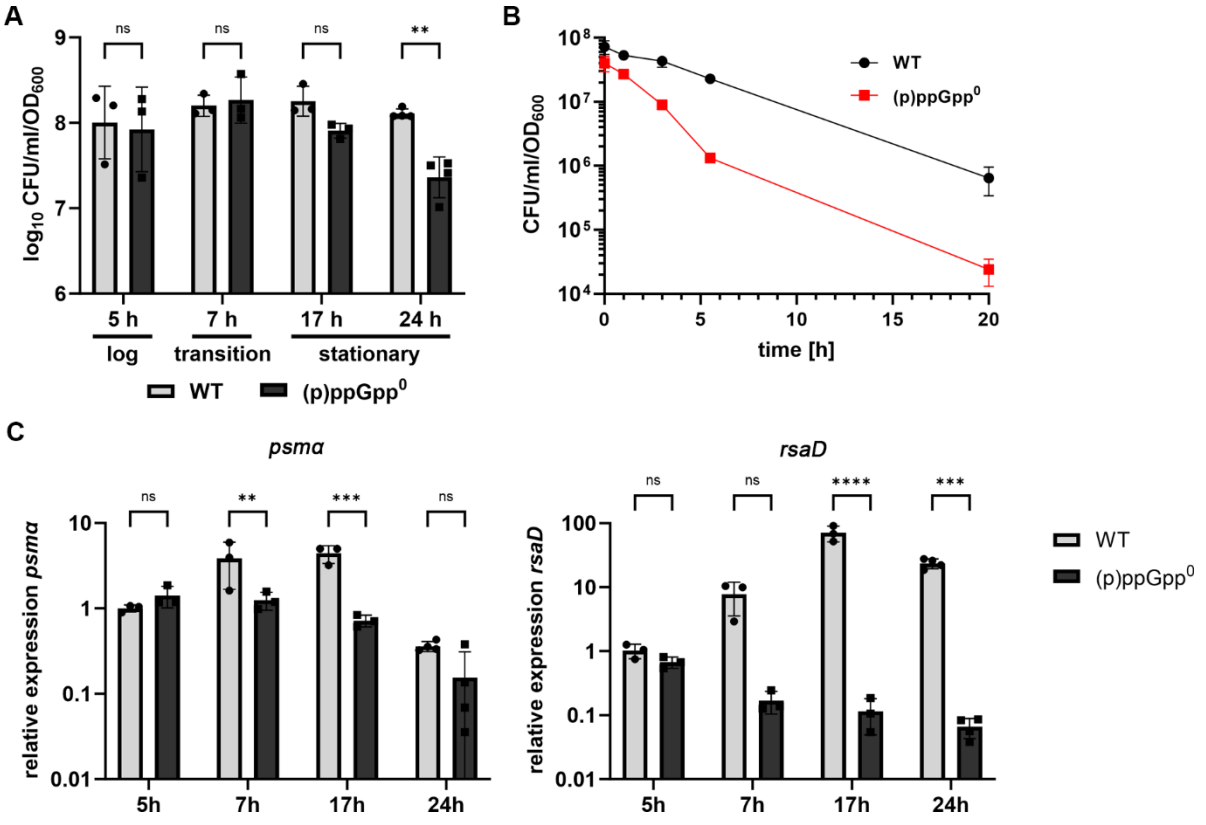

Fig. 10 Decreasing cultuarbility of (p)ppGpp^0 cells during stationary phase and stringent response induction. (A) Culturability of HG001 wildtype (WT) and (p)ppGpp^0 mutant was evaluated during different growth

stages by CFU/ml enumeration normalized to OD_{600}. Data shown are mean of ± SD (n=3 biological replicates). Statistical significance was determined by a two-way analysis of variance (ANOVA) with Šidák's post-test performed on log_{10} transformed data (**p-value <0.01, ns >0.05). (B) Bacteria grown to exponential growth phase were treated with sub-inhibitory concentrations of mupirocin (0,125 µg/ml) and culturability was determined by CFU/ml enumeration normalized to OD_{600}. Data shown are mean of ± SD (n=3 biological replicates). (C) Bacterial cells were harvested during different growth phases and total RNA was isolated. *psmα and rsaD* transcript levels were determined by RT-qPCR and normalized to *gyrB* expression by ΔΔCt method. Data are shown as mean ± SD (n=3 biological replicates). Statistical significance was determined by two-way analysis of variance (ANOVA) with Šidák's post-test (***p-value <0.001, **p-value <0.01, *p-value <0.05, ns >0.05).

6.2.2 (p)ppGpp-dependent GTP homeostasis is essential for stationary phase culturability independent of CodY

(p)ppGpp has been shown to play an important role in controlling GTP homeostasis [54, 55]. Therefore, we assessed the viability of different mutants in which GTP metabolism was disrupted after growth to the late stationary phase. The pleiotropic repressor CodY, when loaded with GTP, inhibits expression of several biosynthesis-related gene clusters and virulence genes. CodY target genes are de-repressed under stringent conditions when (p)ppGpp is synthesized and consequently intracellular GTP levels drop [27]. We therefore hypothesized that the decreased survival of the $(p)ppGpp^0$ mutant might be ascribed to diminished expression of CodY target genes. However, deletion of *codY* did not increase the culturability of the $(p)ppGpp^0$ mutant (Fig. 11B). PurR is a repressor of the purine operon [62, 123], and in *B. subtilis,* it is allosterically activated via (p)ppGpp [64] (Fig. 11A). Thus, deletion of *purR* in the $(p)ppGpp^0$ mutant will likely lead to increased GTP levels. This mutant was even less culturable in the stationary phase, indicating that elevated GTP levels are detrimental to bacterial culturability (Fig. 11B). Accordingly, lowering the GTP pool should promote bacterial survival. Indeed, deletion of the GTP synthesis operon *guaBA* rescued the defect in the $(p)ppGpp^0$ mutant (Fig. 11B). We next manipulated levels of GTP via supplementation with guanine, which feeds into the salvage pathway of GTP synthesis (Fig. 11A). This resulted in a dose-dependent decrease in the culturability of the $(p)ppGpp^0$ mutant (Fig. 11C).

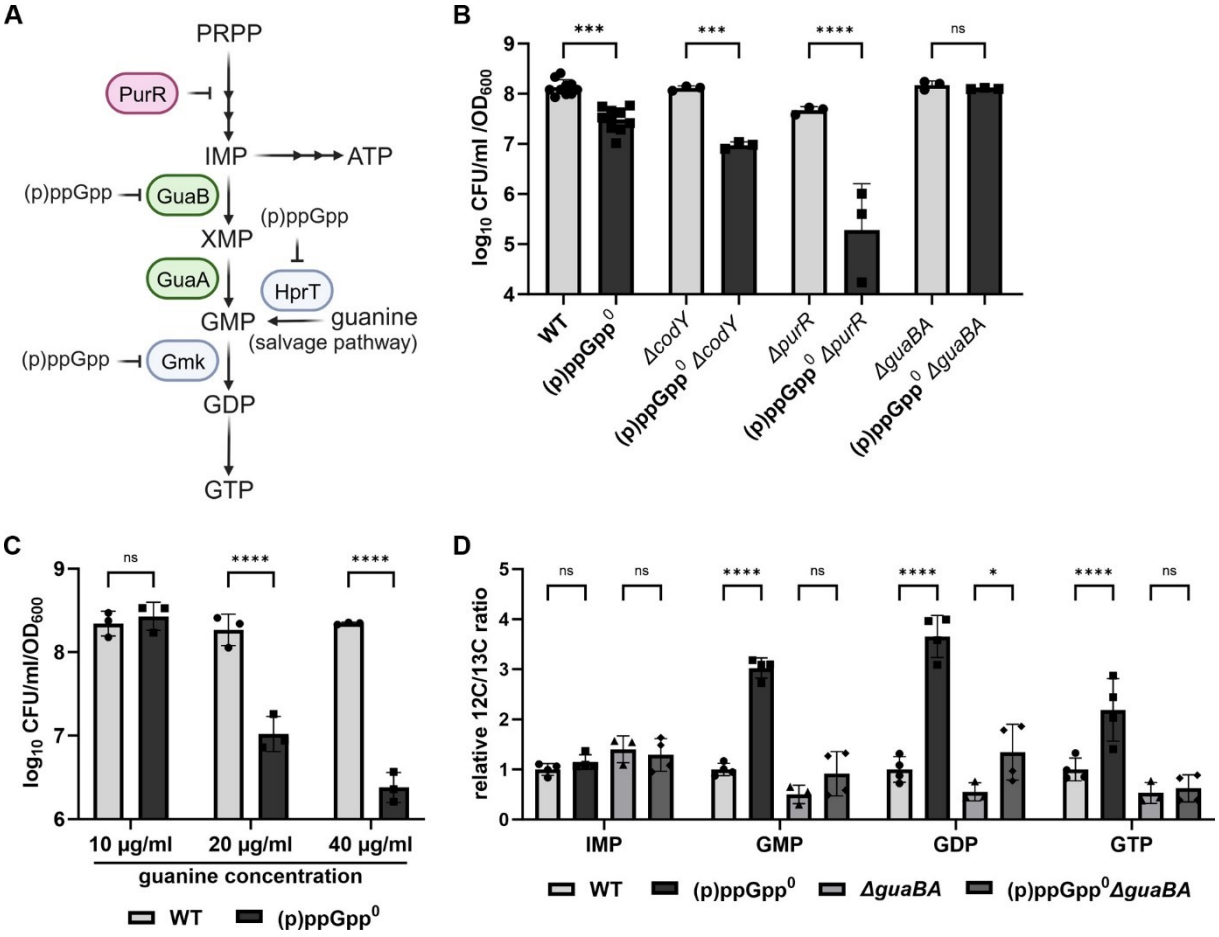

Fig. 11 (p)ppGpp is essential for GTP homeostasis during stationary phase starvation. (A) The purine biosynthesis pathway is regulated by PurR repression and by (p)ppGpp repressing the biosynthetic enzymes GuaB, HrpT and Gmk. (B) Strains were grown in CDM for 24h and survival was determined by CFU/ml enumeration normalized to OD_{600}. Data shown are mean ± SD (n≥3). Statistical significance was determined by one-way analysis of variance (ANOVA) with a Tukey's post-test on log_{10} transformed data (****p-value <0.0001, ***p-value <0.001, ns >0.05). (C) HG001 wildtype (WT) and (p)ppGpp^0 were grown in CDM with varying concentrations of guanine for 24h and culturability was determined by CFU/ml enumeration normalized to OD_{600}. Data shown are mean ± SD (n=3). Statistical significance was determined by two-way way analysis of variance (ANOVA) with a Šidák post-test (****p-value <0.0001, ns >0.05). (D) Relative nucleotide levels were determined from late stationary phase cells by LC-MS/MS and normalized to OD_{600}. Data shown are mean ± SD (n=4). Statistical significance was determined by two-way analysis of variance (ANOVA) with a Tukey's post-test (****p-value <0.0001, ***p-value <0.001, **p-value <0.01, ns >0.05).

We used LC-MS/MS analysis to confirm the anticipated nucleotide levels in the (p)ppGpp^0 and *ΔguaAB* mutants. As expected, levels of all guanine nucleotides (GMP, GDP and GTP) were significantly elevated in the (p)ppGpp^0 mutant in the late stationary phase (Fig. 11D). Lower levels were observed in wildtype, likely due to (p)ppGpp-dependent inhibition of biosynthetic enzymes, including GuaB (Fig. 11A). Consistently, deletion of *guaBA* also decreased guanine pools in (p)ppGpp^0 (Fig. 11D). Levels of ATP decreased in (p)ppGpp^0 independent of deletion of *guaBA* (Fig. 12A & Fig. 11E), but levels of the other nucleotides differed only slightly between

the strains (Fig. 12A-C). These results confirm a clear correlation between elevated guanine nucleotide levels during stationary phase starvation and decreased culturability.

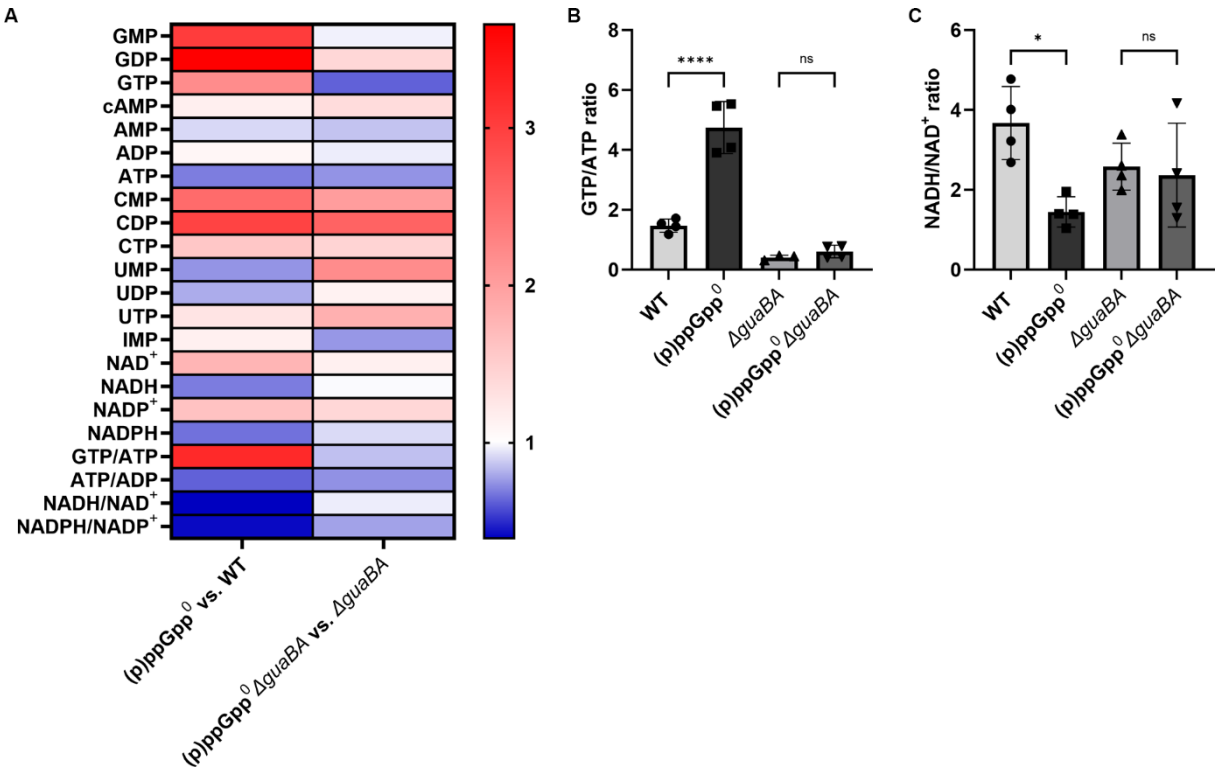

Fig. 12 Determination of nucleotide levels in late stationary phase cells. (A) Metabolites were extracted and nucleotides were measured and quantified via LC-MS/MS from cells grown to late stationary growth phase (24h). Shown are relative nucleotide levels as determined by comparing (p)ppGpp^0 vs. WT and (p)ppGpp^0 *ΔguaBA* vs. *ΔguaBA*. (B) The GTP/ATP ratio and (C) the NADH/NAD$^+$ ratio were calculated from the relative nucleotide levels. Data shown are mean of ± SD (n=4 biological replicates). Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey's post-test (****p-value <0.0001, *p-value <0.05, ns >0.05).

6.2.3 (p)ppGpp-deficient cells enter a division-incompetent but viable state during starvation

To further investigate the cause and mechanism of the decreased culturability of the (p)ppGpp^0 mutant, we used different viability assays based on membrane integrity or metabolic activity. Despite the large decrease in CFUs during the stationary phase (Fig. 9B & Fig. 10A), only a small percentage of (p)ppGpp^0 mutant cells were stained with the membrane-impermeable dye propidium iodide, and there was no significant difference between wildtype and the (p)ppGpp^0 mutant (Fig. 13A). Analysis of the metabolic activity of late stationary phase cells by RedoxSensor™ Green staining, which monitors bacterial reductase activity, revealed slightly reduced metabolic activity in the (p)ppGpp^0 mutant (Fig. 13B). However, the decrease in metabolic activity was less than expected from the difference in CFU counts between wildtype and (p)ppGpp^0 during the late stationary phase (only approximately 10% culturable (p)ppGpp^0 mutant). Resazurin conversion (Fig. 13C), an indicator of cellular redox potential, also revealed

slightly decreased respiratory activity in the (p)ppGpp^0 mutant compared to wildtype and the *guaBA*-negative strain. To further confirm the alterations in the redox state of the mutant cells, we directly measured the NADH/NAD$^+$ ratio (Fig. 13D) and ATP level (Fig. 13E). Both parameters were significantly decreased in the (p)ppGpp^0 mutant. The effect was less prominent in the *guaBA* mutant background, indicating that the change in redox state is mainly a consequence of changes in the GTP pool.

The discrepancy between the decreased CFU counts and low propidium iodide staining, together with the decreased metabolic activity in the late stationary phase, suggest that the (p)ppGpp^0 cells did not immediately lose viability. It is more likely that the cells entered a dormant state, slowing their metabolic activity and halting cell division, resulting in the observed large decrease in CFU.

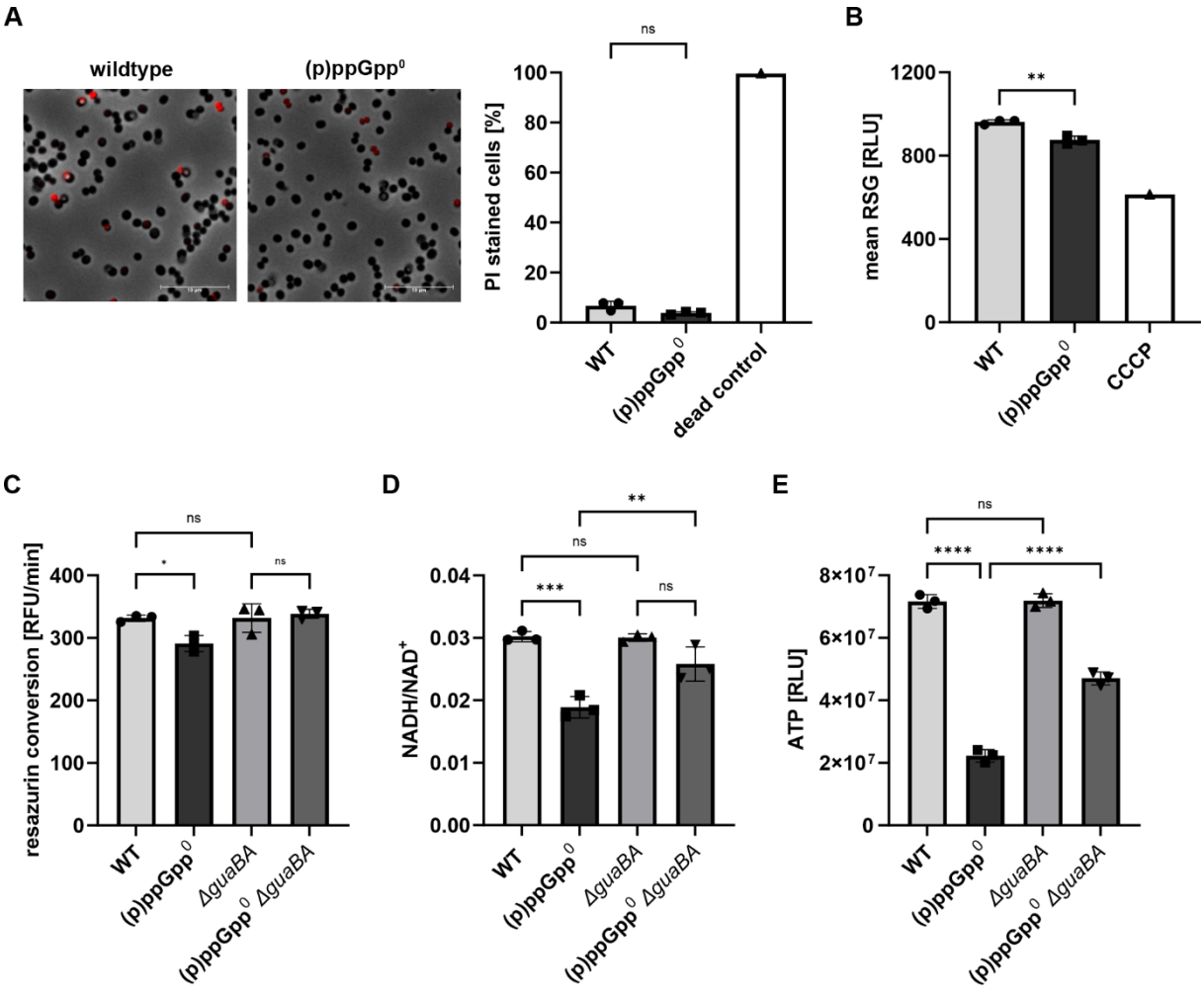

Fig. 13 The (p)ppGpp^0 mutant maintains membrane integrity but displays decreased metabolic activity. Strains were grown to late stationary phase (24h) in CDM. (A) Representative fluorescence microscopy images of propidium iodide (PI)-stained HG001 wildtype (WT) and (p)ppGpp^0 are shown. The percentage of propidium iodide stained cells was measured by flow cytometry. For dead control bacteria were permeabilized with 70% EtOH. Data shown are mean ± SD (n=3 biological replicates, n=1 for dead control). Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey's post-test (ns >0.05). Scale bar: 10 µm (B) Bacteria were

stained with RedoxSensor™ Green reagent and analysed by flow cytometry to monitor metabolic activity. Data shown are mean of RSG fluorescence ± SD (n=3 biological replicates, n=1 for CCCP control). Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey's post-test (**p-value <0.01). (C) Redox potential was accessed by resazurin conversion over time in HG001 wildtype, (p)ppGpp^0, *ΔguaBA* and (p)ppGpp^0 *ΔguaBA*. Data shown are mean ± SD (n=3 biological replicates). Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey's post-test (*p-value <0.05, ns >0.05). (D) NADH/NAD$^+$ ratio and (E) relative ATP levels were determined. Data shown are mean of ± SD (n=3 biological replicates). Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey's post-test (****p-value <0.0001, ***p-value <0.001, **p-value <0.01, ns >0.05).

6.2.4 The division-incompetent state of (p)ppGpp^0 cells is not the result of increased translation during the stationary phase

(p)ppGpp is known to efficiently inhibit protein synthesis [12, 124] and thereby may prevent accumulation of protein aggregates [125]. We measured protein synthesis at different time points during growth by incorporating the puromycin analogue O-propargyl-puromycin (OPP) into newly synthesized proteins. As expected, in the (p)ppGpp^0 mutant, translation was significantly greater in the late exponential and early stationary growth phases (7 h and 9 h) (Fig. 14A). However, in the late stationary growth phase (24 h), complete shutdown of protein synthesis was observed, indicating that this occurred independently of (p)ppGpp. Delayed or absent translation inhibition can lead to formation of protein aggregates [125]. However, only a slight increase in protein aggregates was observed in the (p)ppGpp^0 mutant, as assessed by densitometric quantification of Coomassie-stained protein aggregates isolated from wildtype and (p)ppGpp^0 late stationary phase cultures (Fig. 15A & B).

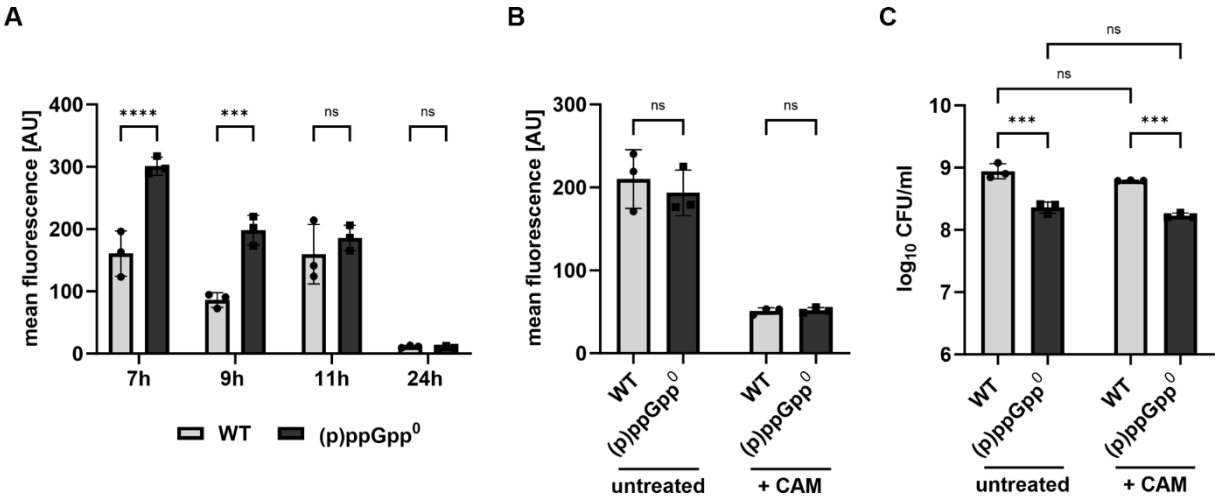

Fig. 14 Increased translation of (p)ppGpp^0 does not contribute to entrance into dormant state. (A) Protein synthesis was monitored at different timepoints during growth in wildtype (WT) and (p)ppGpp^0 cells by labelling with the puromycin analogue OPP and subsequent flow cytometry analysis. Data shown are mean of ± SD (n=3 biological replicates). Statistical significance was determined by two-way analysis of variance (ANOVA) with Šidák's post-test (****p-value <0.0001, ***p-value <0.001, ns >0.05). (B) Wildtype and (p)ppGpp^0 cells grown to transition phase were treated with 100x MIC chloramphenicol (+ CAM), and protein synthesis by OPP incorporation and (C)

culturability were assessed by CFU/ml enumeration. Data shown are mean of ± SD (n=3 biological replicates). Statistical significance was determined by two-way analysis of variance (ANOVA) with Šidák's post-test (on log_{10} transformed data for (C), ***p-value <0.001, ns >0.05).

To further analyse whether increased translation in (p)ppGpp^0 might contribute to entry into a division-incompetent state, we inhibited translation with 100x the MIC of the bacteriostatic antibiotic chloramphenicol. Chloramphenicol treatment almost completely inhibited protein synthesis in both wildtype and (p)ppGpp^0 cells (Fig. 14B). However, chloramphenicol-induced inhibition of translation did not increase the culturability of (p)ppGpp^0 cells (Fig. 14C).

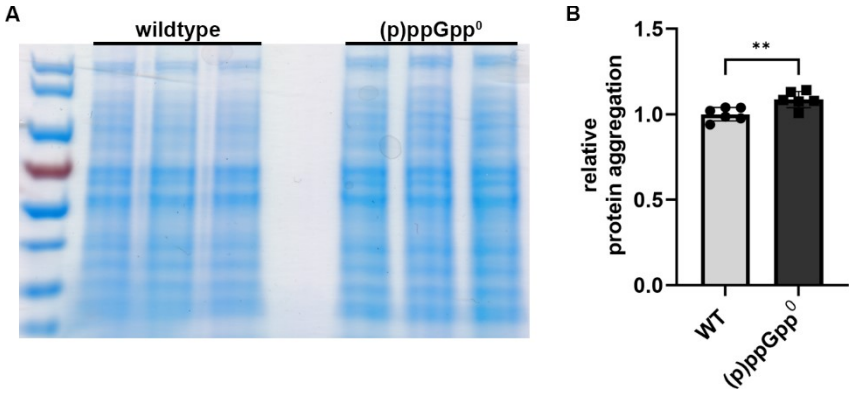

Fig. 15 Quantification of protein aggregates. (A) Protein aggregates were isolated from wildtype and (p)ppGpp^0 cells grown to late stationary phase and anaylsed by SDS-PAGE and Coomassie staining. For each strain, protein aggregates were isolated from three individual biological replicates (n=3). (B) Protein aggregates were quantified by densiometric analysis. Data shown are mean of ± SD (n=6 biological replicates from two individual experiments). Statistical significance was determined by a two-tailed, unpaired t-test (**p-value <0.01).

Thus, translation inhibition appears to be delayed in the (p)ppGpp^0 mutant, leading to slightly increased protein aggregation during the stationary phase. However, this delay in translation inhibition does not seem to be the cause of the non-culturable state of (p)ppGpp^0 cells during stationary phase starvation.

6.2.5 Division-incompetent cell state of (p)ppGpp^0 cells is not the result of oxidative stress during the stationary phase

In *S. aureus, (*p)ppGpp was shown to contribute to tolerance to oxidative stress [101], suggesting that elevated oxidative stress is a potential cause of the "dormant" state of the (p)ppGpp^0 mutant. To test this hypothesis, we analysed culturability under anaerobic and oxygen-limited, static (biofilm) conditions. Under these conditions, the culturability of the (p)ppGpp^0 mutant was not compromised (Fig. 16A). However, treatment of aerobically grown cultures with the ROS scavenger N-acetyl cysteine (NAC) did not have any impact on culturability in the late stationary phase (Fig. 16B). Furthermore, no notable difference in endogenous ROS formation was detected between wildtype and the (p)ppGpp^0 mutant during

the stationary growth phase (Fig. 16C). Thus, (p)ppGpp-deficient cells were impaired only in culturability under aerobic conditions. However, this phenotype could not be linked to elevated ROS levels. Thus, we investigated the contributions of metabolic cues such as aerobic respiration and proton motive force (PMF).

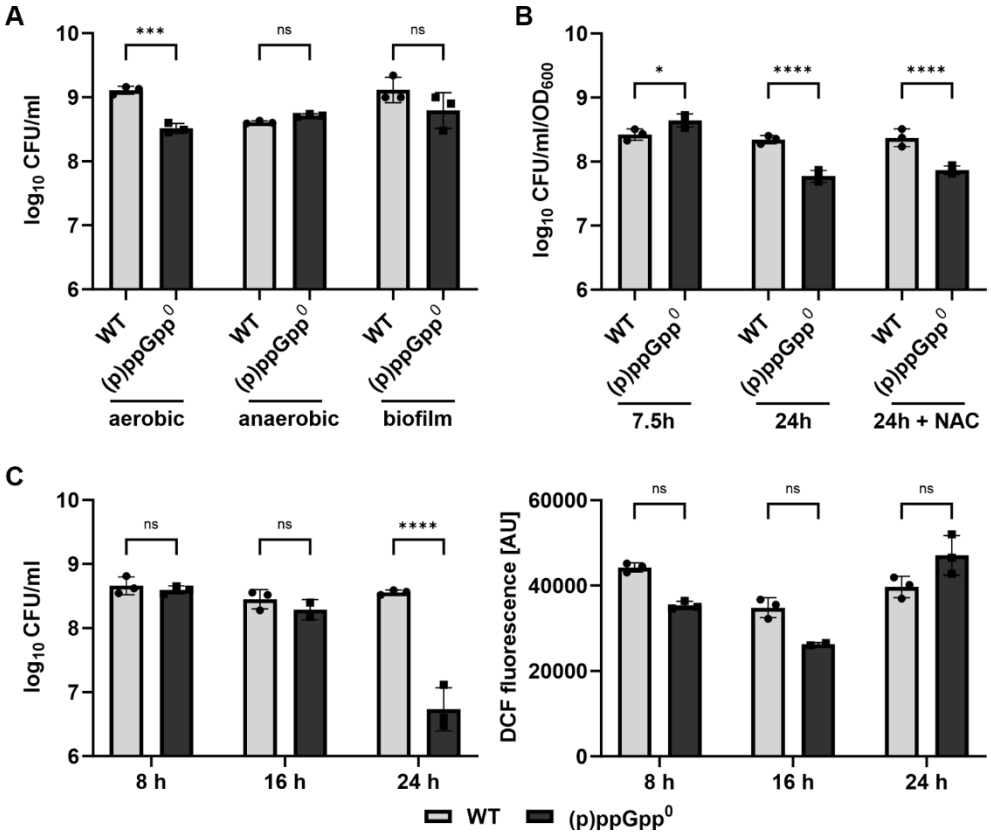

Fig. 16 Oxidative stress in (p)ppGpp^0 does not contribute to entrance into dormant state. (A) HG001 wildtype (WT) and (p)ppGpp^0 strain were grown under aerobic, anaerobic or oxygen-limited, static (biofilm) conditions for 24h and culturability was accessed by CFU/ml enumeration. Data shown are mean ± SD (n=3 biological replicates). Statistical significance was determined by two-way analysis of variance (ANOVA) with Šidák's post-test on $\log_{10}$ transformed data (***p-value <0.001, ns >0.05). (B) Wildtype and (p)ppGpp^0 cells were treated with 12.5 mM N-acetylcysteine (+ NAC) during transition to stationary phase (7.5h) and CFU/ml counts normalized to OD600 were determined before and after treatment. Data shown are mean of ± SD (n=3 biological replicates). Statistical significance was determined by two-way analysis of variance (ANOVA) with Šidák's post-test (****p-value <0.0001, * <0.05). (C) Monitoring of culturability, as determined by CFU/ml enumeration, and intracellular ROS levels, as measured by using the DCFH2-DA dye, which is oxidized to DCF by ROS, from early to late stationary phase. Data are shown as mean ± SD (n=3 biological replicates). Statistical significance was determined by two-way analysis of variance (ANOVA) with Šidák's post-test (****p-value <0.0001, ns >0.05).

6.2.6 Membrane function and architecture are impaired in (p)ppGpp^0 cells

Changes in membrane potential can impact cell vitality by perturbing ATP synthesis and ion transport. We measured membrane potential by staining with the carbocyanine dye $DiOC_2(3)$. During the exponential growth phase, there was no difference in membrane potential between wildtype, (p)ppGpp^0 and *guaBA*-negative cells (Fig. 17A). However, during the late stationary

phase, the membrane potential of the (p)ppGpp^0 mutant was notably lower than that of wildtype. In contrast, there was no significant difference in the level of the *guaBA*-negative strain, indicating that high levels of GTP disturb the membrane potential. The PMF is composed of the electrical potential ΔΨ and the transmembrane proton gradient ΔpH. The intracellular pH was similar between wildtype and the (p)ppGpp^0 mutant (Fig. 17B), indicating that membrane potential ΔΨ mainly reduced in cells with high GTP levels. Analysis of the membrane and cell wall architecture by co-staining with the dye FM4-64X and the BODIPY-vancomycin conjugate revealed irregular staining of the (p)ppGpp^0 cell membrane during starvation in the stationary phase, which was not observed in wildtype cells (Fig. 17C). In contrast, no abnormalities were detected in the cell wall of (p)ppGpp^0 cells during stationary phase starvation compared to wildtype cells (Fig. 17C). These irregularities in the cell membrane of the (p)ppGpp^0 strain, together with increased membrane fluidity, as measured by DPH fluorescence polarization (Fig. 17D), are indicative of membrane patches with increased fluidity, also known as regions of increased fluidity (RIFs).

Taken together, the results show that in the stationary phase, high levels of GTP, as found in the (p)ppGpp^0 mutant, led to decreased membrane potential, changes in cell membrane architecture and increased membrane fluidity. Thus, the decreased culturability of the (p)ppGpp^0 mutant is likely due to loss of membrane potential.

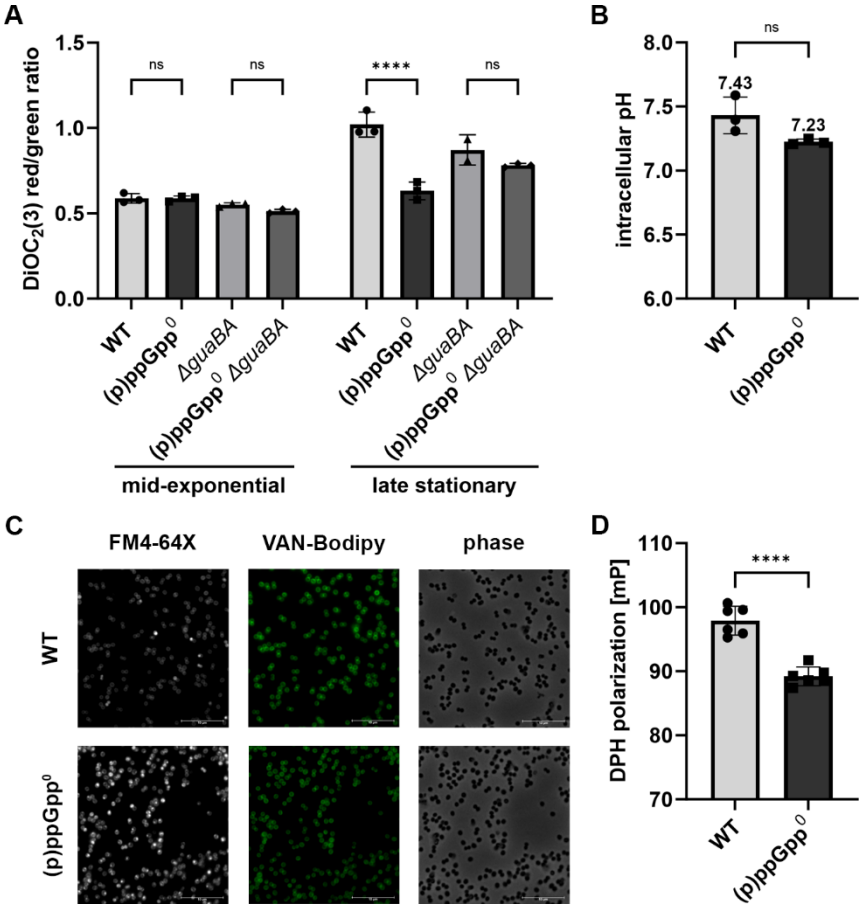

Fig. 17 Membrane function and architecture is altered in the (p)ppGpp^0 mutant. (A) Membrane potential was measured during mid-exponential and late stationary phase by staining with the carbocyanine dye $DiOC_2(3)$. Data shown are mean of ± SD (n=3 biological replicates). Statistical significance was determined by two-way analysis of variance (ANOVA) with Šidák's post-test (****p-value <0.0001, ns >0.05) (B) Intracellular pH was determined by pHRodo Green AM staining of late-stationary phase bacteria. Data shown are mean ± SD (n=3 biological replicates). Statistical significance was determined by a two-tailed, unpaired t-test (ns > 0.05). (C) Representative microscopic images of FM4-64X (cell membrane) and BODIPY-vancomycin conjugate (cell wall) double-stained *S. aureus* from late stationary phase (24h). Scale bar: 10 µm (D) Membrane fluidity was measured by DPH fluorescence polarization in late stationary phase (24h). Data shown are mean ± SD (n=6 biological replicates). Statistical significance was determined by a two-tailed, unpaired t-test (****p-value <0.0001).

6.2.7 Impact of a lack of (p)ppGpp biosynthesis and GTP imbalance on the transcriptome during stationary phase starvation

To better understand how changes in GTP levels might alter membrane potential, we analysed changes in gene expression in wildtype (GTP low), (p)ppGpp^0 mutant (GTP high) and *ΔguaBA* mutant (GTP low) cells after 24 h of growth (Supplementary Tables S1 und S2). Genes with at least a log2-fold change of ≥ 1 and ≤ -1 and a p value ≤ 0.001 were defined as differentially regulated. In total, 235 and 288 genes were significantly upregulated or downregulated, respectively, in the (p)ppGpp^0 mutant compared to wildtype. Such (p)ppGpp-dependent and GTP-independent differences were much less prominent in the Δ*guaBA* background (only 50

and 65 genes up- or downregulated, respectively). To assess (p)ppGpp-independent and GTP-dependent changes in gene expression, the (p)ppGpp^0 *ΔguaBA* mutant was compared to the (p)ppGpp^0 mutant. In total 514 and 628 genes were found to be significantly up- or downregulated, demonstrating that under our experimental conditions, the majority of transcriptomic changes are mediated by differences in GTP levels, as opposed to being direct effects of (p)ppGpp.

Many of the previously identified stringent response genes identified after challenge with amino acid limitation [49] or transcriptional induction of pppGpp synthetases [36] were differentially regulated during stationary phase starvation (see Fig. S4 and S1C). Most CodY target genes were downregulated in the (p)ppGpp^0 mutant [e.g., *rsaD* (-185.8-fold), the *cap* operon (-5,2-fold for *capA*) and *aur* (-65,7-fold)]. However, genes involved in amino acid synthesis and transport, such as *brnQ2* (-6.1-fold), *gltBD* (-14.8-fold and -12.2-fold), *dapABDL* (-12.5-fold for *dapA*), the *opp-3ABCDEF* operon (-20.1-fold for *opp-3B*) and *ilvD* (-7.2-fold), were also downregulated. Furthermore, several genes of the SigB regulon were downregulated in (p)ppGpp^0. For instance, *krtA,* which encodes a potassium transporter, was downregulated 2.1-fold. Similarly, *clpL*, which is involved in protein metabolism, was downregulated 6.1-fold. The *mnh* operon, which is hypothesized to encode a redox-energized complex involved in PMF maintenance [126], was also downregulated 5.1-fold. Furthermore, *groEL* (2.4-fold) and *groES* (4.9-fold), which are part of the CtsR/HrcA regulon, were found to be upregulated, indicative of proteotoxic stress. Both protein complexes were upregulated during proteotoxic stress, which leads to protein misfolding and aggregation [127]. These findings are consistent with the slightly elevated protein aggregates detected in the (p)ppGpp^0 mutant during stationary phase starvation (Fig. 15). Interestingly, phage-encoded genes were upregulated under GTP-high conditions, indicating that phages φ11, φ12 and φ13 are induced in (p)ppGpp^0 during the stationary phase. These observations are in agreement with previous studies that found the ΦSa3 phage to be downregulated under guanine-limited conditions [128].

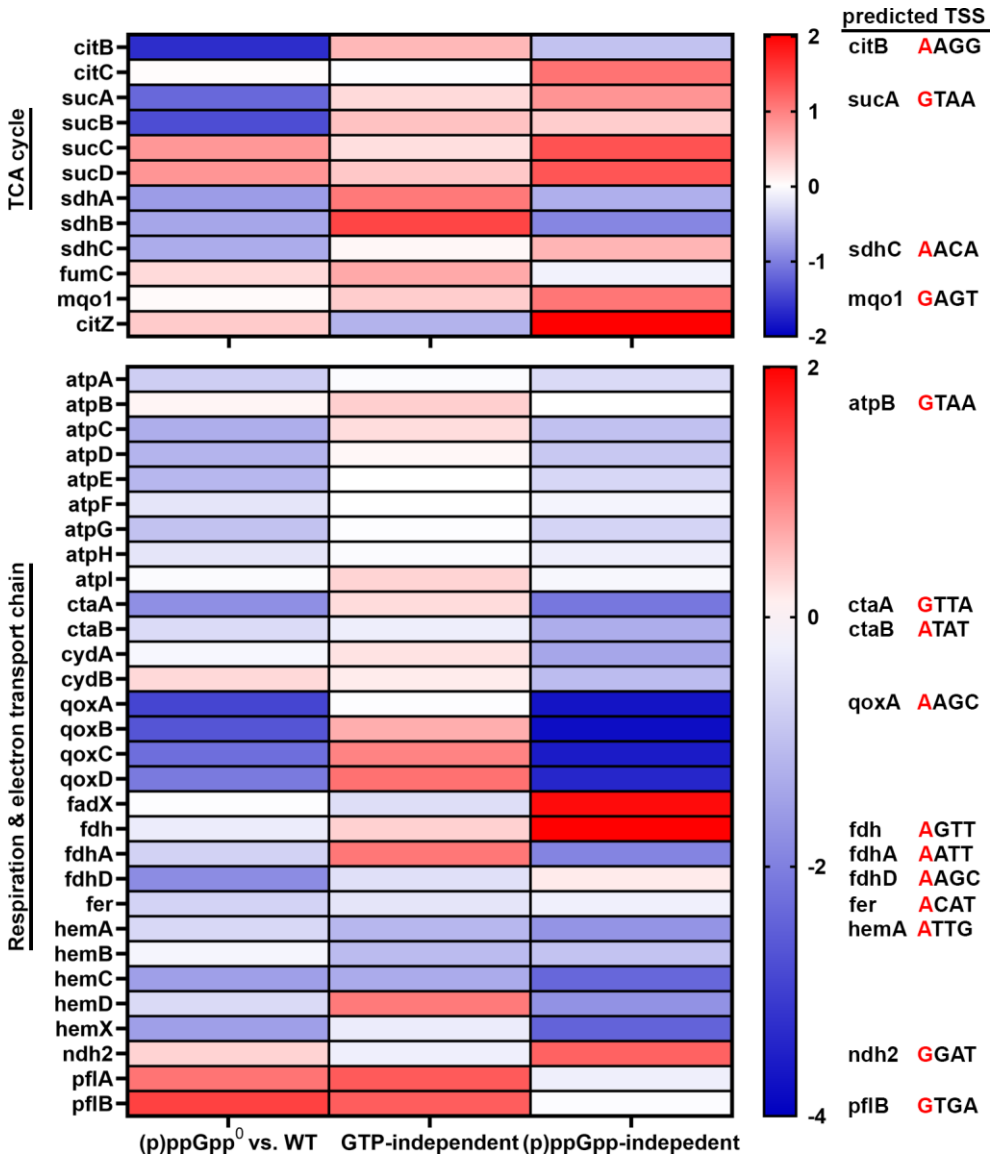

Fig. 18 Transcriptomic changes in TCA cycle and respiration in dormant (p)ppGpp^0 cells. Heatmaps displaying the RNA-seq fold change of selected genes involved in the TCA cycle and electron transport chain, and related to respiration (SEED annotation). Log2 fold changes in relative transcript abundances are colour-coded with red and blue, indicating up- and downregulation, respectively. "GTP-independent" compares the expression levels of (p)ppGpp^0 ΔguaBA vs. ΔguaBA, while "(p)ppGpp-independent" compares the expression levels of (p)ppGpp^0 vs. (p)ppGpp^0 ΔguaBA. The predicted TSS position between nucleotide +1 and +4 is depicted, while the TSS +1 is indicated in red colour. The RNA-seq data shown are from three individual biological replicates (n=3). Full RNA-seq data are available in Data S1 and S2 in the supplementary material.

Notably, the transcriptome further revealed strong downregulation of TCA cycle (*citB*-3.1-fold, *sucA*-2.3-fold and *sucB*-2.6-fold) and aerobic respiration (*qoxABCD* -7.6-fold for *qoxA*, *ctaA* -3.4-fold and ATP synthase e.g. *atpD* -2,3-fold) genes (Fig. 18), which was GTP dependent, as both the TCA cycle and respiration were mostly unaffected in the *guaBA* negative background (Fig. 18). Downregulation of the TCA cycle and respiration may account for the reduced membrane potential observed during stationary phase starvation in the (p)ppGpp^0 mutant (Fig. 17A).

6.2.8 The sensitivity of *qoxABCD* expression to GTP determines the activity of the electron transport chain and PMF

Since the transcriptome analysis of the (p)ppGpp^0 mutant revealed not only downregulation of the TCA cycle but also strongly decreased expression of the terminal oxidase gene *qoxABCD*, a component of the electron transport chain (ETC), we hypothesized that these transcriptional changes lead to decreased PMF.

We verified the RNA-seq data by RT–qPCR and confirmed decreased *qoxA* expression in (p)ppGpp^0 but increased expression in the *guaBA*-negative strain compared to wildtype (Fig. 19B). Expression of rRNA is controlled by the concentration of the transcription-initiating nucleotide (iNTP) within the promoter [53]. Analysis of potential transcriptional start sites upstream of *qoxABCD* predict an ATP as the transcription-initiating nucleotide (based on data from [129]) (Fig. 18). Thus, we mutated the TSS+1 to a GTP in wildtype and (p)ppGpp^0 (named WT PqoxABCD$_{mut}$ and (p)ppGpp^0 PqoxABCD$_{mut}$) (Fig. 19A). This mutated TSS+1 increased *qoxA* expression in the late stationary phase in (p)ppGpp^0, indicating that this promoter is nucleotide sensitive (Fig. 19B). Alteration of the iATP to iGTP also resulted in slightly greater CFU counts than those of (p)ppGpp^0 (Fig. 19C). However, culturability was still lower compared to wildtype, showing that additional factors are likely to contribute to entry into dormancy.

To further demonstrate the importance of the PMF for stationary phase survival, we treated wildtype and (p)ppGpp^0 cultures with sub-inhibitory concentrations of valinomycin during the late stationary phase and evaluated survival after 24 h. Valinomycin is an ionophore that disrupts membrane potential by selectively translocating potassium ions across the membrane. Upon valinomycin treatment, wildtype and (p)ppGpp^0 showed similar reductions in CFU counts compared to untreated cultures (Fig. 19D). Thus, disruption of membrane potential indeed compromises culturability and survival.

Together, these findings illustrate the significance of preserving PMF, particularly $\Delta\Psi$, throughout stationary phase starvation to sustain culturability.

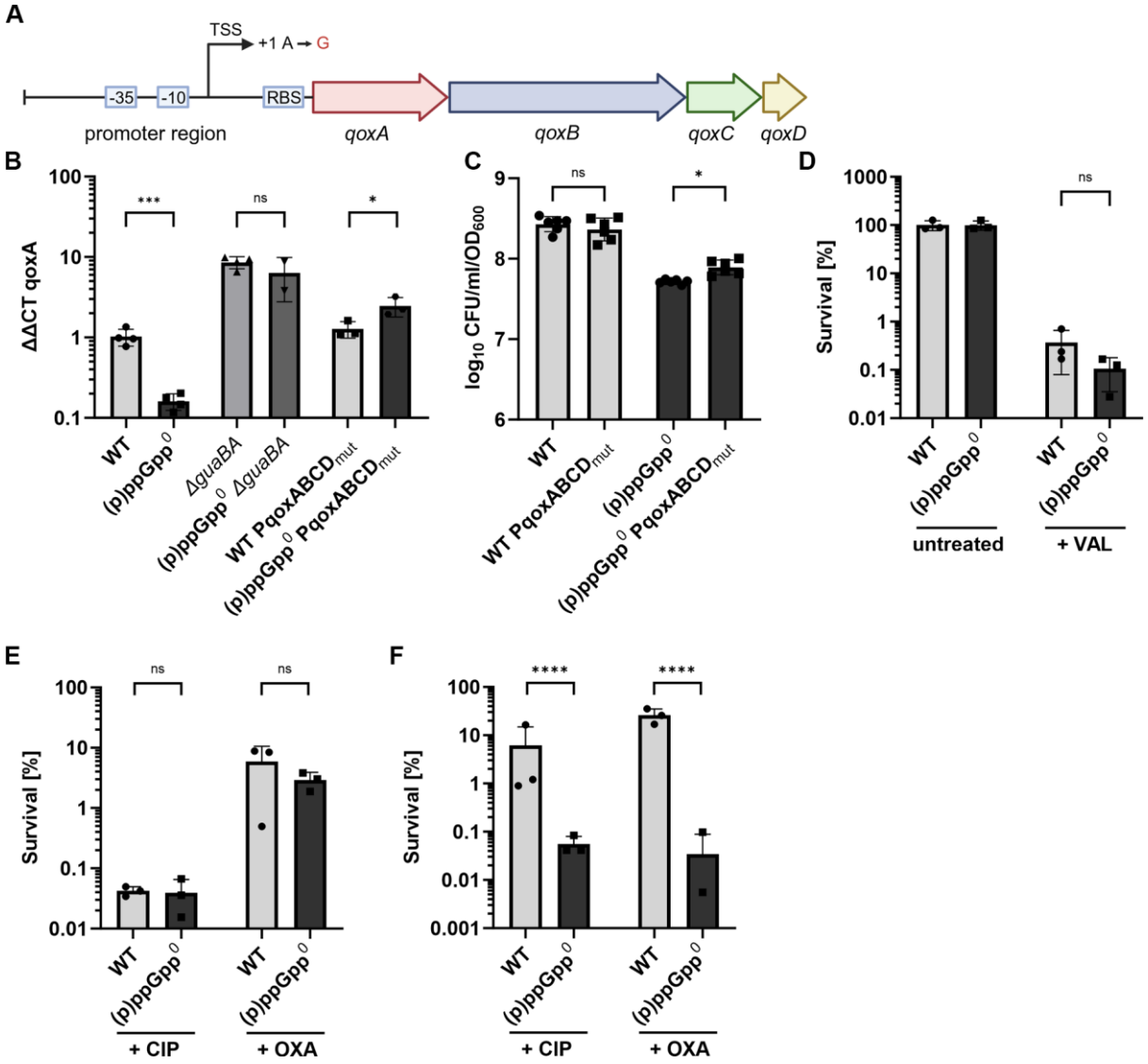

Fig. 19 Decrease in ETC activity contributes to entrance into dormant state and decreased antibiotic tolerance. (A) *qoxABCD* gene operon with promoter region. The predicted initiation nucleotide (TSS +1) iATP was mutated to iGTP. (B) Bacterial cells were harvested during the late stationary growth phase and total RNA was isolated. qoxA transcript levels were determined by RT-qPCR and normalized to *gyrB* expression by ΔΔCt method. Data are shown as mean ± SD (n=3 biological replicates). Statistical significance was determined by two-tailed unpaired t-tests (***p-value <0.001, *p-value <0.05, ns >0.05). (C) Culturability during late stationary phase (24h) was determined by CFU enumeration and normalized to OD600. Data shown are mean of ± SD (n=6 biological replicates from two individual experiments). Statistical significance was determined by a one-way analysis of variance (ANOVA) with Tukey's post-test (*p-value <0.05). (D) Wildtype (WT) and (p)ppGpp^0 cells were treated with a sub-inhibitory concentration of valinomycin (20 µM) during the late stationary growth phase (24h) and viability was assessed by CFU/ml enumeration after 24h of treatment. Survival was calculated in comparison to untreated samples. Data shown are mean of ± SD (n=3 biological replicates). Statistical significance was determined by a two-way analysis of variance (ANOVA) with Šidák's post-test (ns >0.05, based). (E) Mid-exponential phase or stationary phase (F) cells were treated with 100x MIC ciprofloxacin (+ CIP) or oxacillin (+ OXA) and survival was calculated after 3h (E) or 24h (F) of treatment in comparison to untreated cells. Data shown are mean of ± SD (n=3 biological replicates). Statistical significance was determined by a two-way analysis of variance (ANOVA) with Šidák's post-test (****p-value <0.0001, ns >0.05).

6.2.9 Preservation of PMF during starvation is crucial for antibiotic tolerance

It has been demonstrated that maintenance of PMF is essential for the survival of starvation-induced tolerant bacteria [130, 131]. We sought to determine whether the decreased membrane potential in the (p)ppGpp^0 mutant would also result in a reduction of starvation-induced antibiotic tolerance. Therefore, we cultured wildtype and (p)ppGpp^0 cells to exponential or stationary growth phase and exposed them to either 100x MIC oxacillin or ciprofloxacin. No significant difference in antibiotic survival between wildtype and (p)ppGpp^0 cells was observed when treated during exponential growth (Fig. 19E). However, wildtype cells were barely killed by the antibiotics during the stationary phase, confirming the higher antibiotic tolerance of non-growing bacteria [132]. Instead, both antibiotics efficiently decreased the survival of the (p)ppGpp^0 mutant during the stationary phase (Fig. 19F).

7 Discussion

The discussion presented here is part of the accepted research article “Small alarmone synthetases RelP and RelQ of *Staphylococcus aureus* are involved in biofilm formation and maintenance under cell wall stress conditions” and the submitted manuscript “(p)ppGpp-mediated GTP homeostasis ensures the survival and antibiotic tolerance of *Staphylococcus aureus* during starvation by preserving the proton motive force”. The manuscript has been uploaded to the preprint server bioRxiv and might differ from the final published article. The accepted research article and the submitted manuscript can be accessed under the following links:

- https://doi.org/10.1101/2024.02.06.579068
- https://doi.org/10.3389/fmicb.2020.575882

7.1 The role of (p)ppGpp in biofilm formation and antibiotic tolerance in *Staphylococcus aureus*

The small alarmone synthetases RelP and RelQ maintain the biofilm-forming capacity of *S. aureus* when exposed to sub-inhibitory concentrations of vancomycin. Both enzymes are part of the cell wall stress regulon and are transcriptionally induced by vancomycin [27]. They act synergistically, and the *relPQ* double mutant can be complemented via the chromosomal integration of either *relP* or *relQ*. Thus, the (p)ppGpp synthesis expected to occur upon vancomycin treatment supports biofilm growth, whereas without (p)ppGpp, no biofilms are formed in the presence of vancomycin. How (p)ppGpp promotes biofilm formation remains to be elucidated.

(p)ppGpp synthesis results in an immediate decrease in intracellular GTP, leading to the de-repression of the CodY regulon [16]. CodY, when loaded with GTP and/or branched-chain amino acids, represses many metabolism-related genes, the Agr system and *ica* gene expression [56, 133]. The impact of CodY on biofilm formation is probably multifactorial and strain dependent [120, 134]. *codY* mutants have also been reported to aggregate, which may be linked with the interaction of PIA and eDNA on the bacterial surface [135]. However, the involvement of CodY regulation in the observed biofilm maintenance can be excluded, as biofilm formation was not affected in a *codY*-negative background. Furthermore, the study excluded the Agr quorum-sensing system and, thus PSMs (which are strongly dependent on Agr activity), as mediators of the biofilm phenotype. Thus, the main mechanism for biofilm formation under vancomycin stress remains unclear. (p)ppGpp dependent DNA-release by any of the lytic processes (e.g. autolysins, phages) likely contribute to biofilm formation.

Recently, ita study demonstrated that mupirocin, a potent inducer of Rel-dependent (p)ppGpp synthesis, leads to increased biofilm formation [92]. Similar to our results, the mupirocin-induced biofilm forms independent of PIA and PSMs and is largely composed of eDNA. Thus, it is likely that the observed biofilm-inducing phenotype induced by mupirocin is similar to the vancomycin-dependent biofilm observed in our study. Jin et al. [136] found that mupirocin upregulates *cidA*, encoding a holin-like protein, and that a *cidA* mutant shows reduced eDNA release. Thus, one may speculate that under our assay conditions, the (p)ppGpp-mediated activation of *cidA* may also contribute to (p)ppGpp-promoted biofilm formation.

7.1.1 (p)ppGpp is required for biofilm-related antibiotic tolerance

The sub-inhibitory concentration of vancomycin applied in our standard biofilm assay did not affect bacterial viability, and the MIC in planktonically grown strains did not differ between the analysed strains. When vancomycin was added to preformed biofilms, the biofilms were protected even at up to a concentration 100-fold higher than the MIC. It has been proposed that antibiotic tolerance and persister formation share common characteristics such as a slow- or non-growing phenotype [137]. Here, we showed that biofilm tolerance is at least partly (p)ppGpp dependent since biofilms of the pppGpp^0 strain were significantly better resolved in the presence of high vancomycin concentrations. This seems to contrast with recent results indicating that (p)ppGpp is not involved in persister formation in *S. aureus* [138]. However, the persister assays were performed under relaxed conditions, and thus, the role of (p)ppGpp might have been missed. Nevertheless, (p)ppGpp synthesis was previously shown to contribute to antibiotic tolerance in *S. aureus* [27, 139] and other pathogens [140, 141]. Nguyen et al. [140] suggested that in *Pseudomonas aeruginosa,* the stringent response contributes to antimicrobial tolerance in biofilms by reducing oxidative stress. We recently showed that (p)ppGpp in *S. aureus* activates ROS-detoxifying systems [36], which might contribute to protection against vancomycin.

Due to the role of (p)ppGpp in biofilm formation and antibiotic tolerance, the (p)ppGpp synthesis pathway is thought to be a promising antimicrobial target. Anti-biofilm peptides have been reported to exert their activity via their ability to reduce (p)ppGpp levels in live bacterial cells [78, 94]. A direct mechanism of action involving the binding of (p)ppGpp and promotion of its intracellular degradation was suggested [94]. We confirmed the biofilm-dissolving effect of DJK-5. However, this was not due to the proposed interaction of the peptides with (p)ppGpp because an even stronger inhibitory effect of DJK-5 was observed in the (p)ppGpp^0 mutant. Interestingly, treatment with DJK-5 and a sub-inhibitory vancomycin concentration resulted in significantly higher bacterial killing activity and biofilm inhibition in the (p)ppGpp^0 mutant than the wildtype. Thus, (p)ppGpp protects against bactericidal DJK-5 activity. These findings are in good agreement with a recent re-analysis of the proposed antibiofilm peptide IDR-1018 in

E. coli and *P. aeruginosa* [142]. Genetic disruption of the *relA* and *spoT* genes responsible for (p)ppGpp synthesis moderately sensitizes *E. coli* to IDR-1018, rather than protecting the bacterium[142]. While the IDR-1018 and DJK-5 peptides are potent antimicrobials, they do not specifically disrupt biofilms via direct and specific interaction with the intracellular messenger nucleotide (p)ppGpp. Their alternative mode of action remains to be elucidated. In conclusion, in *S. aureus,* (p)ppGpp supports biofilm formation under cell wall stress conditions and increases tolerance against vancomycin and the anti-biofilm peptide DJK-5.

7.2 The role of (p)ppGpp-mediated GTP homeostasis during starvation for survival and antibiotic tolerance of *Staphylococcus aureus*

In the second part of this thesis, I could show that bacterial culturability in the stationary phase is dependent on (p)ppGpp-driven GTP depletion. While (p)ppGpp is not required for growth and survial during nutrient-rich conditions in the exponential growth phase, we initially observed a decrease in survival in the stationary phase of an *S. aureus* HG001 (p)ppGpp^0 mutant, as determined by CFU counts. As previous studies in *B. subtilis* and *S. aureus* have shown [54, 65, 66], guanine nucleotide levels were significantly elevated during nutrient starvation. The mechanism that leads to cell death induced by GTP dysregulation is not yet fully understood. Therefore, this study focused on characterizing the mechanisms that lead to cell death. Instead, we found that the *S. aureus* (p)ppGpp^0 mutant enters a dormancy state with strongly reduced culturability and metabolism rather than experiencing cell death. While the permeability of the cell membrane was unaffected, we observed decreased membrane potential and alterations in the cell membrane architecture of the (p)ppGpp^0 strain. Uncontrolled, high levels of GTP in the (p)ppGpp^0 mutant decrease membrane potential, at least in part, via transcriptional downregulation of nucleotide-sensitive genes of the TCA cycle and the respiratory chain. Maintenance of respiratory chain activity by (p)ppGpp/low GTP levels is also responsible for the tolerance of stationary phase cells to bactericidal antibiotics. By increasing the activity of the electron transport chain (increased expression of complex IV (*qoxABCD*) by mutating the TSS+1), we were able to partially restore the culturability of the (p)ppGpp^0 mutant under nutritional stress.

In summary, (p)ppGpp is essential for maintaining GTP homeostasis under nutritional stress and thereby preserve electron transport chain activity and PMF to ensure culturability of *S. aureus*. (Fig. 20).

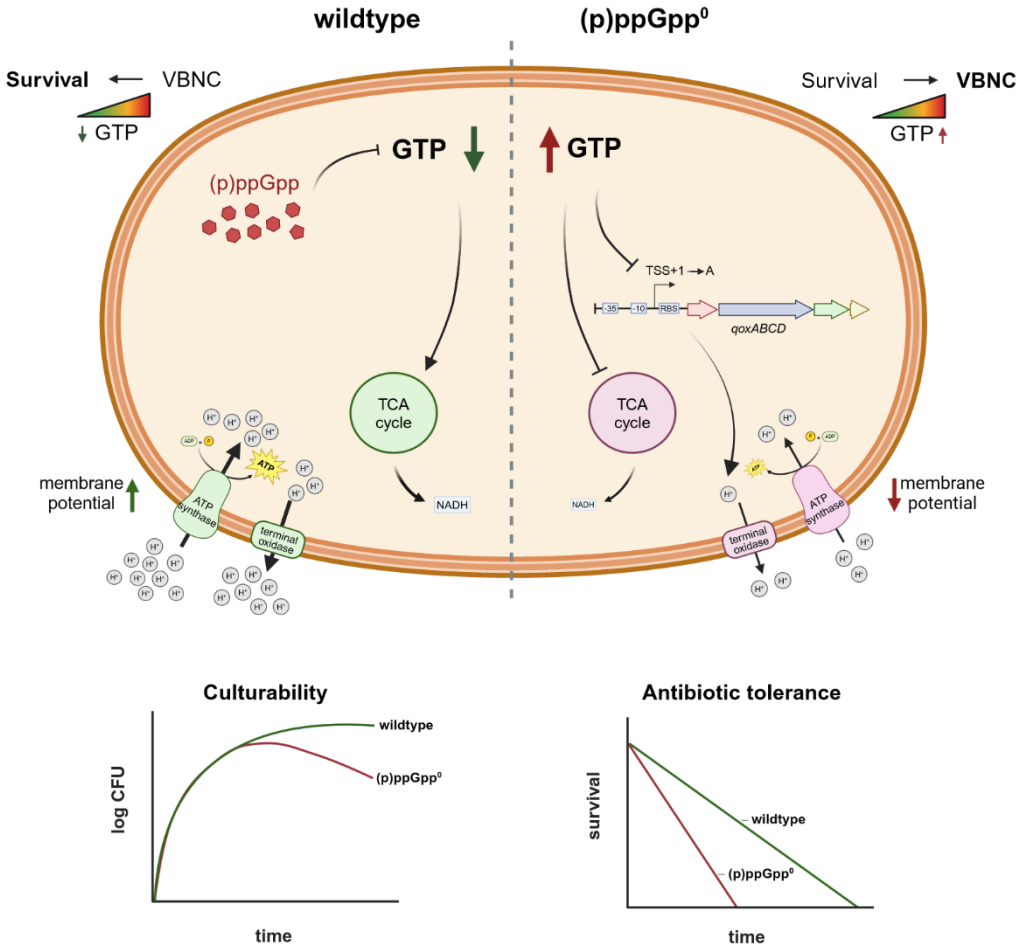

Fig. 20 (p)ppGpp-dependent regulation of GTP homeostasis enables maintenance of culturability. During stationary phase starvation, (p)ppGpp synthesis contributes to regulation of intracellular GTP levels and thereby maintains membrane potential and ensures culturability and survival during antibiotic treatment. In a (p)ppGpp-deficient mutant, GTP homeostasis is disturbed leading to decreased expression of the TCA cycle and electron transport chain (ETC) genes including the terminal oxidase *qoxABCD*. This in turn leads to a decreased membrane potential, which causes entrance into a division-incompetent but viable (VBNC) state, accompanied by reduced antibiotic tolerance.

7.3 The (p)ppGpp-dependent GTP homeostasis determines long-term bacterial culturability

The defect in culturability of the (p)ppGpp^0 mutant is clearly linked to an uncontrolled increase in GTP levels upon entry into the stationary phase. Culturability correlated inversely with changes in the GTP level induced by deletion of *purR* or *guaAB* or by guanine feeding. Mutation of *guaAB* rescued the defect in the (p)ppGpp^0 mutant. A high guanine concentration did not interfere with the survival of wildtype, indicating that (p)ppGpp can also control GTP levels provided by the salvage pathway. Direct (p)ppGpp-dependent alterations in translation or replication machinery [12, 64] are likely to also be less prominent in a *guaAB*-negative background. Thus, we conclude that the level of GTP homeostasis is the main determinant of bacterial culturability in stationary phase cells.

Control of guanine metabolism by (p)ppGpp is conserved in several Firmicutes species, such as *B. subtilis*, [54], *E. faecalis* [39] and *S. aureus* [55]. Recently, reduced long-term survival of the *S. aureus* USA300 (p)ppGpp^0 mutant [66] was linked to disrupted GTP homeostasis since suppressor mutants with alterations in *gmk* were selected during long-term culture. In *B. subtilis*, low GTP levels reduce the growth rate but promote survival during amino acid starvation [54, 65] and cell viability during fatty acid starvation [24]. Elevated GTP levels in a (p)ppGpp^0 mutant of *B. subtilis* promoted the bactericidal effect of otherwise bacteriostatic antibiotics [26].

7.4 Disturbance of the GTP homeostasis during nutrient stress leads to induction of a VBNC state

Previously, it was proposed that (p)ppGpp deletion in *B. subtilis* results in “death-by-GTP” and that this cell death might be due to unrestricted translation [54]. However, in *S. aureus*, uncontrolled translation could not be linked to diminished bacterial survival since translation inhibitors did not restore culturability of the (p)ppGpp^0 mutant. Furthermore, our results do not support the hypothesis that high GTP levels trigger a classical “cell death” programme. The (p)ppGpp^0 mutant of *S. aureus* was nonculturable but negative for propidium iodide uptake and maintained basal metabolic activity and transcription, albeit with significantly reduced ATP levels. This is indicative of the previously defined state of VBNC (viable but nonculturable) or a deep dormant-like state [143]. It was proposed that ATP depletion is involved mainly in formation of VBNC state cells [125, 144], in which intracellular energy (ATP) is the main factor maintaining the culturability of bacteria on solid media. In some gram-negative bacteria, (p)ppGpp was shown to prevent VBNC state formation in response to certain stress conditions [145, 146]. In (p)ppGpp^0 mutants of *B. subtilis*, fatty acid starvation leads to membrane rupture and, consequently, cell death [24]. The authors proposed a model whereby during fatty acid starvation, a (p)ppGpp-dependent decrease in GTP levels halts growth, preventing collapse of membrane potential and loss of membrane integrity. However, nonculturable *B. subtilis* (p)ppGpp^0 mutants died, as indicated by propidium iodide staining [24, 26]. Thus, there might be major differences between GTP-dependent cell death pathways in *S. aureus* and *B. subtilis*.

7.5 How do elevated GTP levels lead to VBNC state formation?

The RNAseq analysis revealed that TCA cycle genes and genes coding for major components of the electron transport chain, including the terminal oxidase (*qoxABCD*) and the F_0F_1 ATP synthase, were significantly downregulated in the (p)ppGpp^0 mutant during the stationary phase. Accordingly, ATP levels, the NADH/NAD$^+$ ratio and membrane potential were decreased in the (p)ppGpp^0 mutant. In Firmicutes, (p)ppGpp-dependent transcriptional changes are regulated mainly by the GTP-dependent transcriptional repressor CodY [27] or

by the availability of the NTP substrate for transcription initiation [53, 55, 147]. We could exclude any influence of CodY on induction of the VBNC state. We mutated the predicted initiation of NTP transcription in the promoter region of *qoxABCD,* the most prominently downregulated component of the electron transport chain. By using this approach, we were able to restore wildtype levels of *qoxA* expression in the (p)ppGpp^0 mutant. Furthermore, the culturability of the (p)ppGpp^0 mutant was partially restored by this single nucleotide exchange. Expression of other genes involved in respiration and generation of membrane potential was downregulated in the (p)ppGpp^0 mutant, which were also initiated with iATP; these genes may further contribute to the observed phenotype.

7.6 PMF maintenance during starvation is crucial for antibiotic tolerance

I could show that a (p)ppGpp^0 mutant grown to the stationary phase lost antibiotic tolerance to the β-lactam oxacillin and the fluoroquinolone antibiotic ciprofloxacin. Non-growing wildtype bacteria are tolerant to these antibiotics, as shown previously [132]. However, Conlon et al. did not find evidence that (p)ppGpp is involved in antibiotic persistence or tolerance. The authors proposed that ATP depletion is important for the observed tolerance of stationary phase cells [132]. This seems to contrast our findings that (p)ppGpp-dependent maintenance of low GTP/high ATP levels protected the cells. This discrepancy might be explained by the experimental design. The growth medium used by Conlon et al. [132] likely does not induce a stringent response and/or might contain low amounts of guanine. Thus, under these conditions, the intracellular GTP pool might not be elevated in the (p)ppGpp^0 mutant. In agreement with our results, previous studies have shown that PMF is essential for starvation-induced tolerance in both gram-negative and gram-positive bacteria, including S*. aureus* [130, 131, 148]. Wan et. al propose a model in which maintaining a basic PMF level upon encountering starvation is required to fuel efflux activities of β-lactam antibiotics and establish a tolerance phenotype. However, no effect of (p)ppGpp deficiency on antibiotic tolerance could be observed during exponential growth phase confirming previous results for antibiotic tolerance towards ciprofloxacin from our group [27]. Further, GTP depletion was shown to be a key metabolic effector of antibiotic persistence in *B. subtilis* [149] supporting our theory that actually elevated GTP levels might negatively affect tolerance to antibiotics. Hence, these findings support the idea of applying PMF inhibitors to combat antibiotic-tolerant bacteria, including *S. aureus* [150, 151].

8 Conclusion and future perspectives

This work investigated the role of (p)ppGpp synthesis and the stringent response during the biofilm and non-growing lifestyles of *S. aureus*, as well as their importance for antibiotic tolerance under these conditions.

The synthesis of (p)ppGpp in *S. aureus* is crucial for biofilm formation and biofilm-mediated antibiotic tolerance, particularly under cell wall stress conditions. Although the biofilm-dispersing peptide DJK-5 did not show a (p)ppGpp-inhibiting effect, it exhibited strong activity in eradicating biofilm and inhibiting biofilm formation in an *in vitro* biofilm assay. In addition, the combination of the anti-biofilm peptide and vancomycin demonstrated bactericidal activity against *S. aureus* biofilms, making it a promising therapeutic approach for combating biofilm-related persistent infections.

Furthermore, it could be demonstrated that (p)ppGpp-mediated GTP homeostasis is crucial for survival and culturability during nutrient starvation. By transcriptional regulation of vital metabolic pathways (TCA cycle and oxidative phosphorylation), (p)ppGpp is required to maintain protonmotive force and cell membrane function of *S. aureus* to ensure culturability under adverse environmental conditions. Furthermore, it has been demonstrated that preserving PMF during a non-growing state is crucial for the development of starvation-induced antibiotic tolerance. These results represent a significant advancement in comprehending the molecular foundation of bacterial tolerance to bactericidal antibiotics during metabolic shutdown in the human pathogen *S. aureus*. Therefore, disrupting bacterial PMF could be an effective strategy for eradicating antibiotic-tolerant bacteria.

9 References

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Appendix

Accepted publications

1. **Small alarmone synthetases RelP and RelQ of *Staphylococcus aureus* are involved in biofilm formation and maintenance under cell wall stress conditions**

   Andrea Salzer, Daniela Keinhörster, Christina Kästle, Benjamin Kästle and Christiane Wolz

   Front Microbiol. 2020 Sep 18;11:575882. doi: 10.3389/fmicb.2020.575882.

2. **Inducible expression of (pp)pGpp synthetases in *Staphylococcus aureus* is associated with activation of stress response genes**

   Petra Horvatek, Andrea Salzer, Andrew Magdy Fekry Hanna, Fabio Lino Gratani, Daniela Keinhörster, Natalya Korn, Marina Borisiova, Christoph Mayer, Dominik Rejman, Ulrike Mäder and Christiane Wolz

   PLoS Genet. 2020 Dec 30;16(12):e1009282. doi: 10.1371/journal.pgen.1009282.

3. **Role of (p)ppGpp in antibiotic resistance, tolerance, persistence and survival in Firmicutes**

   Andrea Salzer and Christiane Wolz

   Microlife. 2023 Mar 11;4:uqad009. doi: 10.1093/femsml/uqad009.

Submitted manuscripts

1. **(p)ppGpp-mediated GTP homeostasis ensures survival and antibiotic tolerance of *Staphylococcus aureus* during starvation by maintaining the proton motive force**

   Andrea Salzer, Sophia Ingrassia, Lisa Sauer, Johanna Rapp, Ronja Dobritz, Jennifer Müller, Hannes Link and Christiane Wolz

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Small Alarmone Synthetases RelP and RelQ of *Staphylococcus aureus* Are Involved in Biofilm Formation and Maintenance Under Cell Wall Stress Conditions

*Andrea Salzer, Daniela Keinhörster, Christina Kästle, Benjamin Kästle and Christiane Wolz**

Interfaculty Institute of Microbiology and Infection Medicine, University of Tübingen, Tübingen, Germany

The stringent response is characterized by the synthesis of the alarmone (p)ppGpp. The phenotypic consequences resulting from (p)ppGpp accumulation vary among species, and for several pathogenic bacteria, it has been shown that the activation of the stringent response strongly affects biofilm formation and maintenance. In *Staphylococcus aureus*, (p)ppGpp can be synthesized by the RelA/SpoT homolog Rel upon amino acid deprivation or by the two small alarmone synthetases RelP and RelQ under cell wall stress. We found that *relP* and *relQ* increase biofilm formation under cell wall stress conditions induced by a subinhibitory vancomycin concentration. However, the effect of (p)ppGpp on biofilm formation is independent of the regulators CodY and Agr. Biofilms formed by the strain HG001 or its (p)ppGpp-defective mutants are mainly composed of extracellular DNA and proteins. Furthermore, the induction of the RelPQ-mediated stringent response contributes to biofilm-related antibiotic tolerance. The proposed (p)ppGpp-inhibiting peptide DJK-5 shows bactericidal and biofilm-inhibitory activity. However, a non-(p)ppGpp-producing strain is even more vulnerable to DJK-5. This strongly argues against the assumption that DJK-5 acts via (p)ppGpp inhibition. In summary, RelP and RelQ play a major role in biofilm formation and maintenance under cell wall stress conditions.

Keywords: stringent response, (p)ppGpp, biofilm, *Staphylococcus aureus*, cell wall stress

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****Correspondence:***
Christiane Wolz
christiane.wolz@uni-tuebingen.de

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INTRODUCTION

Biofilms are sessile microbial communities attached to surfaces and embedded in an extracellular matrix. Biofilm-forming staphylococci cause many device-related or chronic infections (Kong et al., 2006; O'Gara, 2007; Speziale et al., 2014; Mccarthy et al., 2015; Paharik and Horswill, 2016; Figueiredo et al., 2017; Moormeier and Bayles, 2017; Arciola et al., 2018; Otto, 2018). Depending on the composition of the biofilm matrix, staphylococcal biofilms are classified as *ica*-dependent or *ica*-independent (O'Gara, 2007). *Ica*-dependent biofilms are characterized by polysaccharide intercellular adhesin (PIA) also known as poly-N-acetyl glucosamine (PNAG), which is synthesized by enzymes encoded by the *icaADBC* operon (Götz, 2002). In *ica*-independent biofilms, proteins

(Speziale et al., 2014) and extracellular nucleic acids (eDNA) (Moormeier and Bayles, 2017) are the main matrix components. Biofilm formation is a three-step process that includes initial attachment to the surface, biofilm maturation due to intercellular aggregation and bacterial cell detachment. Detachment is mediated by the enzymatic degradation of matrix components by proteases, nucleases or a group of small amphiphilic α-helical peptides, known as phenol-soluble modulins (PSMs) (Otto, 2018).

The nature and extent of biofilms are highly variable between different strains and growth conditions. Mounting evidence suggests that subinhibitory antibiotic concentrations can promote biofilm formation (Rachid et al., 2000; Kaplan et al., 2012; Liu et al., 2018; Ranieri et al., 2018; Jin et al., 2020) by inducing eDNA release and thus shifting the composition of the biofilm matrix toward a higher eDNA content (Brunskill and Bayles, 1996; Mlynek et al., 2016; Schilcher et al., 2016). Multiple regulatory mechanisms are involved in the molecular switch from a planktonic to a biofilm lifestyle, such as transcriptional regulation via SarA, SaeRS, CodY or the quorum-sensing system Agr (Kong et al., 2006; Boles and Horswill, 2008; Beenken et al., 2010; Stenz et al., 2011; Cue et al., 2012; Mrak et al., 2012; Abdelhady et al., 2014; Atwood et al., 2015; Paharik and Horswill, 2016).

For several bacterial species, it has been demonstrated that the activation of the stringent response also affects biofilm formation (Balzer and Mclean, 2002; Taylor et al., 2002; Lemos et al., 2004; Aberg et al., 2006; Chavez De Paz et al., 2012; He et al., 2012; Sugisaki et al., 2013; De La Fuente-Nunez et al., 2014; Azriel et al., 2016; Xu et al., 2016; Diaz-Salazar et al., 2017; Liu et al., 2017; Colomer-Winter et al., 2018, 2019). The stringent response is characterized by the synthesis of guanosine-tetra-phosphate (ppGpp) and guanosine-penta-phosphate (pppGpp), collectively referred to as (p)ppGpp. The accumulation of (p)ppGpp affects gene expression, protein translation, enzyme activation and replication (Wu and Xie, 2009; Liu et al., 2015; Steinchen and Bange, 2016; Bennison et al., 2019). In many pathogenic bacteria, (p)ppGpp determines virulence or antibiotic tolerance/persistence (Dalebroux et al., 2010; Hauryliuk et al., 2015; Harms et al., 2016; Wood and Song, 2020). *Staphylococcus aureus* harbors three genes encoding (p)ppGpp synthetases, Rel, RelP, and RelQ (Wolz et al., 2010). The (p)ppGpp synthetase activity of the bifunctional Rel enzyme can be induced by tRNA-synthetase inhibitors such as mupirocin or serine-hydroxamate or by amino acid deprivation (Geiger et al., 2010). Rel usually shows strong hydrolase activity, which is essential to detoxify (p)ppGpp produced by RelP or RelQ (Gratani et al., 2018). RelP and RelQ only contain a synthase domain (Geiger et al., 2014) and are part of the VraRS cell wall stress regulon (Kuroda et al., 2003). Thus, they are transcriptionally induced (e.g., after vancomycin treatment) and contribute to tolerance toward cell wall-active antibiotics such as ampicillin or vancomycin (Geiger et al., 2014).

There is some evidence that the stringent response might trigger biofilm formation in *S. aureus* based on the observation that treatment with mupirocin (Sritharadol et al., 2018; Jin et al., 2020) or serine hydroxamate (De La Fuente-Nunez et al., 2014) results in increased biofilm formation. Moreover, the anti-biofilm peptides IDR-1018 and DJK-5 have been suggested to directly interact with (p)ppGpp, preventing its signaling effects and, thus, biofilm formation (De La Fuente-Nunez et al., 2014, 2015).

Here, we aimed to investigate the role of the stringent response mediated by the two small alarmone synthetases RelP and RelQ in biofilm formation. Under cell wall stress conditions induced by a subinhibitory vancomycin concentration, both RelP and RelQ are crucial for biofilm development. Moreover, (p)ppGpp synthesis prevents biofilm eradication by vancomycin. (p)ppGpp-mediated biofilm formation was shown to be independent of the major stringent response mediator CodY and the main biofilm regulator Agr. The anti-biofilm peptide DJK-5 could prevent biofilm formation of wild type and (p)ppGpp-defective mutants. However, the (p)ppGpp^0 strain is even more vulnerable to DJK-5. Thus, DKJ-5 does not act on biofilm formation via (p)ppGpp inhibition.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

The strains and plasmids used in this study are listed in **Supplementary Table S1**. For overnight cultures, the strains were grown with appropriate antibiotics (10 μg/ml erythromycin, 100 μg/ml spectinomycin) at 37°C and 200 rpm. For biofilm analyses, the following media were used: chemically defined medium (CDM; Pohl et al., 2009), tryptic soy broth (TSB, Oxoid) with 3% NaCl and 0.5% glucose, and BM2 glucose [0.4% (w/v) glucose, 62 mM potassium phosphate buffer, pH 7.0, 7 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 mM $FeSO_4$, 0.5% casamino acids] (De La Fuente-Nunez et al., 2014).

Strain Construction

For *relP* complementation, the pCG833 plasmid was constructed. The complete *relP* operon with its native promoter was amplified by PCR using the primers pCG833gibfor and pCG833gibrev and cloned via Gibson assembly into the BamHI-digested integration vector pCG3. The oligonucleotides used in these procedures are listed in **Supplementary Table S2**. Due to showing toxicity in *E. coli*, the plasmid was directly transformed into *S. aureus* Cyl316 by electroporation. The integration of the plasmid into the genome was verified by PCR using the scv1, scv21, pCG3intfor and pCG3intrev oligonucleotides followed by sequencing. The integrated plasmid was transduced into the target strains using Φ11 phage.

For *relQ* complementation, the pCG216 plasmid was constructed. *relQ* with its native promoter was amplified by PCR with the primers BamHrelQkompl-for and BamHrelQkomp-rev and ligated with the BamHI-digested pCG3 vector backbone. The plasmid was verified by PCR with relQdigfor and relQdigrev. The plasmid was transformed into Cyl316 and verified by PCR using the primers relQfor and relQrev for integration of the plasmid. The integrated plasmid was then transduced via phage Φ11 in the target strains.

codY and *agr* mutations were transduced into the (p)ppGpp strain using the lysates of RN4220-21 (Pohl et al., 2009) and RN6911 (Kornblum et al., 1990), respectively.

Colony-Forming Unit (CFU) and Minimal Inhibitory Concentration (MIC) Determination

For CFU measurements, biofilm-grown bacteria (24 h, 37°C) were resuspended by thorough pipetting. The bacterial suspension (biofilm resolved and planktonic) was serially diluted in phosphate-buffered saline (PBS), and 10 µl aliquots were spotted onto TSA plates for CFU determination. The MIC was determined by serial microdilution and *E*-tests.

Biofilm Assay

For the static biofilm assay, 1 ml medium was inoculated in a 24-well polystyrene cell culture plate (Greiner) to obtain an OD_{600} of 0.05. After 24 h of static incubation at 37°C, the wells were washed twice with 1 ml phosphate-buffered saline (PBS, pH 7.4, Gibco). The biofilm was dried at 40°C for 30 min. For biofilm staining, 200 µl of crystal violet (80 µg/ml in distilled water) was added to the wells, followed by incubation at RT for 5 min. The wells were then washed twice with 1 ml distilled water and dried at 40°C for 30 min. Biofilm quantification was performed by A_{600} determination (microplate reader, Tecan Infinite 200 and Tecan Spark). To account for the different distributions of biofilms within a single well, measurements were performed 100 times within one well, and the average was calculated. If required, a sub-inhibitory concentration of vancomycin (0.78 µg/ml) or 5 µg/ml of the anti-biofilm peptide DJK-5 (De La Fuente-Nunez et al., 2015) were added. To test the biofilm eradication capacity, preformed biofilms (37°C, 8 h) were incubated for an additional 16 h in the presence of vancomycin at concentrations ranging from 1 to 100 µg/ml. Biofilm staining and CFU determination were performed as described above.

Biofilm Composition

Biofilms were washed twice with PBS and treated with 1 mg/ml proteinase K (AppliChem, 37°C, 4 h), 0.1 mg/ml DNase (Sigma-Aldrich, 37°C, 4 h) or with 40 mM sodium periodate ($NaIO_4$) (24 h, 4°C). The biofilms were then washed twice with PBS, dried and stained as described above.

RESULTS

(p)ppGpp Synthetases Show no Significant Effect on Biofilm Formation Under Non-stress Conditions

Recently, it was proposed that the stringent response facilitates biofilm formation in several pathogens, including *S. aureus* (De La Fuente-Nunez et al., 2014). However, basal medium 2 (BM2) used in this study allowed hardly any *S. aureus* growth, resulting in a final OD_{600} below 1 after overnight growth. Therefore, we first analyzed the impact of (p)ppGpp on biofilm formation using different media. These media included tryptic soy broth (TSB) with the addition of 0.5% glucose and 3% NaCl, which is widely used for *S. aureus* biofilm analyses (Lade et al., 2019), and CDM (Pohl et al., 2009), used to define the stringent response phenotype in *S. aureus*. For discrimination between the Rel- and RelP/RelQ-mediated stringent response, a rel_{syn} mutant (mutation in the synthetase domain, leaving hydrolase activity unaltered), a *relP, relQ* double mutant and a (p)ppGpp^0 mutant in which all three synthetases were non-functional were included in the analysis. Independent of the (p)ppGpp synthetases, all strains showed the strongest biofilm formation in CDM. Interestingly, in the prototypic biofilm medium TSB, biofilm formation was lower than in CDM (**Figure 1A**). However, no significant difference in biofilm formation was observed between the different strains in either medium. In BM2, a trend toward a slightly higher biofilm-forming capacity in the wild type compared to the mutant strains was observed, indicating that under these nutrient-limited conditions, the stringent response might be slightly activated. Thus, (p)ppGpp synthetases have no or little influence on the biofilm formation ability under non-stressed conditions.

RelP and RelQ Regulate Biofilm Formation Under Cell Wall Stress

Subinhibitory concentrations of vancomycin were shown to transcriptionally activate *relP* and *relQ* (Geiger et al., 2014). We thus speculated that the vancomycin-induced stringent response may interfere with biofilm formation. The minimal inhibitory concentration (MIC) (1 µg/ml) of vancomycin in planktonically grown bacteria did not differ between the analyzed strains. At a subinhibitory concentration of vancomycin (0.78 µg/ml), the *relPQ* double mutant and the (p)ppGpp^0 mutant showed significantly reduced biofilm formation compared to the wild type and the rel_{syn} mutant (**Figures 1B,C**). Under vancomycin treatment, the wild type formed an almost uniform thick layer of biofilm. In contrast, the *relPQ* mutant and the (p)ppGpp^0 strain showed significantly decreased biofilm formation, with some cell aggregates remaining after the washing procedure (**Figures 1B,C**). The bacterial survival of the tested strains was not impaired by the subinhibitory concentrations of vancomycin (**Figure 1D**). Thus, the RelP- and/or RelQ-dependent changes in biofilm formation were not due to growth inhibition or bacterial killing.

RelP and RelQ Synergistically Affect Biofilm Formation

To determine which of the synthetases impacts biofilm formation under vancomycin conditions, single *relP* and *relQ* mutants were analyzed. Both *relP* and *relQ* contributed to biofilm formation in the presence of vancomycin. They act synergistically, since the *relPQ* double mutant showed the lowest biofilm formation (**Figure 2**). The *relPQ* double mutant phenotype could be complemented by the integration of either *relP* or *relQ* into the chromosome (**Figure 2**). Thus, the vancomycin-dependent induction of either *relP* or *relQ* is sufficient to sustain *S. aureus* biofilm formation under cell wall stress conditions.

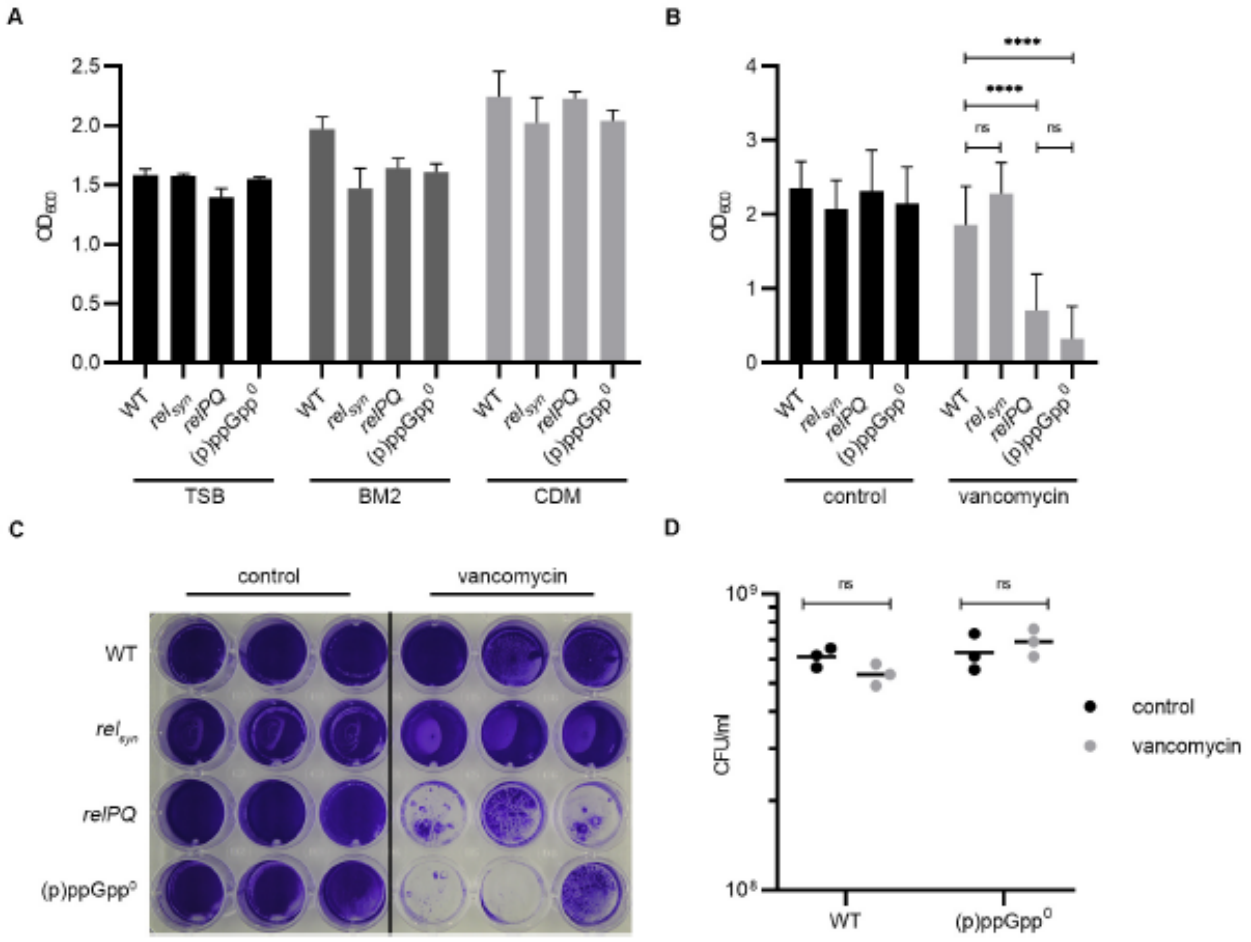

FIGURE 1 | (p)ppGpp synthases effect biofilm formation under cell wall stress conditions. **(A)** Biofilm formation in TSB (+3% NaCl, +0.5% glucose), BM2 and CDM after 24 h. **(B)** Biofilm formation under uninduced and vancomycin-stress (subinhibitory vancomycin 0.78 μg/ml) conditions in CDM after 24 h. Three separate experiments were performed with biological triplicates each. Error bars represent the standard deviation, statistical significance based on ordinary one-way ANOVA (ns: not significant, ****: $P < 0.0001$). **(C)** Representative plate stained with crystal violet. **(D)** CFU was determined from resolved biofilm and planktonic bacteria after 24 h of static incubation.

The Biofilm Composition Is Not Affected by the Stringent Response

The biofilm matrix is composed of PIA, proteins or eDNA (O'Gara, 2007; Paharik and Horswill, 2016). We analyzed which matrix components were involved in the observed RelP/Q-dependent biofilm alterations under vancomycin treatment. Preformed biofilms were treated with sodium periodate, proteinase K or DNase to selectively degrade PIA, proteins or eDNA matrix components, respectively (Seidl et al., 2008). Without vancomycin, the biofilms formed by the wild type or (p)ppGpp^0 stain were almost completely degraded by proteinase K and DNase treatment, whereas sodium periodate had no effect on the biofilm matrix (**Figures 3A,B**). To ensure that vancomycin does not impact the biofilm composition, we additionally examined the matrix components after vancomycin treatment. Again, the biofilms consisted almost exclusively of proteins, and eDNA and sodium periodate treatment did not degrade the biofilm matrix. Thus, under the conditions applied here, HG001 forms *ica*-independent biofilms, and the biofilm composition is not altered by the stringent response.

The Stringent Response Induces Biofilm Formation Independent of Agr and CodY

The quorum-sensing system Agr (especially the target genes *psm*) (Otto, 2018) and the transcriptional regulator CodY (Stenz et al., 2011) have been identified as key controllers of biofilm structure and detachment. (p)ppGpp synthesis results in the derepression of the CodY regulon and upregulation of Agr-dependent *psm* genes (Geiger et al., 2012). Thus, we hypothesized that Agr and/or CodY activity could interfere with the observed (p)ppGpp-dependent biofilm. However, the mutation of *agr* or *codY* did not impact biofilm formation (**Figure 4**). Thus, under our assay conditions, biofilm formation occurs independent of CodY or Agr. Strain specific effects of Agr (Yarwood et al., 2004) or CodY (Stenz et al., 2011) on biofilm formation were described previously.

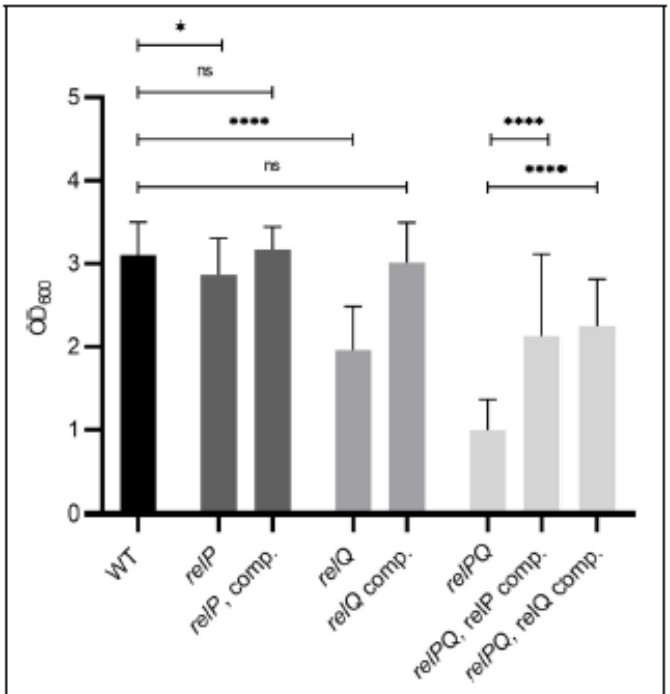

FIGURE 2 | RelP and RelQ affect biofilm formation synergistically. Biofilm formation under vancomycin-stress (0.78 μg/ml vancomycin) in CDM. Strains: HG001 wild type, *relP* and *relQ* single mutants, *relPQ* double mutant and complemented strains. Three separate experiments were performed with biological triplicates each. Error bars represent the standard deviation, statistical significance based on ordinary one-way ANOVA (ns: not significant, *: $P < 0.1$, ****: $P < 0.0001$).

(p)ppGpp Contributes to Biofilm Antibiotic Tolerance

Biofilms are normally more tolerant to high concentrations of antibiotics than planktonic cultures. We hypothesized that the stringent response contributes to biofilm antibiotic tolerance in *S. aureus*. Therefore, biofilm antibiotic tolerance was compared between the wild type and the isogenic (p)ppGpp^0 mutant. Preformed biofilms were exposed to increasing concentrations of vancomycin for 16 h. At the MIC (1 μg/ml for planktonically grown bacteria), vancomycin did not result in biofilm dispersal. However, at concentrations 10- and 100-fold higher than the MIC, the biofilm produced by the (p)ppGpp^0 strain was significantly reduced, whereas the biofilm produced by the wild type was more resistant to vancomycin treatment (**Figures 5A,B**). Thus, (p)ppGpp contributes to biofilm-related antibiotic tolerance.

The Anti-Biofilm Peptide DJK-5 Exerts Its Effects Independent of (p)ppGpp

Recently, the peptides IDR-1018 (De La Fuente-Nunez et al., 2014) and DJK-5 (De La Fuente-Nunez et al., 2015) were proposed to prevent biofilms due to the specific targeting of intracellular (p)ppGpp. If correct, the peptides are expected to inhibit biofilm formation under stringent conditions in the wild type but not in the pppGpp^0 background. We confirmed that DJK-5 interferes with biofilm formation in *S. aureus* (**Figures 6A,B**). However, without vancomycin treatment, biofilm formation by the wild type and pppGpp^0 strains was equally affected by DJK-5, indicating that the effect was independent of (p)ppGpp. The combination of a subinhibitory vancomycin concentration and DJK-5 resulted in the complete inhibition of biofilm formation in the pppGpp^0 strain. This can be explained by bacterial killing of the pppGpp^0 strain through the synergistic action of vancomycin and DJK-5 (**Figure 6C**). Thus, (p)ppGpp synthesis in the wild type obviously protects the strain from the action of DJK-5. These findings are in contrast to the assumption that the biofilm-inhibiting activity of DJK-5 is exerted via (p)ppGpp inhibition, as proposed by De La Fuente-Nunez et al. (2014, 2015).

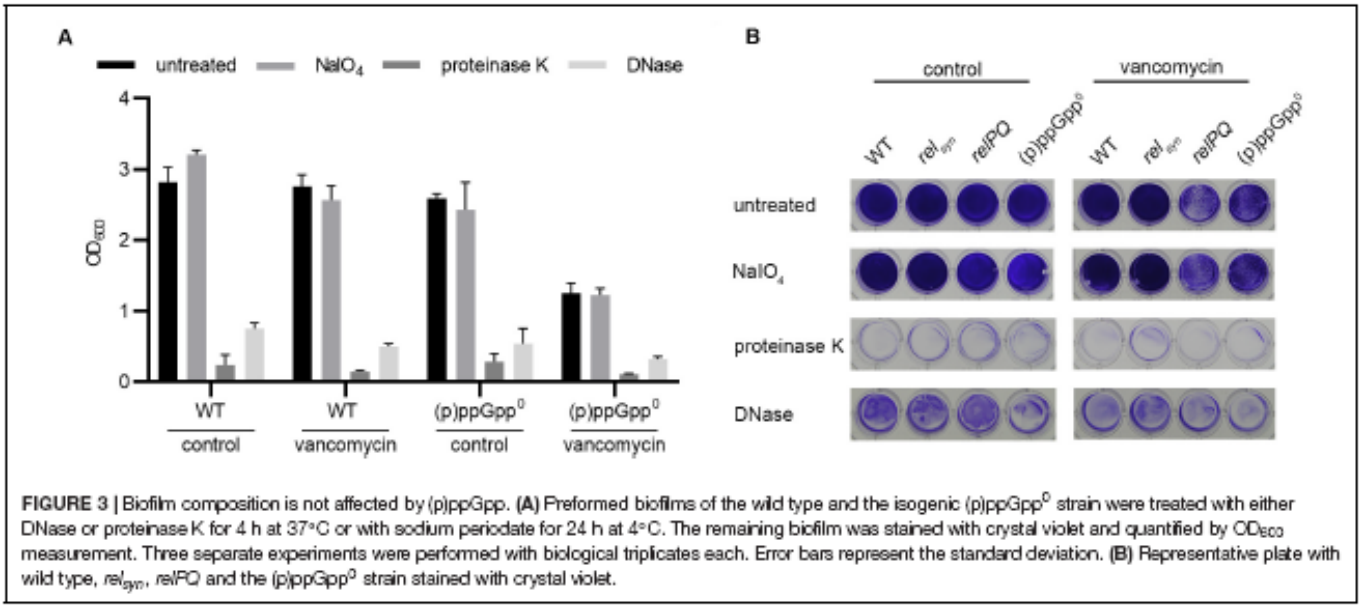

FIGURE 3 | Biofilm composition is not affected by (p)ppGpp. **(A)** Preformed biofilms of the wild type and the isogenic (p)ppGpp^0 strain were treated with either DNase or proteinase K for 4 h at 37°C or with sodium periodate for 24 h at 4°C. The remaining biofilm was stained with crystal violet and quantified by OD_{600} measurement. Three separate experiments were performed with biological triplicates each. Error bars represent the standard deviation. **(B)** Representative plate with wild type, rel_{syn}, *relPQ* and the (p)ppGpp^0 strain stained with crystal violet.

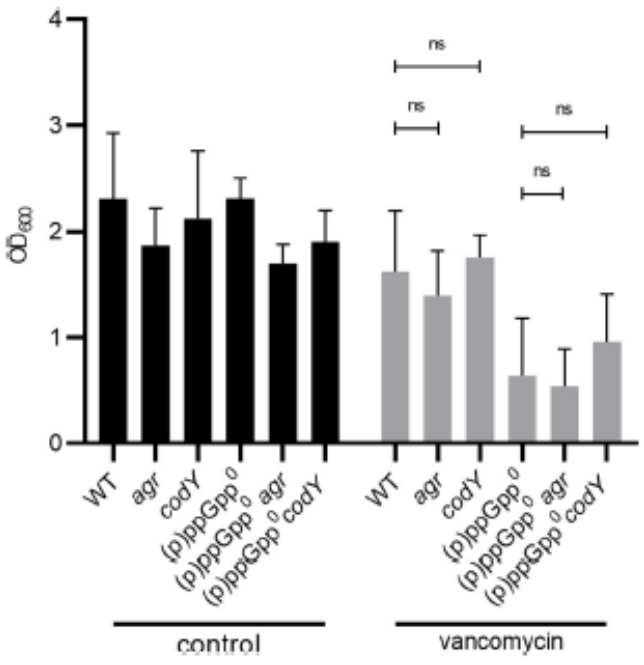

FIGURE 4 | Biofilm formation under stringent conditions is independent of CodY and Agr. Biofilm formation under non-stressed and vancomycin-stress (0.78 μg/ml vancomycin) in CDM, 24 h. Three separate experiments were performed with biological triplicates each. Error bars represent the standard deviation, statistical significance based on ordinary one-way ANOVA (ns: $P > 0.05$).

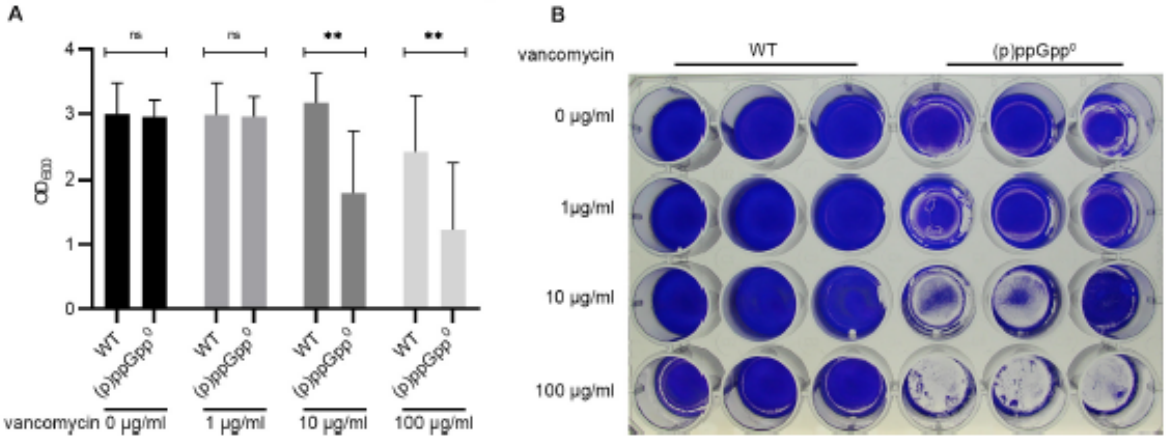

FIGURE 5 | (p)ppGpp contributes to biofilm related antibiotic tolerance. **(A)** Preformed biofilms (8 h) were exposed to increasing concentrations of vancomycin for 16 h. Three separate experiments were performed with biological triplicates each. Error bars represent the standard deviation, statistical significance based on ordinary one-way ANOVA (ns: not significant, **: $P < 0.01$). **(B)** Representative plate stained with crystal violet.

DISCUSSION

Here, we show that the small alarmone synthetases RelP and RelQ maintain the biofilm-forming capacity of *S. aureus* when exposed to subinhibitory concentrations of vancomycin. Both enzymes are part of the cell wall stress regulon and are transcriptionally induced by vancomycin (Geiger et al., 2014). They act synergistically, and the *relPQ* double mutant can be complemented via the chromosomal integration of either *relP* or *relQ*. Thus, the (p)ppGpp synthesis expected to occur upon vancomycin treatment supports biofilm growth, whereas without (p)ppGpp, no biofilms are formed in the presence of vancomycin. How (p)ppGpp promotes biofilm formation remains to be elucidated. (p)ppGpp results in an immediate decrease in intracellular GTP and derepression of the CodY regulon (Geiger et al., 2010). When CodY is loaded with GTP and/or branched-chain amino acids, it represses many metabolism-related genes, the Agr system and *ica* gene expression (Majerczyk et al., 2008; Pohl et al., 2009). The impact of CodY on biofilm formation is probably multifactorial and strain dependent

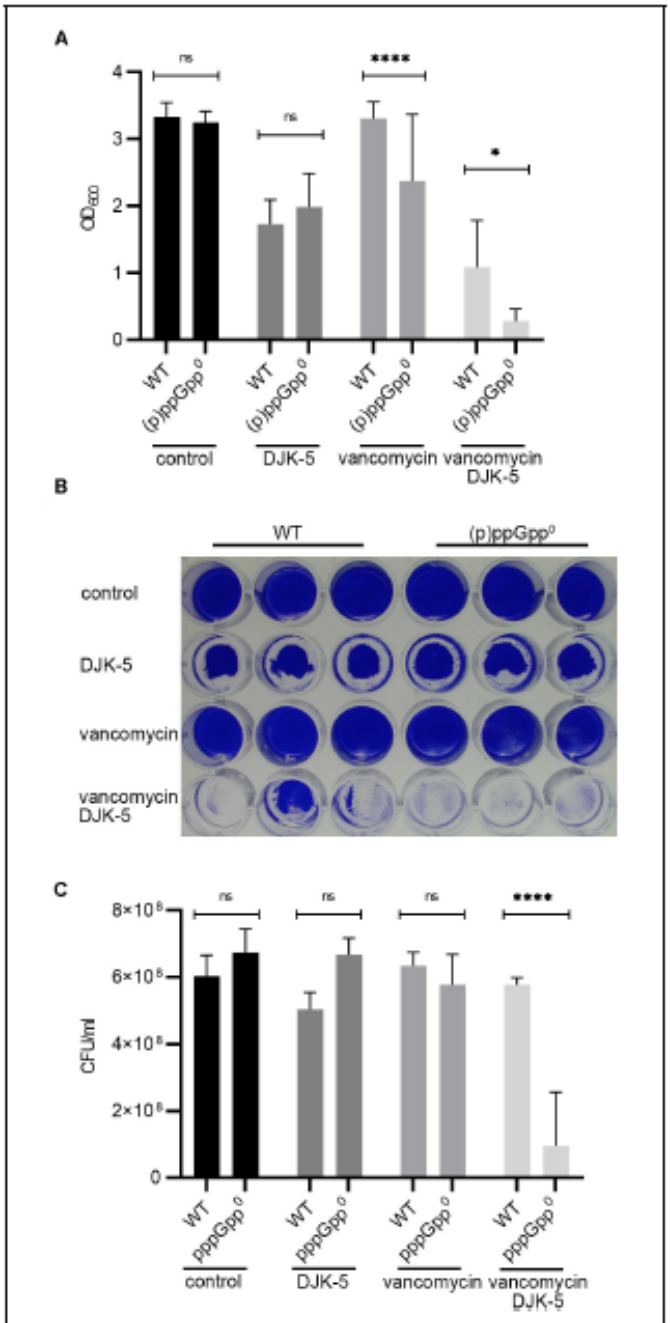

FIGURE 6 | DJK-5 interferes with biofilm formation under relaxed and stringent conditions. **(A)** Biofilms grown with or without vancomycin (0.78 μg/ml) and/or the anti-biofilm peptide DJK-5 (5 μg/ml) in CDM for 24 h. **(B)** Representative plate stained with crystal violet. **(C)** CFU of planktonic and biofilm bacteria determined in parallel after static growth for 24 h. Three separate experiments were performed with biological triplicates each. Error bars represent the standard deviation, statistical significance based on ordinary one-way ANOVA (ns: not significant, *: $P < 0.1$, ****: $P < 0.0001$).

(Stenz et al., 2011; Atwood et al., 2015). *codY* mutants have also been reported to aggregate, which can be linked with the interaction of PIA and eDNA on the bacterial surface (Mlynek et al., 2020). However, we can exclude the involvement of CodY regulation in the observed biofilm maintenance, since biofilm formation was not altered in a *codY*-negative background. Additionally, the Agr quorum-sensing system and, thus PSMs (which are strongly dependent on Agr activity), were excluded as mediators of the biofilm phenotype. Thus, the main mechanism for biofilm formation under vancomycin stress remains to be elucidated. (p)ppGpp dependent DNA-release by any of the lytic processes (e.g., autolysins, phages) likely contributes to biofilm formation. Recently, it was shown that mupirocin, a strong inducer of Rel-dependent (p)ppGpp synthesis, causes increased biofilm formation (Jin et al., 2020). Similar to our results, the mupirocin-induced biofilm forms independent of PIA and PSMs and is largely composed of eDNA. Thus, it is likely that the observed biofilm-inducing phenotype induced by mupirocin is similar to the vancomycin-dependent biofilm observed in our study. Jin et al. (2020) found that mupirocin upregulates *cidA*, encoding a holin-like protein, and that a *cidA* mutant shows reduced eDNA release. Thus, one may speculate that under our assay conditions, the (p)ppGpp-mediated activation of *cidA* may also contribute to (p)ppGpp-promoted biofilm formation.

The subinhibitory concentration of vancomycin applied in our standard biofilm assay did not affect bacterial viability, and the MIC in planktonically grown strains did not differ between the analyzed strains. When vancomycin was added to preformed biofilms, the biofilms were protected even at up to a concentration 100-fold higher than the MIC. It has been proposed that antibiotic tolerance and persister formation share common characteristics such as a slow- or non-growing phenotype (Waters et al., 2016). Here, we showed that biofilm tolerance is at least partly (p)ppGpp dependent, since biofilms of the $pppGpp^0$ strain were significantly better resolved in the presence of high vancomycin concentrations. This seems to contrast with recent results indicating that (p)ppGpp is not involved in persister formation in *S. aureus* (Conlon et al., 2016). However, the persister assays were performed under relaxed conditions, and thus, the role of (p)ppGpp might have been missed. Nevertheless, (p)ppGpp synthesis was previously shown to contribute to antibiotic tolerance in *S. aureus* (Geiger et al., 2014; Bryson et al., 2020) and other pathogens (Nguyen et al., 2011; Bernier et al., 2013). Nguyen et al. (2011) suggested that in *Pseudomonas aeruginosa*, the stringent response contributes to antimicrobial tolerance in biofilms by reducing oxidative stress. We recently showed that (p)ppGpp in *S. aureus* activates ROS-detoxifying systems (Horvatek et al., 2020), which might contribute to protection against vancomycin.

Due to the role of (p)ppGpp in biofilm formation and antibiotic tolerance, the (p)ppGpp synthesis pathway is thought to be a promising antimicrobial target. Anti-biofilm peptides have been reported to exert their activity via their ability to reduce (p)ppGpp levels in live bacterial cells (De La Fuente-Nunez et al., 2014, 2015). A direct mechanism of action involving the binding of (p)ppGpp and promotion of its intracellular degradation was suggested (De La Fuente-Nunez et al., 2015). We confirmed the biofilm-dissolving effect of DJK-5. However, this was clearly not due to the proposed interaction of the peptides with (p)ppGpp because an even stronger inhibitory effect of DJK-5 was observed in the $(p)ppGpp^0$ mutant. Interestingly, treatment

with DJK-5 and a subinhibitory vancomycin concentration resulted in significantly higher bacterial killing activity and biofilm inhibition in the (p)ppGpp^0 mutant than the wild type. Thus, (p)ppGpp protects against bactericidal DJK-5 activity. These findings are in good agreement with a recent re-analysis of the proposed antibiofilm peptide IDR-1018 in *E. coli* and *P. aeruginosa* (Andresen et al., 2016). Genetic disruption of the *relA* and *spoT* genes responsible for (p)ppGpp synthesis moderately sensitizes *E. coli* to IDR-1018, rather than protecting the bacterium (Andresen et al., 2016). While the IDR-1018 and DJK-5 peptides are potent antimicrobials, they do not specifically disrupt biofilms via a direct and specific interaction with the intracellular messenger nucleotide (p)ppGpp. Their alternative mode of action remains to be elucidated.

CONCLUSION

In conclusion, in *S. aureus*, (p)ppGpp supports biofilm formation under cell wall stress conditions and increases tolerance against vancomycin and the anti-biofilm peptide DJK-5.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.575882/full#supplementary-material

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PLOS GENETICS

RESEARCH ARTICLE

Inducible expression of (pp)pGpp synthetases in *Staphylococcus aureus* is associated with activation of stress response genes

Petra Horvatek[1], Andrea Salzer[1], Andrew Magdy Fekry Hanna[1], Fabio Lino Gratani[1,2], Daniela Keinhörster[1], Natalya Korn[1], Marina Borisova[1], Christoph Mayer[1], Dominik Rejman[3], Ulrike Mäder[4], Christiane Wolz[1]*

1 Interfaculty Institute of Microbiology and Infection Medicine, University of Tuebingen, Germany, **2** Quantitative Proteomics & Proteome Center Tuebingen, University of Tuebingen, Germany, **3** Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Prague, Czech Republic, **4** Interfaculty Institute for Genetics and Functional Genomics, University Medicine Greifswald, Greifswald, Germany

* christiane.wolz@uni-tuebingen.de

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Abstract

The stringent response is characterized by the synthesis of the messenger molecules pppGpp, ppGpp or pGpp (here collectively designated (pp)pGpp). The phenotypic consequences resulting from (pp)pGpp accumulation vary among species and can be mediated by different underlying mechanisms. Most genome-wide analyses have been performed under stress conditions, which often mask the immediate effects of (pp)pGpp-mediated regulatory circuits. In *Staphylococcus aureus*, (pp)pGpp can be synthesized via the RelA-SpoT-homolog, Rel_{Sau} upon amino acid limitation or via one of the two small (pp)pGpp synthetases RelP or RelQ upon cell wall stress. We used RNA-Seq to compare the global effects in response to induction of the synthetase of *rel-Syn* (coding for the enzymatic region of Rel_{Sau}) or *relQ* without the need to apply additional stress conditions. Induction of *rel-Syn* resulted in changes in the nucleotide pool similar to induction of the stringent response via the tRNA synthetase inhibitor mupirocin: a reduction in the GTP pool, an increase in the ATP pool and synthesis of pppGpp, ppGpp and pGpp. Induction of all three enzymes resulted in similar changes in the transcriptome. However, RelQ was less active than Rel-Syn and RelP, indicating strong restriction of its (pp)pGpp-synthesis activity *in vivo*. (pp)pGpp induction resulted in the downregulation of many genes involved in protein and RNA/DNA metabolism. Many of the (pp)pGpp upregulated genes are part of the GTP sensitive CodY regulon and thus likely regulated through lowering of the GTP pool. New CodY independent transcriptional changes were detected including genes involved in the SOS response, iron storage (e.g. *ftnA*, *dps*), oxidative stress response (e.g., *perR*, *katA*, *sodA*) and the *psmα1–4 and psmβ1-2* operons coding for cytotoxic, phenol soluble modulins (PSMs). Analyses of the *ftnA*, *dps* and *psm* genes in different regulatory mutants revealed that their (pp)pGpp-dependent regulation can occur independent of the regulators PerR, Fur, SarA or CodY. Moreover, *psm* expression is uncoupled from expression of the quorum sensing system Agr, the main known *psm* activator. The expression of central genes of the oxidative stress response protects the bacteria from anticipated ROS stress derived from

analysis, decision to publish, or preparation of the manuscript.

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PSMs or exogenous sources. Thus, we identified a new link between the stringent response and oxidative stress in *S. aureus* that is likely crucial for survival upon phagocytosis.

Author summary

Most bacteria make use of the second messenger (pp)pGpp to reprogram bacterial metabolism under nutrient-limiting conditions. In the human pathogen *Staphylococcus aureus*, (pp)pGpp plays an important role in virulence, phagosomal escape and antibiotic tolerance. Here, we analyzed the immediate consequences of (pp)pGpp synthesis upon transcriptional induction of the (pp)pGpp-producing enzymes Rel, RelP or RelQ. (pp)pGpp synthesis provokes immediate changes in the nucleotide pool and severely impacts the expression of hundreds of genes. A main consequence of (pp)pGpp synthesis in *S. aureus* is the induction of ROS-inducing toxic phenol soluble modulins (PSMs) and simultaneous expression of the detoxifying system to protect the producer. This mechanism is likely of special advantage for the pathogen after phagocytosis.

Introduction

The stringent response is characterized by the synthesis of the alarmones pGpp, ppGpp and pppGpp, here collectively named (pp)pGpp. (pp)pGpp interferes with many cellular processes, including transcription, replication and translation [1,2,3,4,5,6,7,8,9,10]. Depending on the species, the stringent response is crucial for diverse biological processes, including differentiation, biofilm formation, antibiotic tolerance, production of secondary metabolites or virulence [8,11]. It is now clear that there are fundamental differences between the stringent response initially characterized in *Eschericia coli* and the stringent response in Firmicutes [7,9]. Differences have been observed in the enzymes involved in the synthesis and degradation of the messengers and in the downstream effects of (pp)pGpp.

(pp)pGpp is synthesized by RelA-SpoT-homologs (RSHs) by transferring pyrophosphate originating from ATP to the 3´ OH group of GTP, GDP or GMP. Long RSH enzymes are present in nearly all bacteria and show a conserved molecular architecture composed of a C-terminal sensory region and an N-terminal enzymatic region with distinct (pp)pGpp hydrolase and synthetase domains [12]. Firmicutes, such as *Staphylococcus aureus*, possess one long RSH enzyme, Rel_{Sau} and in addition two small alarmone synthetases (SAS), RelP and RelQ. Amino acid limitation is the only condition known to induce a Rel_{Sau}-mediated stringent response phenotype [13]. Under non-inducing conditions, Rel_{Sau} is primarily in a hydrolase-On/synthetase-Off conformation even when the C-terminal sensory region is deleted [14]. The strong hydrolase activity of Rel_{Sau} makes the enzyme essential for the detoxification of (pp)pGpp produced by RelP or RelQ [13].

RelP and RelQ are part of the VraRS cell-wall stress regulon [15] and are thus transcriptionally induced, e.g., after vancomycin treatment [16]. Thereby, they contribute to tolerance towards cell-wall active antibiotics such as ampicillin or vancomycin. Recently, structural and mechanistic characterization revealed that RelQ from *Bacillus subtilis* and *Enterococcus faecalis* form tetramers [17,18]. RelQ activity is strongly inhibited through the binding of single-stranded RNA. pppGpp binding leads to disassociation of the RelQ:RNA complex and its activation [18]. In contrast, RelP activity is inhibited by both pppGpp and ppGpp, activated by Zn^{2+} and insensitive to inhibition by RNA [19,20]. For the *S. aureus* enzymes it could be

shown that RelQ, but not RelP are allosterically stimulated by the addition of pppGpp, ppGpp or pGpp [21]. Thus, although highly homologous, RelP and RelQ seem to have different functions within the cell. One can assume that different post-translational regulatory mechanisms are in play to fine-tune (pp)pGpp synthesis under different growth conditions.

In *S. aureus*, the stringent response plays important roles in virulence [13], phagosomal escape [22] and antibiotic tolerance [8,16,23,24,25,26,27]. The enzymes HprT and Gmk involved in GTP synthesis, putative GTPases (RsgA, RbgA, Era, HflX, and ObgE) and DNA primase were identified as (pp)pGpp target proteins [23,28]. (pp)pGpp binding inhibits enzyme activities, resulting in lowering of the GTP pool and inhibition of the translation apparatus and replication. Of note, in contrast to *E. coli*, (pp)pGpp from Firmicutes does not interfere with RNA polymerase activity [10]. Instead, in these organisms, (pp)pGpp regulates transcription via an indirect mechanism that strongly relies on the lowering of the intracellular GTP pool [22,28,29,30]. A decrease in the GTP level leads to the repression of nucleotide-sensitive, GTP-initiating promoters, e.g., those of rRNA genes [30,31]. Low GTP levels also affect the CodY regulon. The transcription factor CodY, when loaded with GTP and branched-chain amino acids, acts mainly as a repressor of many genes involved in amino acid synthesis and virulence [32,33]. The global transcriptional effects of (pp)pGpp have been examined previously in several Firmicutes, such as *B. subtilis* [34], *Streptococcus pneumoniae* [35], *Enterococcus faecalis* [36], *Streptococcus mutans* [37] and *S. aureus* [22]. These studies were based on the comparison of the wild-type and *rel* mutant strains under conditions mimicking amino acid starvation. Of note, these stress conditions are accompanied by profound physiological changes, which are only partially mediated by (pp)pGpp. For instance, amino acid limitation leads to the stabilization of many transcripts independent of (pp)pGpp [13]. Thus, from these analyses, it is difficult to draw firm conclusions on the primary transcriptional changes imposed by (pp)pGpp synthesis. Recently, one study tried to circumvent this drawback by transcriptional induction of (pp)pGpp synthetase in *E. coli* and gained major new insights [38].

Here, we aimed to compare the Rel-, RelQ- and RelP-mediated effects on nucleotide pools, transcription and functional consequences without imposing nutrient starvation or stress. Therefore, the Rel synthetase (Rel-Syn, N-Terminal region of Rel with mutated hydrolase), RelP and RelQ were expressed from an anhydrotetracycline (ATc)-inducible promoter in a (pp)pGpp^0 strain in which the enzymatic domains of all three synthetases were mutated. Through RNA-Seq analyses, we identified new (pp)pGpp-regulated genes, many of which are involved in the oxidative stress response, iron storage and the synthesis of phenol-soluble modulins (PSMs). Thus, (pp)pGpp synthesis contributes not only to PSM-derived ROS production but also to protection from these toxic molecules.

Results

Changes in the nucleotide pools after transcriptional induction of *rel-Syn* and *relQ*

We first compared the stringent response imposed by mupirocin (isoleucyl-tRNA synthase inhibitor [39]) with the genetic induction of (pp)pGpp synthetases. To analyse Rel dependent (pp)pGpp synthesis without stress, we cloned the N-terminal region of Rel with inactivated hydrolase domain [14] designated as Rel-Syn. *Rel-Syn* or *relQ* were expressed using an ATc-inducible expression system in a (pp)pGpp^0 strain background. Strain (pp)pGpp^0 is unable to synthesize (pp)pGpp due to mutations in all three (pp)pGpp synthetases (full deletion of *rel*, synthetase mutations in *relP* and *relQ* [14]). Strains were grown to an early exponential growth phase and gene expression was induced by ATc for 30 min. Sub-inhibitory concentration of ATc resulted in similar induction of *rel-Syn* or *relQ*, (7.0 and 6,7 $\log_2$ fold increase compared

to un-induced, respectively, S1 Data). ATc treatment did not influence gene expression in strains containing the empty control plasmid pCG248 (S1 Fig).

Quantification of the nucleotide pools after mupirocin treatment or induction of *rel-syn* or *relQ* revealed that induction of *rel-syn* resulted in similar levels of pppGpp, ppGpp, and pGpp as induction of the stringent response in the wild type by mupirocin (Fig 1). In Firmicutes, (pp)pGpp inhibit several enzymes involved in purine nucleotide synthesis and transport [7]. Accordingly, the concentrations of guanine nucleotides GTP and GDP was negatively correlated to (pp)pGpp synthesis (Fig 1). Of note, (pp)pGpp synthesis leads to concomitant increase of adenine nucleotides (ATP, ADP and AMP) and a slight increase in the adenylate energy charge. In the (pp)pGpp^0 strain, mupirocin treatment resulted in significant increase of GTP

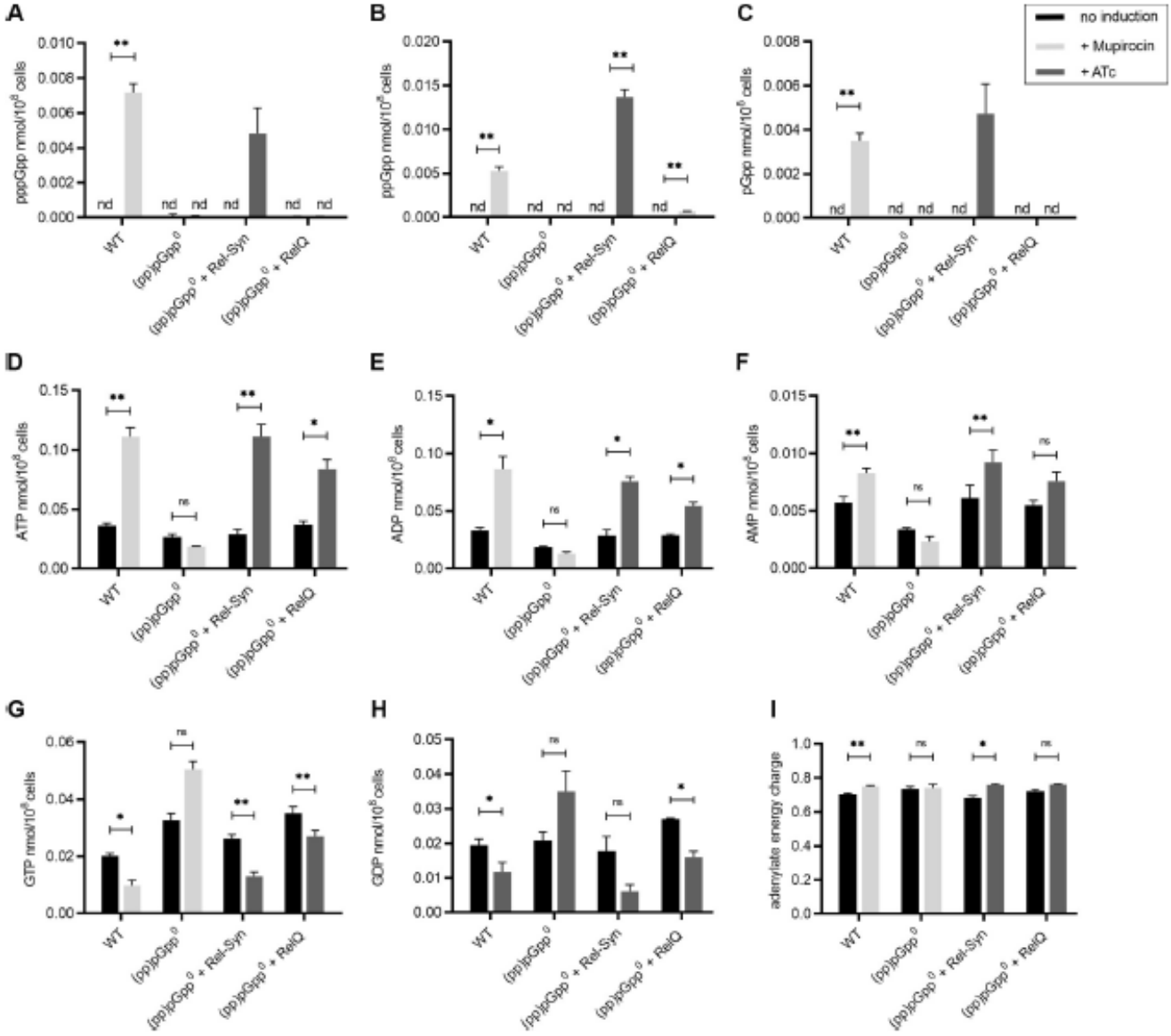

Fig 1. Changes in the nucleotide pool after mupirocin treatment or transcriptional induction of *rel-Syn* or *relQ*. Strain HG001 and derivatives were grown to OD_{600} = 0.3 and treated for 30 min with or without 0.125 μg/ml mupirocin (lightgrey) or 0.1 μg/ml ATc (darkgrey). Nucleotide analyses were performed using mass spectrometry (ESI-TOF) in negative ion mode. Error bars represent SEM (n = 3) from three biological replicates. The adenylate energy charge was calculated by [ATP] + 0.5 [ADP]/[ATP] + [ADP] + [AMP]. Statistical significance was determined by two-tailed Student´s T-test, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$.

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and decrease ATP concordant with previous results [30]. In summary, Rel-mediated (pp)pGpp synthesis imposed by mupirocin or genetic *rel-syn* induction resulted in similar changes of the nucleotide pool characterised by reduction of guanine nucleotides and accumulation of adenine nucleotides. After induction of *relQ*, similar changes in the nucleotide pools were detectable. However, the effect of *relQ* induction on the nucleotide pools was significantly lower than that of *rel-syn* induction or mupirocin treatment. Thus, *rel-syn* induction probably mirrors stress conditions (e.g. upon mupirocin treatment), whereas *relQ* expression more likely reflect basal low level (pp)pGpp synthesis.

Impact of (pp)pGpp synthesis on the transcriptome

We next analysed the impact of (pp)pGpp synthesis after induction of *rel-syn* or *relQ* on mRNA abundance. RNA-Seq data revealed that 1074 genes or sRNAs were significantly affected by either Rel-Syn (total: 478 up, 551 down) or RelQ (total: 155 up, 97 down) with a large overlap in genes affected by Rel-Syn and RelQ (Fig 2A and S1 Data). However, consistent with the nucleotide measurements (Fig 1), the effect of *relQ* induction was less prominent (S1 Table). We compared the RNA-Seq data with previous microarray analyses obtained after stringent response imposed by transferring bacteria to amino acid limiting conditions (-Leu, -Val) [22]. Most of the previously identified stringent response genes were confirmed by the RNA-Seq analysis (Fig 2A). Of note, in the present analysis, only genes with at least three-fold differences and a significance level of $p < 0.001$ were included in the analysis shown in Fig 2 and S2 Data. Despite the higher stringency in the analysis, the present analysis revealed far more (pp)pGpp-regulated genes. Genes were classified into functional categories using the SEED annotation (http://pubseed.theseed.org). (pp)pGpp induction resulted in the downregulation of many genes involved in protein synthesis (e.g ribosomal proteins RplA-T) and RNA/DNA (e.g. purine biosynthesis, gyrase) metabolism consistent with previous results that the stringent response mainly leads to the shutdown of translation and replication [22] (Fig 2B). Many of the genes upregulated by Rel-Syn and RelQ are part of the CodY regulon (S1 Data) and thus likely regulated by lowering of the GTP pool. The sRNA, RsaD was recently described to be directly repressed by CodY [40]. Concordantly, *rsaD* was found strongly up-regulated upon *rel-Syn* and *relQ* induction (8.5 and 6.0 $\log_2$ fold change, respectively) and used as read-out for a prototypic CodY target is subsequent experiments. *AlsS* was identified as RsaD repressed target gene [40] and as expected downregulated upon *rel-Syn* expression. Thus, the genetic approach proved to gain reliable, physiologically relevant results as validated by comparison with previous studies on (pp)pGpp mediated transcriptional changes. We further focused on so far unknown genes/phenotypes which were induced during stringent conditions. Therefore, we concentrated on genes that were strongly affected by *rel-syn* induction (Fig 2C and S1 Data) but not known to be under the control of CodY. Interestingly, many of these genes were assigned to iron acquisition/metabolism (upregulation of genes involved in iron storage; downregulation of genes involved in siderophore biosynthesis and iron transport), stress response (*dps, sodA, katA ahpC, uspA1/2, asp23, ptpA*, and *msrA2)*, and virulence (upregulation of *psmα/ß*; downregulation of *agr*). Phage-encoded genes were also upregulated, indicating phage-inducing conditions. This is in line with the upregulation of *recA* and *lexA*. For further analysis, we selected *ftnA, dps, agr* and *psmα* as read-out for (pp)pGpp-mediated CodY independent activities.

Comparison of the mupirocin-induced stringent response and induction of *rel-Syn*

We compared the expression of the selected genes after induction of the stringent response via mupirocin and after *rel-Syn* induction by Northern blot analysis (Fig 3A) and qRT-PCR (Fig 3B).

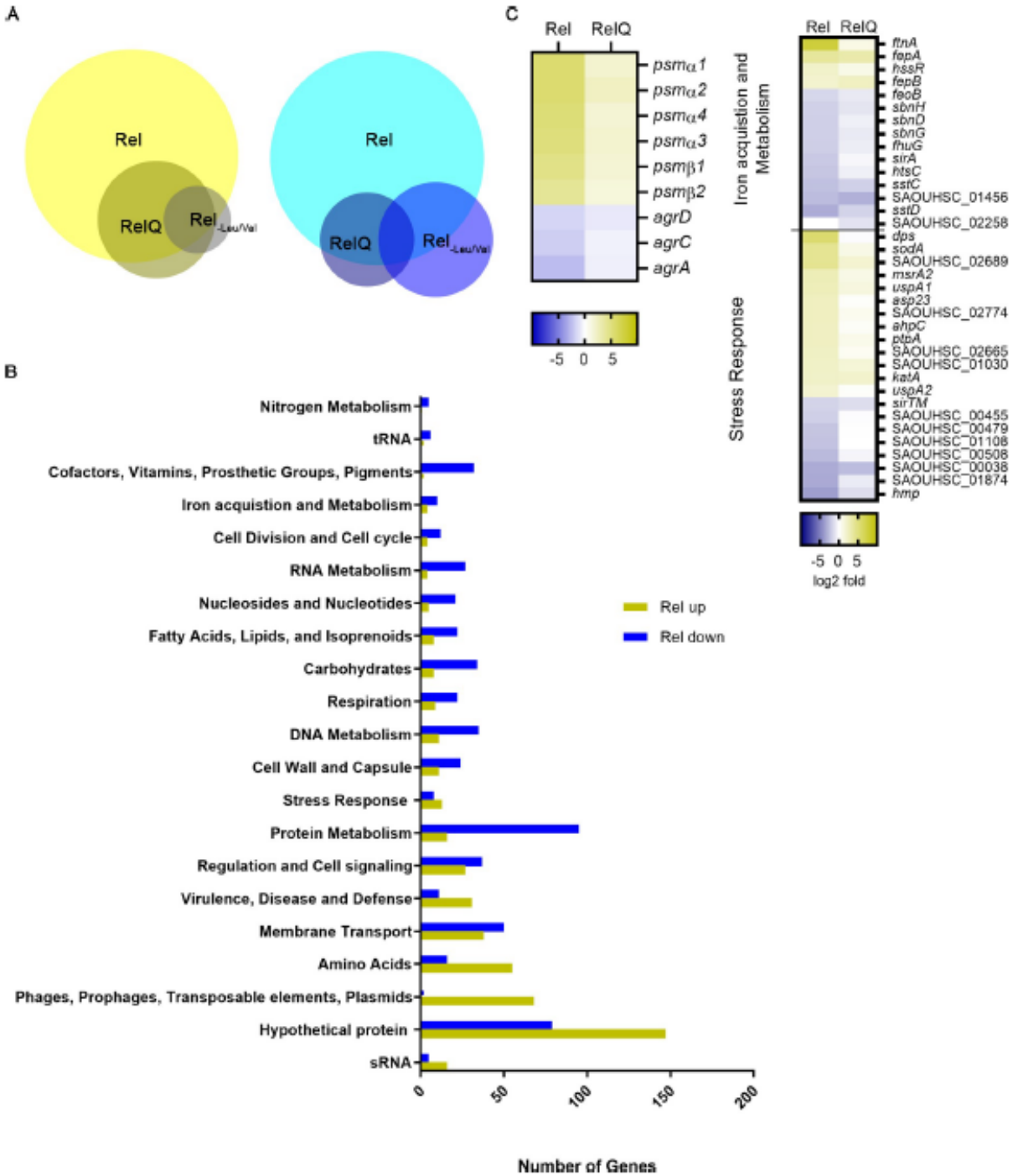

Fig 2. Global changes in gene expression upon transcriptional induction of *rel-Syn* or *relQ*. (pp)pGpp^0 with inducible *rel-Syn* or *relQ* was grown to OD_{600} = 0.3 and treated for 30 min without or with 0.1 μg/ml ATc. A. Venn diagrams showing genes or sRNAs upregulated (yellow) or downregulated (blue) after induction in comparison to uninduced cultures (< 3-fold difference, p< 0.001). Previously, described stringent genes [22] are indicated as $Rel_{Leu/Val}$. B. Genes with significant changes after induction of *rel-syn* (< 3-fold difference, p< 0.001) according to functional categories. C. Heatmap representing Rel-Syn-dependent up- and downregulated genes assigned to the functional categories iron acquisition and metabolism, stress response and Agr-related genes.

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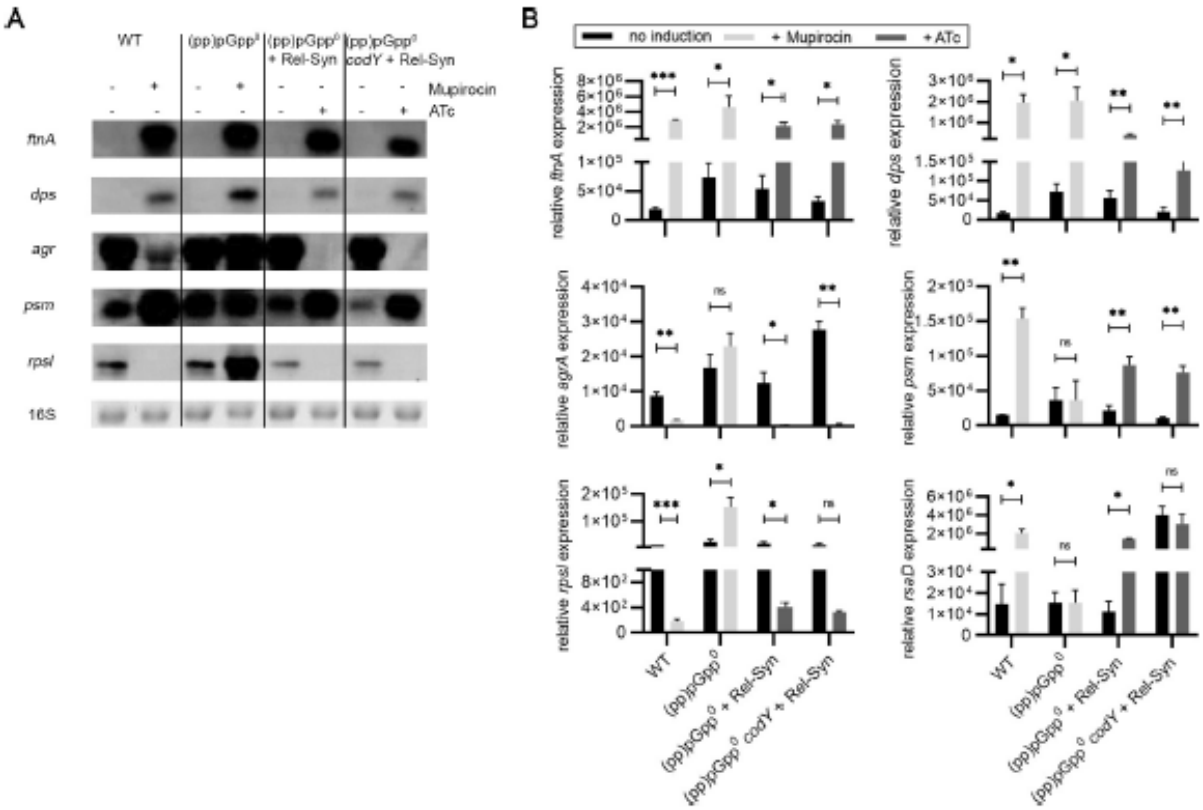

Fig 3. Correlation of mupirocin-induced stringent response and transcriptional induced *rel-syn* for selected CodY-independent genes. Strain HG001 and derivatives were grown to OD_{600} = 0.3 and treated for 30 min with or without 0.125 μg/ml mupirocin or 0.1 μg/ml ATc (mutant strains with inducible *rel-Syn*). A. For Northern blot analysis, RNA was hybridized with digoxigenin-labelled probes specific for *ftnA*, *dps*, *psm*, *agrA or rpsI*. The 16S rRNA detected in ethidium bromide-stained gels is indicated as a loading control in the bottom lane. B. Quantification of mRNA by qRT-PCR based on three biological replicates. Statistical significance was determined by two-tailed Student's T-test, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$.

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We verified the upregulation of *ftnA*, *dps*, *psm* and the sRNA *rsaD* under both conditions. Mupirocin also resulted in *ftnA* and *dps* activation in the (pp)pGpp^0 strain indicating additional (pp)pGpp-independent effects of mupirocin on the expression of these genes. (pp)pGpp-mediated activation of *psm* expression is clearly not correlated to *agr* expression. Agr is the main activator required for *psms* expression [41]. Notably, the expression of the *agr* operon was even lower upon (pp)pGpp synthesis (S1 Data and Fig 3A and 3B), indicating that (pp)pGpp induces *psm* expression independent of Agr. Induction of *rel-Syn* in wild type background resulted in minor changes in gene expression compared to induction in the (pp)pGpp^0 strain (S2 Fig). This emphasizes the strong hydrolase activity of Rel as present in the wild type leading to rapid hydrolysis of (pp)pGpp [14].

CodY-independent activation of gene expression upon *rel-Syn* induction

(pp)pGpp synthesis leads to the lowering of the GTP pool and subsequently to de-repression of CodY target genes. Indeed, many of the genes that were upregulated in response to *rel-Syn* induction belong to the CodY regulon (S1 Data). However, the selected marker genes are not supposed to be regulated via CodY. For further validation we induced *rel-Syn* in a *codY* negative (pp)pGpp^0 strain (Fig 3). The CodY target *rsaD* was confirmed to be de-repressed in the *codY* negative background [40]. However, other selected genes showed similar expression

patterns in *codY*-positive and *codY*-negative backgrounds (Fig 3A and 3B). Thus, (pp)pGpp impacts the expression of these genes independent of CodY.

Induction of *rel-Syn*, *relQ* and *relP* in *S. aureus* USA300

To confirm that the observed gene expression pattern is not restricted to strain HG001 and not due to potential second site mutations in the (pp)pGpp^0 strain we repeated key experiments in strain USA300. Therefore a (pp)pGpp^0 strain was constructed by sequential mutation of *relP*, *relQ* and *rel*. *rel-Syn*, *relQ* and *relP* were induced from the ATc inducible expression vectors as described. The transcriptional changes imposed by *rel-Syn* induction recapitulated the findings of *rel-Syn* induction in strain HG001 (Fig 4). We also verified that induction of *relQ* only slightly effects marker gene expression (Fig 4). However, induction of *relP* was highly effective, resulting in an expression pattern comparable to induction of *rel-Syn*. The strong effect of *relP* induction could be due to the fact that in contrast to *relP* it does not require pppGpp activation [20]

Rel-Syn induction influences the oxidative stress response and virulence independent of PerR, Fur or SarA

Some of the prominent (pp)pGpp-activated genes are known to be under the control of other global regulators, such as PerR, Fur and SarA [42]. We speculated that (pp)pGpp dependent

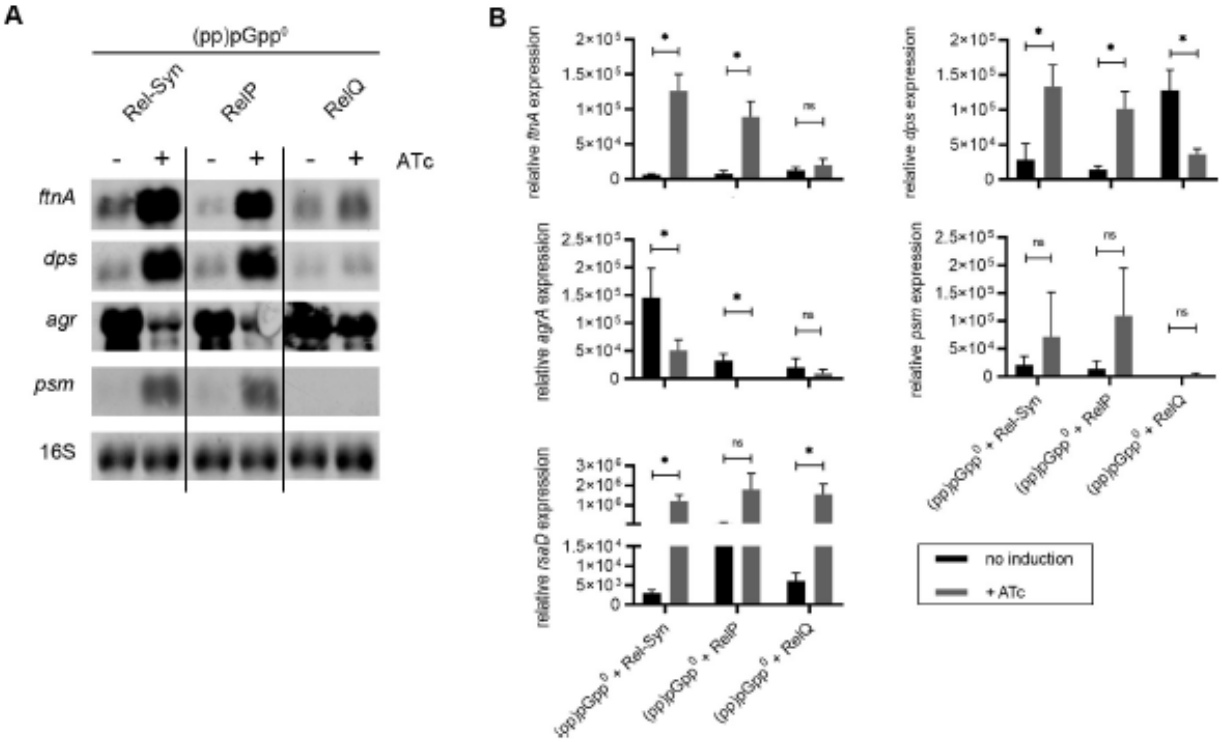

Fig 4. Gene expression following induction of *rel-Syn*, *relQ* or *relP* in USA300 strain background. A. Strain USA300 and derivatives were grown to OD_{600} = 0.3 and treated for 30 min without or with 0.1 μg/ml ATc (mutant strains with inducible *rel-Syn*, *relP* or *relQ*). For Northern blot analysis, RNA was hybridized with digoxigenin-labelled probes specific for *ftnA*, *dps*, *psmα* or *agrA*. The 16S rRNA detected in ethidium bromide-stained gels is indicated as a loading control in the bottom lane. B. Quantification of mRNA by qRT-PCR based on three biological replicates. Statistical significance was determined by two-tailed Student´s T-test, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$.

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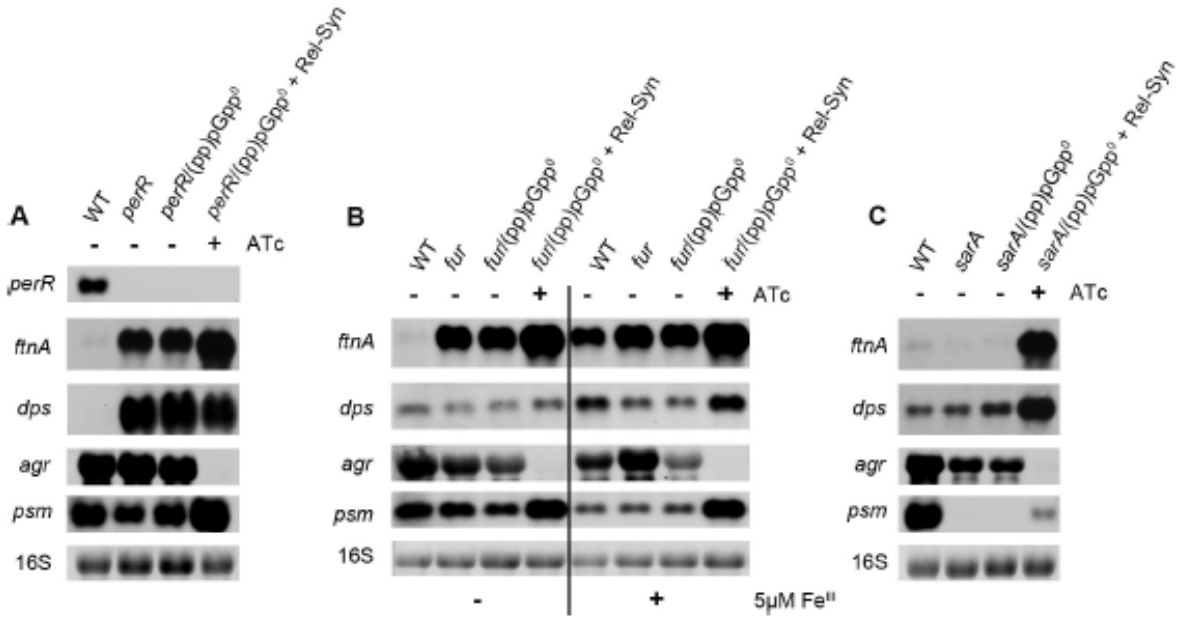

Fig 5. (pp)pGpp-dependent transcriptional changes independent of PerR, Fur or SarA. Strain HG001 and derivatives were grown to OD_{600} = 0.3 and treated for 30 min without or with 0.1 μg/ml ATc (mutant strains with inducible *rel-Syn*). For Northern blot analysis, RNA was hybridized with digoxigenin-labelled probes specific *for ftnA*, *dps*, *psmα* or *agrA*. The 16S rRNA detected in ethidium bromide-stained gels is indicated as a loading control in the bottom lane. For quantitative results see S3 Fig.

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gene expression may somehow be mediated via these global regulators. Therefore, we analysed *rel-Syn* induction in *per*, *fur* and *sarA* mutants (Figs 5A and S3A).

The (pp)pGpp controlled genes *ftnA*, *dps*, *ahpC*, *katA* and *perR* (S1 Data) are likely controlled via binding of PerR to a conserved PerR-binding motif (based on the public databases RegPrecise [43] and Aureowiki [44]). As expected, *ftnA* and *dps* were both upregulated in the *perR* mutants. Inducing *rel-Syn* in *perR*/(pp)pGpp^0 strain showed further increase in *ftnA*, supporting that (pp)pGpp acts in addition and independent of PerR. For *dps*, the *perR* mutation also resulted in high expression, which was slightly decreased by (pp)pGpp. *PerR* deletion resulted in a slight decrease in *psm* expression, which was compensated by *rel-syn* induction. Thus, (pp)pGpp also affects gene expression in a *per*-negative background.

We found that many of the genes affected by Rel-Syn are indicative of iron overload conditions (e.g. upregulation of *ftnA* and *dps* (Fig 2D). We induced *rel-Syn* in a *fur*/(pp)pGpp^0 background under low and high iron conditions (Figs 5B and S3). Independent of the availability of iron, *ftnA*, *dps* and *psm* were upregulated and *agr* was downregulated after *rel-Syn* induction in the *fur*-negative background indicating that (pp)pGpp regulation is not determined by iron availability or *fur* regulation.

SarA was shown to activate transcription of the *agr* operon [45,46] and proposed to be involved in oxidative stress sensing via a single Cys9 residue [47,48,49]. *sarA* was found to be significantly upregulated by Rel-Syn (S1 Data). *ftnA* and *dps* expression was not influenced by *sarA* mutation (Figs 5C and S3). *agr* and *psm* expression was downregulated in the *sarA* mutant, consistent with the proposed activation of the *agr* system by SarA [45]. Induction of *rel-syn* in the *sarA* mutant again showed the typical induction of *ftnA*, *dps* and repression of *agr*. Interestingly, *psm* expression remained hardly detectable in the *SarA* mutant. In the *sarA* mutant the very low Agr activity is likely not sufficient to allow for *psm* expression. Taken together, the results do not support the hypothesis that any of the candidate regulators function as a central hub for the observed (pp)pGpp dependent gene alterations.

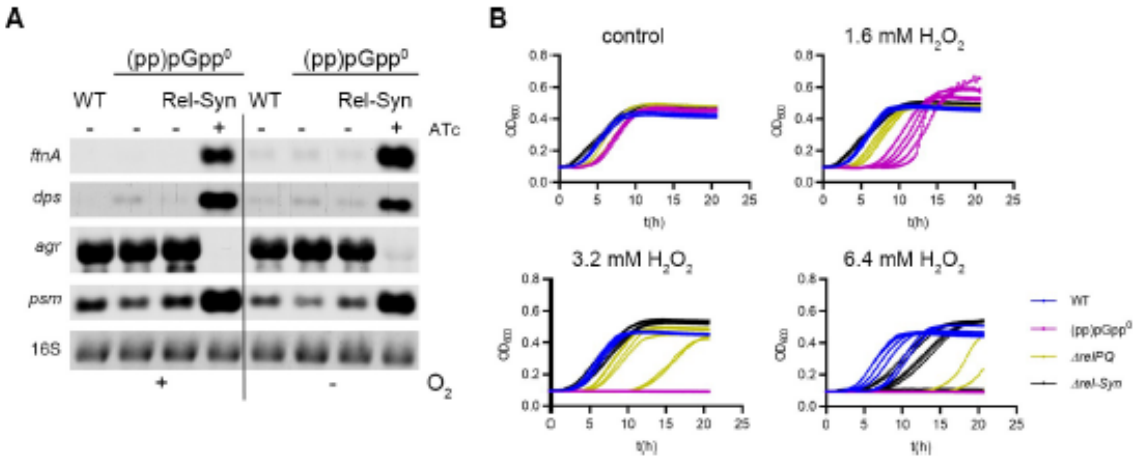

Fig 6. Functional link between stringent response and oxidative stress. A. Strain HG001 and derivatives were grown with shaking aerobically or anaerobically to OD_{600} = 0.3 and treated for 30 min without or with 0.1 μg/ml ATc (mutant with inducible *rel-Syn*). For Northern blot analysis, RNA was hybridized with digoxigenin-labelled probes specific for *fthA*, *dps*, *psm* or *agrA*. The 16S rRNA detected in ethidium bromide-stained gels is indicated as a loading control in the bottom lane. B WT, (pp)pGpp^0, Δ*relPQ* and *rel-Syn* mutants were diluted from overnight culture to an OD = 0.1, challenged with different H_2O_2 concentrations, and growth was monitored over time.

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(pp)pGpp is involved in oxidative stress resistance

Recently, it was shown that PSMs lead to the production of reactive oxygen species (ROS) [50]. One might speculate that under stringent conditions PSM-mediated ROS production triggers the expression of the oxidative stress genes. In this case, induction of *rel-Syn* should not result in the induction of these genes under anaerobic conditions, where ROS cannot be produced. However, *rel-Syn* induction resulted in the same transcriptional pattern regardless of whether bacteria were grown with or without oxygen (Fig 6A). We also analysed whether ROS would result in activation of the stringent response. However, H_2O_2 treatment did not affect transcription of stringent response genes such as *fntA*, *dps*, *psm* or *rpsI* (S5 Fig). Thus, (pp)pGpp-mediated gene alterations of the selected marker genes are not a consequence of ROS formation.

These data indicate that (pp)pGpp simultaneously activates ROS-producing PSMs as well as ROS defence systems to prepare cells to withstand oxidative stress. To verify this hypothesis, we challenged wild type and mutant strains deficient in (pp)pGpp synthesis with H_2O_2. The (pp)pGpp^0 strain was indeed more sensitive to oxidative stress. The minimal inhibitor concentration (MIC) of H_2O_2 to inhibit growth of the wild type was 6.4 mM and 3.2 mM for the (pp)pGpp^0 strain. Moreover, the (pp)pGpp^0 strain was more efficiently killed after 1 h or 2 h incubation with H_20_2 (S4 Fig). Under the assay conditions, the basal (pp)pGpp might be derived from any of the (pp)pGpp synthetases. We analyzed a *relPQ* mutant and a *rel-Syn* mutant in which the synthetase domain of Rel was mutated. Both strains showed an intermittent phenotype in which the MIC varied between 3.2 and 6.4 mM when biological replicates were analyzed. To follow up on these ambiguities, we monitored growth after addition of H_2O_2 (Fig 6C). There was high variation in the lag time between biological replicates. Replicates of the *relPQ* or *rel-Syn* mutant showed a delayed lag phase, and some of the replicates could not grow. In cases where no growth was detectable the cultures were sterile as determined by colony counting. The delay of the lag phase was more prominent for the *relPQ* mutant than for the *rel-Syn* mutant. Nevertheless, none of the (pp)pGpp^0 replicates could resume growth, and this result was consistent with the reproducible lowered MIC of this strain, indicating that (pp)pGpp indeed protects against oxidative stress.

We next speculated that under H_20_2 conditions may induce stringent response in wild type bacteria. However, treatment of bacteria with H_2O_2 even at MIC concentrations did not induce stringent marker genes (S5 Fig). This indicates that the basal level of (pp)pGpp produced in wild type bacteria is sufficient to confer resistance.

Discussion

We chose a genetic approach to define the early transcriptional response upon (pp)pGpp synthesis without the need to apply additional stress conditions. As expected from previous studies, [22,51] (pp)pGpp synthesis resulted in severe downregulation of the translational machinery and de-repression of CodY target genes. Many additional (pp)pGpp-regulated genes that are presumably important for the survival of *S. aureus* during starvation conditions were identified. Regulation of these genes might occur indirectly through other so far ill-defined regulatory circuits or via changes in the nucleotide pool. Here, we focused mainly on genes that were found to be activated upon (pp)pGpp synthesis in a CodY-independent manner, particularly *psm*, *ftnA* and *dps*. The (pp)pGpp-dependent activation of these genes can also occur in strains missing the prototypic proteinaceous transcriptional regulators PerR, Fur, or SarA. These regulators are well known to be involved in the regulation of the selected genes. However, they need to be activated through oxidative stress and/or iron [42]. The RNAseq data revealed that transcription of the repressor *perR* is also upregulated upon (pp)pGpp synthesis indicating a feedback regulation to dampen the response. In summary, (pp)pGpp functions as a complementary, immediate message, allowing cells to react to adverse conditions such as amino acid starvation or cell wall stress. Under these conditions, upcoming oxidative stress seems to be anticipated, and (pp)pGpp prepares the cells for survival, e.g. ROS challenges (Fig 7). Indeed, a $(pp)pGpp^0$ strain is more sensitive to H_2O_2. Both Rel and RelP/RelQ contribute to the protective effect.

(pp)pGpp leads to *psm* activation

One of the most prominent effects of (pp)pGpp synthesis is the upregulation of *psmα* and *psmβ*, confirming previous results [22,52]. PSMs are a family of amphipathic, alpha-helical peptides that have multiple roles in staphylococcal pathogenesis and contribute to a large extent to the pathogenic success of virulent staphylococci [53,54]. (pp)pGpp-dependent *psm* expression within neutrophils was shown to be crucial for survival after phagocytosis [22].

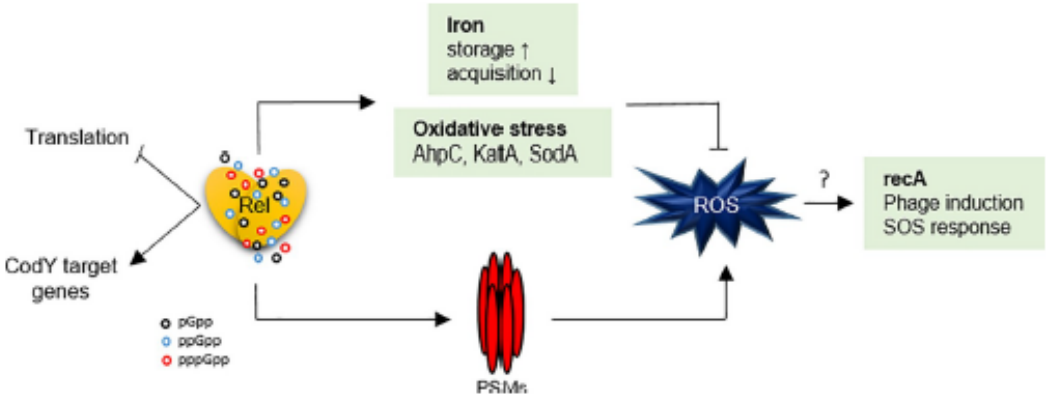

Fig 7. (pp)pGpp protects from oxidative stress. (pp)pGpp leads to upregulation of oxidative stress and iron storage genes. These upregulated genes are beneficial to counteract endogenous (PSMs) or exogenous (e.g., H_2O_2) ROS. Upregulation of SOS and phage genes might be a consequence of ROS accumulation, e.g., by PSMs.

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However, PSMs also interact with the membrane of the producer, promote the release of membrane vesicles from the cytoplasmic membrane via an increase in membrane fluidity [55,56], reduce persister formation [57,58] and are involved in self-toxicity via ROS formation [50]. Interestingly, (pp)pGpp-dependent *psm* activation was not correlated with the activation of the quorum sensing Agr system, the main regulator required for *psm* expression [41]. Agr was even repressed under (pp)pGpp-inducing conditions. Previously, analysis of a clinical isolate overproducing (pp)pGpp also indicated that (pp)pGpp leads to *agr* inhibition [24]. Thus, (pp)pGpp-mediated *psm* activation is clearly uncoupled from *agr* expression. Recently, the sRNA Teg41 (S131) [59] and the transcriptional regulator MgrA [60], Rsp [61] or Rbf [62] were found to interfere with *psm* expression. However, it is unlikely that they mediate the (pp)pGpp regulatory effect because transcription of these regulators was unaltered based on our RNA-Seq analysis (S1 Data). Thus, the molecular mechanism by which (pp)pGpp leads to *psm* activation has to be elucidated. It is likely that the accompanying changes in the ATP/GTP ratios are crucial for this activation pattern. *psm* promoters might be sensitive to the concentration of the initiating nucleoside triphosphate (iNTP). The +1 position (e.g., G or A) of sensitive genes dictates whether transcriptional initiation/elongation requires high GTP or ATP levels, respectively [29]. Various sequence combinations determine whether a promoter is sensitive to iNTP [63]. Such sequence motifs are hard to predict within *psm* promoters. However, both the *psmα* and *psmß* operons start with an A at the +1 position [41], which might explain the higher expression due to the increased ATP levels following (p)ppGpp synthesis.

(pp)pGpp and oxidative stress response

Genes whose expression is indicative of iron overload conditions were also highly affected by (pp)pGpp. We recently, could confirm that the (pp)pGpp^0 strain of strain USA300 has elevated free iron levels contributing to oxidative stress and increased ROS production [64]. A similar effect was reported for *Vibrio cholera* [65]. Here, the expression of the iron transporter FbpA was repressed via (pp)pGpp, resulting in a reduction of intracellular free iron required for the ROS-generating Fenton reaction. This contributed to reducing antibiotic-induced oxidative stress and thus tolerance, and this is also true for *S. aureus* [64]. In addition to interfering with iron metabolism, other genes involved in oxidative stress were activated by (pp)pGpp. A link between the stringent response and oxidative stress response has been observed in different organisms, although the underlying mechanisms and outcome might be highly diverse. (pp)pGpp-dependent upregulation of superoxide dismutase (SOD) was described in *B. suis* [66] and *P. aeruginosa* [67]. SOD was shown to be the key factor responsible for (pp)pGpp-mediated multidrug tolerance in *P. aeruginosa* [67]. Moreover, (pp)pGpp-deficient strains are often found to be more sensitive to oxidative stress [68,69,70]. However, in *E. faecalis*, a (pp)pGpp^0 strain grew faster and to a higher growth yield than its parent in the presence of H_2O_2 [71].

Here, we show that the stringent response in *S. aureus* leads to the activation of ROS-inducing toxins and simultaneous expression of the detoxifying system to protect the producer. This is likely a special advantage for the pathogen once it encounters neutrophils and elevated ROS. (pp)pGpp-dependent PSM synthesis is required to escape from within cells after phagocytosis [22,72]. The upregulation of the oxygen stress programme will help protect the cell from endogenous as well as exogenous ROS.

Comparison of Rel-Syn, RelQ and RelP activity

We compared the *in vivo* activity of Rel-Syn, RelQ and RelP. RelP showed similar effect on gene expression as Rel-Syn, whereas RelQ was far less active (Fig 4). Thus, under our growth conditions RelQ activity seems to be restricted *in vivo*. Comparison of RelP and RelQ from

other organisms revealed that RelQ is inhibited through RNA binding and auto-activated by (pp)pGpp [19,20]. We analyzed RelQ activity in an (pp)pGpp background under non-stress conditions where RelQ activity is likely restricted via RNA binding and/or the missing basal (pp)pGpp provided by other synthetases. The conditions that would relieve this restriction remain to be determined. A recent biochemical analysis of purified RelP and RelQ from *S. aureus* confirmed that RelQ, but not RelP, was allosterically-stimulated by the addition of pppGpp, ppGpp or pGpp [21]. Moreover, in vitro both enzymes were shown to efficiently synthesize pGpp in addition to pppGpp and ppGpp. In our *in vivo* analysis we could detect considerable quantities of pGpp derived from Rel activity (Fig 1). However, *in vitro* Rel seems to preferentially synthesize pppGpp [21]. One may speculate that pGpp detected *in vivo* might be enzymatically derived from NuDiX hydrolase, NahA, an enzyme that in *B. subtilis* efficiently produces pGpp by hydrolyzing (p)ppGpp [73]. In this organism, pGpp potently regulates the purine biosynthesis pathway but in contrast to (p)ppGpp does not interact with the GTPases. It was proposed that the different nucleotides may fulfill different roles through fine-tuning in signal transduction. Moreover, the different levels of (pp)pGpp in the cell may also dictate different outcomes. There is now growing evidence that basal level of (pp)pGpp (often below detection limit) are involved to maintain balanced growth whereas high levels are indicative for stress conditions resulting in severe reprogramming of the cell and growth arrest [74,75]. In this context, the induction of *rel-Syn* likely mirrors stress conditions, whereas *relQ* induction with low detectable (pp)pGpp synthesis more resembles the proposed basal level. From the RNA-Seq analysis we found no clear quantitative difference between both conditions. However, many of the genes affected by RelQ could be assigned to the CodY regulon. This illustrates the high sensitivity of CodY towards subtle changes of the GTP pool imposed by (pp)pGpp synthesis.

Different (pp)pGpp mode of actions result in similar outcome

Recently, genome-wide direct effects on transcription from ppGpp binding to its two sites on RNA polymerase were assessed in *E. coli* [38]. Similar to our approach, ppGpp was produced without concurrent starvation by conditional expression of a RelA variant lacking its autoinhibitory domain. The Rel variant was induced for 5–10 min in strains with or without the two binding sites for ppGpp on RNAP. It could be shown that transcriptional changes are in large part due to ppGpp-RNAP interference. However, (pp)pGpp does not bind to RNAP in Firmicutes. We anticipated that for full stringent response in *S. aureus* significant changes of the nucleotide pool have to occur and that transcriptional changes are not directly linked to (pp)pGpp in this organism. Therefore, we analysed transcriptional changes after 30 min of *rel-Syn* induction to allow the adjustment of the nucleotide pool. We assume that many transcriptional changes are caused by the observed changes in the nucleotide pool. Alternatively, at least some of them might be linked to secondary effects such as inhibition of growth or translation. In the future, time course experiments and concurrent proteome analyses will help to further clarify this issue. Nevertheless, despite major difference in the experimental set-up and the different underlying regulatory mechanisms between *E. coli* and *S. aureus* both approaches revealed a surprisingly similar outcome: First, in both organism the genetic approaches revealed many more (pp)pGpp-regulated genes in comparison to traditional analyses applying concurrent starvation. Second, observed (pp)pGpp-mediated transcriptional changes are highly similar: i.) large number of genes related to nucleotide, protein, and RNA metabolism, translation, and DNA synthesis are negatively regulated by ppGpp; ii) amino acid biosynthesis genes are highly upregulated and iii.) genes for the responses to DNA damage, general stress and

oxidants responded to ppGpp in both organisms. Thus, stringent response has evolved in different organisms to fulfil similar functions by use of highly different mechanisms.

Materials and methods

Strains and growth conditions

Strains and plasmids are listed in S1 Table. For strains carrying a resistance gene a concentration of 10 μg/ml chloramphenicol, 10 μg/ml erythromycin or 100 μg/ml ampicillin was used only for overnight cultures. *S. aureus* strains were grown overnight in chemical defined medium (CDM) [32], diluted to an optical density (OD_{600}) of 0.05 and grown until the early exponential phase OD_{600} = 0.3 with shaking (220 rpm, 37˚C). Gene expression in strains carrying a plasmid with an ATc-inducible promoter was induced at OD_{600} = 0.3 with 0.1 μg/ml ATc for 30 min. We chose 30 min induction to allow adjustment of the anticipated changes of the nucleotide pool. For anaerobic growth the strains were diluted to an OD_{600} = 0.05 in hungate tubes (Chemglass), completely filled with CDM. ATc was applied using a syringe at OD_{600} = 0. 3. For OD measurements and RNA isolation, aliquots were drawn with a syringe.

Generation of (pp)pGpp^0 mutant in USA300 JE2

For the USA300 (pp)pGpp^0 mutant (USA300-229-230-263), lysates were prepared from RN4220 strains containing the mutagenesis vectors pCG229, pCG230 and pCG263, respectively (S1 Table). After plasmid transduction of USA300 JE2, mutagenesis was performed as previously described [76]. To avoid toxic accumulation of (pp)pGpp the genes were mutated in the order *relP*, *relQ* and finally *rel*. Mutations were verified by PCR using oligonucleotides listed in S2 Table.

Generation of *perR*, *fur*, *sarA* and *psmα*/β (pp)pGpp^0 mutants in HG001

Φ11 lysates were generated from transposon mutants NE665 (*perR*), NE99 (*fur*) and NE1193 (*sarA*) from the NARSA transposon library [77] to transduce *S. aureus* strains HG001 and (pp)pGpp^0. *psmα* or *psmβ* mutations were transduced using Φ11 lysates from previously described mutants [22]. All transductants were verified by PCR using oligonucleotides listed in S2 Table.

RNA isolation, Northern Blot analysis and qRT-PCR analysis

RNA isolation and Northern blot analysis were performed as described previously [78]. Briefly, bacteria were pelleted and resuspended in 1 ml TRIzol (Thermo Fisher Scientific) and lysed using zirconia/silica beads (0.1mm diameter) and a high speed homogenizer. RNA was isolated following the recommended procedure by TRIzol manufacturer. For RNA-Seq analysis RNA from the aqueous phase was further purified following the RNA-isolation protocol by Amp Tech ExpressArt RNA ready. Transcripts on the Northern blot were detected by dioxigenin-labeled probes, which were generated by a DNA-labelling PCR-Kit (Roche Life Science). Relative quantification of *ftnA*, *dps*, *agrA*, *α-type psms*, *rsaD* and *rpsI* transcripts by qRT-PCR was performed using the Quantstudio3 (Applied Biosystems) and the QuantiFast SYBR Green RT-PCR Kit (Qiagen). Briefly, 5 μg of total RNA were DNase-treated and diluted 1:10 for qRT-PCR. Relative quantities of transcripts were calculated by a standard curve for each gene generated using 6-fold serial dilution of HG001 wild type RNA. Primers for qRT-PCR are listed inS2 Table.

In vivo nucleotide extraction

Nucleotides were isolated based on published protocol [79]. Briefly, strains were grown in CDM overnight, diluted to an OD_{600} = 0.05 and grown in CDM until an OD_{600} = 0.3. Strains

were split and treated with or without 0.1 μg/ml ATc for 30 min at 37˚C and 220 rpm shaking. 100 ml bacterial cultures were harvested and transferred into 50 ml centrifuge tubes half filled with ice and centrifuged (5 min, 5000 x g, 4˚C). Pellets were immediately frozen in liquid nitrogen and stored at -80˚C until usage. Samples were thawed on ice and resuspended in 2M formic acid and incubated for 30 min. Resuspended bacteria were lysed by high speed homogenizer using zirconia/silicia beads (0.1 mm diameter) and kept on ice for 30 min. The aqueous phase was collected and mixed with 3 ml 50 mM NH_4OAc (pH 4.5), loaded on columns (OASIS Wax cartridge 3xcc) and centrifuged (5000 x g, 5min, 4˚C). Columns were pre-treated first with pure 3 ml methanol and then with 3 ml 50 mM NH_4OAc (pH 4.5). Samples were washed first with 3 ml 50 mM NH_4OAc (pH 4.5) followed by a washing step with 3 ml methanol. Elution was performed with 1 ml of 20% methanol, 10% NH_4OH. Eluted nucleotides were flash frozen in liquid nitrogen and lyophilized overnight. Lyophilized nucleotides were resuspended in 100 μl ddH_2O and analyzed via HPLC-MS. pppGpp and ppGpp standard molecule were purchased Jena Biosciences. pGpp was synthesized starting from conveniently protected guanosine and employing both phosphoramidite and phosphotriester methods (S1 Methods).

In vivo and *in vitro* analysis of (pp)pGpp via HPLC-MS

Nucleotide quantification was performed as described [14]. Briefly, nucleotides were analyzed using ESI-TOF (micrO-TOF II, Bruker) mass spectrometer connected to an UltiMate 3000 High-Performance Liquid Chromatography (HPLC). 5 μl of standards or samples were injected onto SEQuant ZIC-pHILIC column (Merck, PEEK 150 x 2.1 mm, 5 μm). MS analysis was performed in negative-ion mode over the mass range from 200 to 1,000 m/z. MS calibration was done by using a sodium formate solution as the tune mix. Nucleotide standards of AMP (346.0552 m/z), ATP (426.0215 m/z), GTP (521.9828 m/z), pGpp (521.9828m/z), ppGpp (601.9491 m/z) and pppGpp (681.9155 m/z) were diluted 10 times 1:1 from 1 mM until a concentration of 1.95 μM and analyzed by HPLC-MS as previously described [14]. Extracted ion chromatogram (EIC) spectra of all standards were presented in DataAnalysis (Bruker) and the area under the curve (AUC) of the respective EICs was calculated in GraphPad Prism 5 (baseline was set to 150). The obtained AUC values of the diluted standards were used to generate a calibration curve. For absolute nucleotide quantification, the AUC of the samples was plugged into the AUC values of the calibration curve and the concentration of the respective nucleotides in the samples was determined. Nucleotide identification was verified by matching the retention times and m/z values of detected peaks in the samples to the measured nucleotide standards. To separate pGpp from GTP (S6 Fig) we used an expectation–maximization (EM). The relative amount of the first chemical component in the mixture is calculated as $I_1 = \sum_{k;\mu_k \leq t_c} \lambda_k$. The second component was expressed as $I_2 = 1 - I_1$.

The obtained concentrations of the adenosine nucleotides ATP, ADP and AMP in each sample were used to calculate the adenylate energy charge as described [80].

H_2O_2 killing assay

Strains were grown over night in CDM, diluted in fresh CDM to an $OD_{600} = 0.1$ and growth followed for 24 hours with different H_2O_2 concentrations in a microplate reader (Infinite M200, Tecan). H_2O_2 killing was determined by incubation of bacteria grown from an overnight culture to $OD_{600} = 0.3$ followed by incubation with 80 mM H_20_2 for 1 or 2 h. MIC determination was performed according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines using CDM medium (at least three biological replicates for each strain).

RNA-Seq analysis

Strains were grown in triplicates to OD_{600} = 0.3, split into treated (ATc, 0.1 μg/ml) and untreated control and grown for 30 min, 37˚C. Purified RNA was sent to Vertis Biotechnologie AG Freiburg for RNA Sequencing based on Illumina Next Seq 500 system. RNA was examined by a capillary electrophoresis on a Shimadzu MultiNA microchip followed by rRNA depletion using Ribo-Zero rRNA removel Kit from Illumina. RNA was converted to cDNA by fragmenting RNA samples by ultrasound and ligating an oligonucleotide adapter to the 3'end of the RNA. Using M-MLV reverse transcriptase first strand cDNA was created using 3' adapter as primer. The 5'Illumina TruSeq sequencing adapter was ligated to the 3'end of the purified (Agencourt AMPure XP kit) cDNA and PCR was performed. Samples were pooled in equimolar amounts and fractionated in a size range of 200–500 bp using a preparative agarose gel and Illumina sequencing was performed using 75 bp reads. RNA-Seq analysis was performed using CLC Genomic Workbench (Qiagen). Reads were trimmed (TrueSeq-Antinsense Primer AGATCGGAAGAGCACACGTCTGAACTCCAGTCA) and mapped to the reference genome of HG001 (NZ_CP018205.1). Differential gene expression was performed comparing Rel-Syn or RelQ versus the (pp)$pGpp^0$ mutant. Venn diagrams were performed comparing Rel-Syn vs. control and RelQ vs. control. Genes with at least 3-fold difference and a p-value ≥0.001 were defined as differentially regulated compared to the untreated control. Annotation of genes are according to recent "Aureowiki" annotation of strain 8325 (http://aureowiki.med.uni-greifswald.de/Main_Page) [44]. Of the previously identified RNA segments [81] those annotated as indep, s-indep, and indep-NT were regarded as potential regulatory RNAs (sRNAs) and included in the analysis.

Supporting information

S1 Fig. ATc treatment of strains containing empty vector pCG248. Strain HG001 and derivatives were grown to OD_{600} = 0.3 and treated for 30 min with or without 0.1 μg/ml ATc (mutant strains with inducible *rel-Syn*, *relQ* or empty vector). For Northern blot analysis, RNA was hybridized with digoxigenin-labelled probes specific for *sodA*, *ftnA*, *dps*, *per*, *agrA* or *psm*. The 16S rRNA detected in ethidium bromide-stained gels is indicated as a loading control.
(TIF)

S2 Fig. *Rel-Syn* induction in HG001 WT. Gene expression following *rel-Syn* induction in HG001 wild type. HG001 was grown to OD_{600} = 0.3 and treated for 30 min without or with 0.1 μg/ml ATc. Transcript were quantified by qRT-PCR on equal amount of total RNA. Statistical significance was determined by two-tailed Student´s T-test, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$
(TIF)

S3 Fig. *Rel-Syn* induction in *fur*, *per* and *sar* mutants qRT-PCR results accompanying Fig 5. Quantification of mRNA by qRT-PCR based on three biological replicates. Statistical significance was determined by two-tailed Student´s T-test, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$.
(TIF)

S4 Fig. H_2O_2 kill curves. Strains were grown to OD_{600} = 0.3 and then treated with 80mM H_2O_2 for 1 or 2 h.
(TIF)

S5 Fig. H_2O_2 does not induce stringent response in *S. aureus*. Strains were grown to OD_{600} = 0.3 and treated with mupirocin or H_20_2 for 30 min. RNA was hybridized with digoxigenin-

labelled probes specific for *ftnA*, *dps*, *psm*, *agrA* or *rpsI*. The 16S rRNA detected in ethidium bromide-stained gels is indicated as a loading control in the bottom lane.
(TIF)

S6 Fig. Elution profile of GTP versus pGpp analyzed using ESI-TOF (micrO-TOF II, Bruker) mass spectrometer connected to an UltiMate 3000 high-performance liquid chromatography analyzed using ESI-TOF (nnnO-TOF II, Bruker) mass spectrometer connected to an NNNMate 3000 high-performance liquid chromatography.
(TIF)

S1 Table. Strains and plasmids.
(DOCX)

S2 Table. Oligonucleotides.
(DOCX)

S1 Methods. Synthesis of guanosine 3'-*O*-diphosphate 5'-*O*-phosphate pGpp.
(DOCX)

S1 Data. Expression profile of all genes significantly affected upon induction of *rel-Syn* or *relQ*. Role categories and regulons.
(XLSX)

S2 Data. Expression profile of all genes upon induction of *rel-Syn* or *relQ*.
(XLSX)

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Author Contributions

Conceptualization: Christiane Wolz.

Data curation: Petra Horvatek, Andrea Salzer, Ulrike Mäder.

Formal analysis: Petra Horvatek, Andrea Salzer, Andrew Magdy Fekry Hanna.

Funding acquisition: Christiane Wolz.

Investigation: Petra Horvatek, Andrea Salzer, Fabio Lino Gratani, Daniela Keinhörster.

Methodology: Petra Horvatek, Andrea Salzer, Andrew Magdy Fekry Hanna, Daniela Keinhörster, Natalya Korn, Marina Borisova, Dominik Rejman.

Software: Ulrike Mäder.

Supervision: Fabio Lino Gratani, Marina Borisova, Christoph Mayer, Ulrike Mäder, Christiane Wolz.

Validation: Christoph Mayer.

Visualization: Andrea Salzer.

Writing – original draft: Petra Horvatek, Ulrike Mäder, Christiane Wolz.

Writing – review & editing: Andrea Salzer, Christiane Wolz.

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Short Review

Role of (p)ppGpp in antibiotic resistance, tolerance, persistence and survival in Firmicutes

Andrea Salzer [1,2] and Christiane Wolz [1,2,*]

[1]Interfaculty Institute of Microbiology and Infection Medicine, 72076 Tübingen, Germany
[2]Cluster of Excellence EXC 2124 "Controlling Microbes to Fight Infections", University of Tübingen, 72076 Tübingen, Germany
*Corresponding author. Interfakultäres Institut für Mikrobiologie und Infektionsmedizin, Auf der Morgenstelle 28, 72076 Tübingen, Germany. Tel: +49 7071 2974648; Fax: +49 7071 295165; E-mail: christiane.wolz@med.uni-tuebingen.de
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Abstract

The stringent response and its signalling nucleotides, pppGpp and ppGpp, have been the subject of intense research since the discovery of (p)ppGpp in 1969. Recent studies have revealed that the downstream events that follow (p)ppGpp accumulation vary among species. Consequently, the stringent response as initially characterized in *Escherichia coli* largely differs from the response in Firmicutes (Bacillota), wherein synthesis and degradation of the messengers (p)ppGpp are orchestrated by the bifunctional Rel enzyme with synthetase and hydrolase activity and the two synthetases SasA/RelP and SasB/RelQ. Here we will summarize recent studies supporting the role of (p)ppGpp in the development of antibiotic resistance and tolerance as well as survival under adverse environmental conditions in Firmicutes. We will also discuss the impact of elevated (p)ppGpp levels on the development of persister cells and the establishment of persistent infections. (p)ppGpp levels are usually tightly controlled to allow optimal growth under non-stressed conditions. Upon the onset of certain 'stringent conditions' the sudden increase in (p)ppGpp levels limits growth while exerting protective effects. In Firmicutes, the (p)ppGpp-mediated restriction of GTP accumulation is one major mechanism of protection and survival under stresses such as antibiotic exposure.

Keywords: (p)ppGpp, stringent response, persistence, antibiotic tolerance, antibiotic resistance, stress survival

Introduction

The stringent response or stringent control is a bacterial response to different forms of stress and is characterized by the synthesis of the messenger molecules pppGpp and ppGpp (hereafter referred to as (p)ppGpp). (For recent reviews, see Zhu et al. 2019, Das and Bhadra 2020, Fernandez-Coll and Cashel 2020, Pacios et al. 2020, Sinha et al. 2020, Steinchen et al. 2020, Anderson et al. 2021, Bange et al. 2021, Irving et al. 2021, Pulschen et al. 2021). One main feature of the stringent control is to keep macromolecular synthesis tightly coupled to nutrient availability and growth rate. Accordingly, (p)ppGpp can specifically block replication, translation, and transcription (Steinchen et al. 2020, Anderson et al. 2021, Irving et al. 2021). However, the downstream events that follow (p)ppGpp accumulation may vary among species and result in distinct phenotypic outcomes. There are major differences between the stringent response initially characterized in *Escherichia coli* and the response in Firmicutes. Differences can be seen not only in the phenotypic consequences of (p)ppGpp accumulation but also in the main players involved in the synthesis and degradation of the messengers. The role of (p)ppGpp in bacterial survival, antibiotic resistance, and tolerance as well as persister cell formation has mostly been analysed in Proteobacteria. Here we will discuss whether and to what extent this holds true for Firmicutes.

We will employ the recent consensus definitions to discriminate between antibiotic resistance, tolerance, and persistence (Balaban et al. 2019). Antibiotic resistance is characterized by a higher minimal inhibitory concentration (MIC) due to the genetic carriage of a resistance factor. Antibiotic-resistant bacteria not only survive otherwise lethal doses of antibiotics but can also replicate in the presence of antibiotics.

In contrast, antibiotic tolerance describes the ability to survive transient exposure to lethal concentrations of antibiotics without exhibiting an elevated MIC. Further, antibiotic-tolerant cells not only survive antibiotic treatment but can regrow after the removal of the antibiotic. Antibiotic tolerance can be detected by the overall slower killing of the whole population in time-kill assays and can be quantified by the metric minimum duration for killing (MDK) or by the TDtest, a modified disk-diffusion assay that enables the semi-quantitative evaluation of tolerance level (Gefen et al. 2017, Kotková et al. 2019).

The term antibiotic persistence describes a subpopulation of bacterial persister cells that survives intensive bactericidal antibiotic treatment without being antibiotic resistant, whereas the majority of the initial population is antibiotic susceptible and therefore rapidly killed. This phenomenon is characterized by a biphasic killing curve and therefore antibiotic tolerance and persistence can be distinguished in a time-kill assay (Brauner et al. 2016). Bacterial persister cells are phenotypic variants that enter a dormant or slow-growing state and become transiently antibiotic-tolerant (Lewis 2007, Balaban et al. 2019). Hereof the term 'persistent infections' needs to be distinguished, meaning difficult-to-treat infections that are not cleared by the host immune system. Persistent pathogens are difficult to eradicate with standard antimicrobial therapy as they deploy many regulatory strategies e.g. the stringent response, to compensate unfavourable host environmental conditions.

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(p)ppGpp making, breaking, and function in Firmicutes

(p)ppGpp making

(p)pGpp is synthesized by RelA-SpoT-homologs (RSH), which catalyse the transfer of pyrophosphate originating from ATP to the 3′ OH group of GTP, GDP, or GMP. Long RSH enzymes, characterized by an N-terminal enzymatic domain bearing distinct motifs for (p)ppGpp synthetase or hydrolase activity and a C-terminal regulatory domain, are present in nearly all bacteria and display both (p)ppGpp hydrolytic and biosynthetic activity (Atkinson et al. 2011, Bange et al. 2021). Firmicutes possess one long RSH enzyme, Rel. Amino acid limitation through the accumulation of uncharged tRNAs is so far the only common condition that has been shown to induce the (p)ppGpp synthetase activity of Rel (Geiger et al. 2010, Pausch et al. 2020). Rel can be allosterically activated by (p)ppGpp (Roghanian et al. 2021), and its activity can be modulated by the second messenger c-di-AMP (Corrigan et al. 2015, Peterson et al. 2020, Krüger et al. 2021, Heidemann et al. 2022). In some Firmicutes, Rel directly interacts with the c-di-AMP-binding protein DarB, thereby linking potassium deprivation with a stringent response (Krüger et al. 2021, Ainelo et al. 2023). In *Bacillus subtilis*, lipid starvation (Pulschen et al. 2017), heat shock (Schafer et al. 2020), or certain antibiotics (Yang et al. 2022) can also induce Rel-dependent (p)ppGpp synthesis by so far ill-defined mechanisms.

In addition to the long RSH Rel, Firmicutes possess one or two small alarmone synthetases (SAS), SasA/RelP and SasB/RelQ. The (p)ppGpp synthesis by SAS enzymes is induced through transcriptional activation of the encoding genes and/or post-translational modifications. For example, RelP and RelQ from *Staphylococcus aureus* are part of the VraRS cell-wall stress regulon and are induced after vancomycin treatment (Geiger et al. 2014). In *B. subtilis*, phosphorylation of the response regulator WalR controls the heterogeneous expression of RelP (Libby et al. 2019), while in *Clostridioides difficile*, *relQ* transcription is induced by sublethal concentrations of different antibiotics (clindamycin, metronidazole, vancomycin, and fidaxomicin) (Pokhrel et al. 2020, Poudel et al. 2022). Post-translational activation/inhibition of SAS proteins is also important for function. RelQ from *B. subtilis* and *Enterococcus faecalis* form tetramers (Steinchen et al. 2015, Beljantseva et al. 2017). RelQ activity is strongly inhibited through the binding of single-stranded RNA (ssRNA). (p)ppGpp binding leads to disassociation of the RelQ: RNA complex and activation of RelQ (Beljantseva et al. 2017). RelP activity is inhibited by both pppGpp and ppGpp, activated by Zn^{2+}, and insensitive to inhibition by RNA (Manav et al. 2018, Steinchen et al. 2018). Whereas in *S. aureus*, RelQ seems to be insensitive to ssRNA, and RelP is not allosterically stimulated by the addition of pppGpp, ppGpp, or pGpp (Yang et al. 2019). In this bacterium, RelQ is mainly held in an inactive state when overexpressed (Horvatek et al. 2020). Thus, the expression and activity of RelP and RelQ seem to differ considerably between species, although these two proteins are highly homologous. Post-translational regulatory mechanisms are likely important to fine-tune (pp)pGpp synthesis under different growth conditions.

(p)ppGpp breaking

Rel is so far the only described enzyme to degrade (p)ppGpp in Firmicutes and thus important to reset the system. Under non-inducing conditions, Rel is primarily in a hydrolase-on/synthetase-off conformation (Gratani et al. 2018), while starved ribosomes are sufficient to inhibit the hydrolytic activity (Takada et al. 2021). The strong hydrolase activity of Rel from *S. aureus* makes the enzyme essential for the detoxification of (p)ppGpp produced by RelP or RelQ (Geiger et al. 2010). In *B. subtilis and E. faecalis*, *rel* mutants are viable but severely impaired in growth due to (p)ppGpp overproduction (Srivatsan et al. 2008, Gaca et al. 2013, Ababneh and Herman 2015). The hydrolase NahA of *B. subtilis* efficiently hydrolyses (p)ppGpp to produce pGpp, thereby modulating alarmone composition and function (Yang et al. 2020). The results indicate the existence and physiological relevance of pGpp as a third alarmone, with functions that can be distinct from those of (p)ppGpp.

Targets of (p)ppGpp

In gram-positive pathogens, the stringent response plays important roles in virulence (for reviews, see Kundra et al. 2020) and antibiotic tolerance (discussed below). The global transcriptional effects of (p)ppGpp have been examined previously in several Firmicutes, such as *B. subtilis* (Eymann et al. 2002), *Streptococcus pneumoniae* (Kazmierczak et al. 2009), *E. faecalis* (Gaca et al. 2012), *Streptococcus mutans* (Nascimento et al. 2008), and *S. aureus* (Geiger et al. 2012, Horvatek et al. 2020). Many of the observed downstream effects are mediated via specific binding of (p)ppGpp to target proteins resulting in inhibition of replication (DnaG), ribosome function (RsgA, RbgA, Der, Era, HflX, and ObgE), secretion (Ffh and FtsY), and GTP synthesis (GuaB, HprT, Gmk, GdpP, Pde, PgpH, and PurR) (for reviews, see Steinchen et al. 2020, Anderson et al. 2021, Zegarra et al. 2023). Moreover, (p)ppGpp can control transcription via (p)ppGpp-specific riboswitches (Sherlock et al. 2018).

Of note, in contrast to *E. coli*, (p)ppGpp in Firmicutes does not interfere with RNA polymerase activity (Hauryliuk et al. 2015). Instead, in these bacteria, (p)ppGpp regulates transcription indirectly by strongly lowering the intracellular GTP pool (Krasny et al. 2008, Geiger et al. 2012, Kriel et al. 2012, Kastle et al. 2015). A decrease in the GTP levels leads to the repression of nucleotide-sensitive, GTP-initiating promoters, e.g. those of rRNA genes (Krasny and Gourse 2004, Kastle et al. 2015). Low GTP levels also affect the CodY regulon in most Firmicutes. The transcription factor CodY, when loaded with GTP and branched-chain amino acids, acts mainly as a repressor of many genes involved in amino acid synthesis and virulence (Pohl et al. 2009, Majerczyk et al. 2010, Geiger and Wolz 2014). However, GTP is not a co-factor for CodY in streptococci (Zhu et al. 2019). Recently, it was shown that (p)ppGpp also targets the transcriptional regulator PurR, which adds a further mechanism how the alarmone can modulate transcription (Anderson et al. 2021). The mechanism of activation of the stringent response and the main effect of (p)ppGpp on bacterial physiology in Firmicutes are summarized in Fig. 1.

(p)ppGpp and antibiotic resistance

(p)ppGpp and -lactam resistance in MRSA

Antibiotic resistance represents a growing threat to global health, and the clinical burden of infections caused by antimicrobial-resistant pathogens is immense. Hereby, methicillin-resistant *S. aureus* (MRSA) is of particular concern. In the last few years, several groups have observed connections between methicillin resistance in *S. aureus* and the stringent response (Table 1). Methicillin resistance is conferred by the presence of the *mecA/mecC* gene that encodes the penicillin-binding protein PBP2A, a peptidoglycan transpeptidase with extremely low affinity for methicillin and other β-lactam antibiotics (Hartman and Tomasz 1984). Mupirocin inhibits the bacterial isoleucyl tRNA synthetase and, as

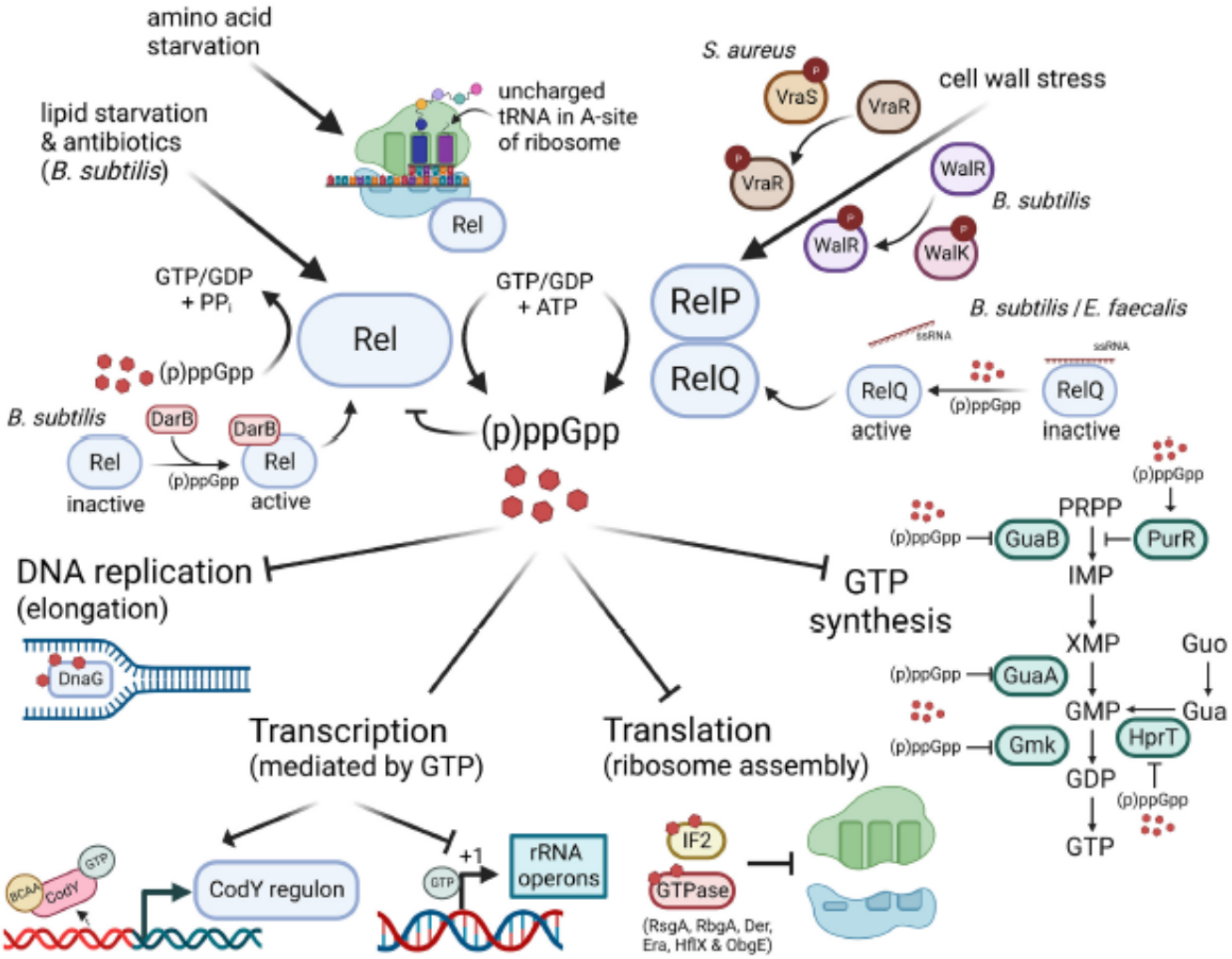

Figure 1. Activation of the stringent response, synthesis of (p)ppGpp, and main effects of (p)ppGpp on bacterial physiology in Firmicutes. Rel-dependent (p)ppGpp synthesis is induced during amino acid starvation (by sensing of stalled ribosomes by Rel), but in *B. subtilis* also during lipid starvation, heat shock, and antibiotic stress. RelP and RelQ are activated during cell wall stress by the two-component systems VraRS in *S. aureus* or WalKR in *B. subtilis*. RelQ is also activated via the (p)ppGpp-induced release of an inactivating ssRNA. The substrates for synthesis are GTP or GDP and ATP, and the reaction is catalyzed by the synthetase domain of Rel and SAS enzymes (RelP/RelQ). (p)ppGpp degradation occurs solely by the hydrolase domain of Rel. Accumulation of (p)ppGpp modulates then bacterial physiology by directly binding to a variety of enzymes or via lowering of the GTP pools. Low intracellular levels of GTP and BCAA relieve the repression of the transcriptional regulator CodY. In addition, GTP depletion represses the transcription of genes, e.g. rRNA operons, requiring GTP as their initiating nucleotide. Created with BioRender.com.

such, induces Rel-dependent (p)ppGpp synthesis. Mupirocin treatment of MRSA strain N315 in combination with the β-lactam antibiotic oxacillin increases the expression of *mecA*, *pbpB*, and *pbpD* and confers resistance towards oxacillin (Aedo and Tomasz 2016). Further, induction of the stringent response converted an initially heterogeneous population with only a small subset of bacterial cells extremely resistant to β-lactams into a homogeneous and highly resistant population (Kim et al. 2013, Aedo and Tomasz 2016). Mwangi et al. used mutant selection and whole genome sequencing to identify two point mutations in *rel* that conferred high-level resistance (Mwangi et al. 2013). Both mutations impaired the hydrolase activity of Rel and thus increased (p)ppGpp levels. The mutant phenotype could be partially rescued by additional loss-of-function mutations within *relQ*. These data indicate that (p)ppGpp produced by either Rel or RelQ can result in enhanced oxacillin resistance. In strain N315 *relQ* deletion alone did not affect the level of β-lactam resistance (Matsuo et al. 2019). However, overexpression of *relQ* did result in an elevated MIC for oxacillin. In *Streptococcus pneumoniae*, inhibition of (p)ppGpp synthesis as well as other interactions in purine metabolism lead to reduced β-lactam and glycopeptide sensitivity manifested by increased relative growth (Leshchiner et al. 2022). In summary, an impact of (p)ppGpp on oxacillin resistance is supported by several studies.

(p)ppGpp-defective bacteria show decreased MIC for diverse antibiotics

(p)ppGpp also influences the sensitivity of bacteria to other antibiotics (Table 1). A (p)ppGpp^0 (common nomenclature for a strain in which all synthetases are mutated) strain of *E. faecalis* showed enhanced sensitivity not only towards the cell-wall targeting antibiotic vancomycin (Abranches et al. 2009), but also to the bactericidal drugs norfloxacin, ampicillin, and chloramphenicol (Gaca et al. 2013). *S. aureus* and *S. pneumoniae rel* mutants were shown to display a significantly higher susceptibility towards mupirocin than the parental strains (Kazmierczak et al. 2009, Geiger et al. 2010). It is assumed that upregulation of genes involved in isoleucine

Table 1. (p)ppGpp mediated antibiotic resistance, tolerance, and persistence in Firmicutes.

	Organism	Genotype/Method of induction	Phenotype	Literature
Antibiotic resistance	*S. aureus*	Upregulation of *mecA* expression during mupirocin treatment (SR induction)	Resistance to β-lactam antibiotics	(Kim et al. 2013, Aedo and Tomasz 2016)
	S. aureus	Increased (p)ppGpp synthase activity of RelQ/decreased (p)ppGpp hydrolase activity of Rel due to point mutations	Increased (p)ppGpp levels are associated with oxacillin resistance	(Mwangi et al. 2013)
	S. aureus	Δ*relQ*	Reduced resistance to β-lactam antibiotics	(Bhawini et al. 2019)
	S. aureus	*relQ* overexpression	Increased MIC for oxacillin	(Matsuo et al. 2019)
	E. faecalis	Δ*relQ*, Δ*relAQ*	Decreased MIC for vancomycin	(Abranches et al. 2009)
	S. aureus/*S. pneumoniae*	Δrel_{syn}	Decreased MIC for mupirocin	(Kazmierczak et al. 2009, Geiger et al. 2010)
	B. subtilis	Δ*umiR*/(p)ppGpp^0	Decreased MIC for tiamulin, retapamulin, lincomycin, iboxamycin, and virginiamycin M1	(Takada et al. 2022)
	S. aureus	Blocking of (p)ppGpp with MIP-NP	Increased efficiency rates for kanamycin and tetracycline	(Chen et al. 2022)
	B. subtilis	Δrel_{syn}, (p)ppGpp^0	Bacteriostatic antibiotics chloramphenicol and tetracycline become bactericidal, decreased MIC for (p)ppGpp^0	(Yang et al. 2022)
	B. subtilis	Δ*rel*, (p)ppGpp^0	Cerulenin treatment is bactericidal	(Pulschen et al. 2017)
	S. aureus	Δrel_{syn}, (p)ppGpp^0	Mupirocin treatment is bactericidal	(Geiger et al. 2010, Geiger et al. 2014)
Antibiotic tolerance	*S. aureus*	Mupirocin treatment	Tolerance to vancomycin	(Singh et al. 2017)
	S. aureus	*ileS* mutation	Tolerance to vancomycin, imipenem, and daptomycin	(Singh et al. 2017)
	S. aureus	Δ*relPQ* and (p)ppGpp^0	Decreased tolerance to vancomycin and ampicillin	(Geiger et al. 2014)
	E. faecalis	Δ*relQ*, Δ*rel* Δ*relQ*	Decreased tolerance to vancomycin	(Abranches et al. 2009)
	E. faecalis/*B. subtilis*	Δ*rel* (p)ppGpp^0	Enhanced tolerance to vancomycin Decreased tolerance to vancomycin after chloramphenicol pretreatment	(Abranches et al. 2009, Yang et al. 2022)
	E. faecalis	Δ*relQ* → low (p)ppGpp	Decreased survival in presence of norfloxacin and ampicillin	(Gaca et al. 2013)
	S. aureus	Nonsense mutation in *rel* → high (p)ppGpp	Enhanced tolerance to ciprofloxacin	(Matsuo et al. 2019)
	S. aureus	Point mutation in *rel*	Enhanced tolerance to daptomycin	(Berti et al. 2018)
	E. faecium	Point mutation in *rel*	Tolerance to vancomycin, linezolid, and daptomycin in biofilm	(Honsa et al. 2017)
	S. aureus	Δ*rsgA* or *rsgA* mutation	Tolerance to β-lactam antibiotics, vancomycin and ciprofloxacin	(Corrigan et al. 2016)
	C. difficile	Inhibition of Rel activity by (p)ppGpp analaog relacin or antisense *rel* RNA	Reduced tolerance to clindamycin and metronidazole	(Pokhrel et al. 2020)

Table 1. Continued

	Organism	Genotype/Method of induction	Phenotype	Literature
	B. subtilis/*E. faecalis*	(p)ppGpp^0, eleveated intracellular GTP levels	Bactericidal effect of chloramphenicol and tetracycline	(Yang et al. 2022)
	S. aureus	Triclosan treatment	Increased tolerance to ampicillin, kanamycin, streptomycin, and ciprofloxacin	(Westfall et al. 2019)
	S. aureus	(p)ppGpp^0	Decreased tolerance to vancomycin in biofilm, increased resolution of biofilm in presence of vancomycin	(Salzer et al. 2020)
	S. aureus	(p)ppGpp^0	Increased biofilm and planktonic culture antibiotic tolerance to ciprofloxacin and vancomycin	(Walsh et al. 2023)
	E. faecium	relA mutation → elevated (p)ppGpp	Increased biofilm antibiotic tolerance to linezolid and daptomycin	(Honsa et al. 2017)
Persistence	*S. aureus*	Δrel_{syn}, (p)ppGpp^0	(p)ppGpp is not required for persister formation	(Conlon et al. 2016)
	B. subtilis	Multisite phosphorylation leads to phenotypic variation in *sasA* expression levels	Increased tolerance to ciprofloxacin in cells expressing high *sasA* levels	(Libby et al. 2019, Diez et al. 2020, Diez et al. 2022)
	B. subtilis	GTP depletion by (p)ppGpp induction	Persistence to vancomycin and ciprofloxacin (tolerance in subpopulation of cells with GTP depletion)	(Fung et al. 2020)

biosynthesis in the wildtype but not in the mutants increases the intracellular levels of isoleucine, which antagonizes mupirocin and thereby enhances the MIC (Geiger et al. 2010).

In *B. subtilis*, (p)ppGpp-mediated signalling was shown to enhance the expression of the ABCF ATPase VmlR—a resistance factor conferring resistance to the translation inhibitors pleuromutilin, linocsamide, and type A streptogramin—during challenge with VmlR-cognate antibiotics (Takada et al. 2022). Significant inhibition of translation is the key to VmlR induction. Accordingly, VmlR mainly functions during the stationary phase, when nutrients are scarce, and bacteria accumulate (p)ppGpp. (p)ppGpp accumulation reduces translation initiation by binding of (p)ppGpp to the initiation factor 2 (IF2) and associated GTPases (Corrigan et al. 2016, Diez et al. 2020). Thus, genetic disruption of *vmlR* or the (p)ppGpp synthesis pathway caused sensitivity to the pleuromutilin tiamulin, lincosamides such as the natural product lincomycin, as well as the more potent semi-synthetic antibiotic clindamycin, the recently developed fully synthetic iboxamycin, and the type A streptogramin virginiamycin M1 (Takada et al. 2022). Moreover, specific binding of molecularly imprinted nanoparticles (MIP-NP) to (p)ppGpp led to a significant increase in antibiotic efficiency rates (kanamycin, oxytetracycline) both in *E. coli* and *S. aureus* (Chen et al. 2022).

(p)ppGpp is needed to prevent the bactericidal effect of antibiotics

Not only is the MIC of a variety of antibiotics lower for (p)ppGpp-defective bacteria, but there have also been several studies showing that antibiotics that exhibit bacteriostatic activity in wild-type cells become bactericidal in (p)ppGpp-deficient cells (Table 1) (Kazmierczak et al. 2009, Geiger et al. 2010, Gaca et al. 2013, Pulschen et al. 2017, Yang et al. 2022). For example, Yang et al. not only observed a decreased MIC for the bacteriostatic antibiotics chloramphenicol and tetracycline but also the bactericidal activity of these antibiotics in (p)ppGpp-defective *B. subtilis* (Yang et al. 2022), similar to what was observed for cerulenin treatment (Pulschen et al. 2017).

Altogether, increased antibiotic resistance towards several antibiotics was observed to correlate with high (p)ppGpp concentrations. (p)ppGpp-mediated resistance might be conferred by up-regulation of resistance genes, e.g. *mecA* (Aedo and Tomasz 2016), slow growth, e.g. via inhibition of translation (Corrigan et al. 2016), and/or (p)ppGpp related changes in the nucleotide pool. The latter is supported by the observation that purine availability and metabolism, which are unbalanced in (p)ppGpp-defective *S. aureus*, can control antibiotic susceptibility towards oxacillin (Nolan et al. 2022). Further studies are needed to clarify which of these putative mechanisms is responsible for the antibiotic resistance observed in the different cases.

(p)ppGpp and antibiotic tolerance

Antibiotic tolerance is an important contributor to antibiotic treatment failure, but it also acts as a precursor to antibiotic resistance (Levin-Reisman et al. 2019, Windels et al. 2019). As most antibiotics target active metabolic processes, a decreased growth rate and reduced metabolic activity contribute to the development of multidrug tolerance. (p)ppGpp leads to an immediate shut-down of replication and translation (for reviews, see Sinha et al. 2020, Zegarra et al. 2023), and high (p)ppGpp levels likely contribute to antibiotic tolerance. This assumption is supported by the following studies (Table 1).

Contribution of small alarmone synthetases (SAS) to antibiotic tolerance

In *S. aureus*, *relP*, and *relQ* expression is specifically induced after treatment with the cell-wall active antibiotics ampicillin and vancomycin (Geiger et al. 2014). The elevated (p)ppGpp levels in turn protect from antibiotic killing, since a *relPQ* double mutant showed a reduced survival rate upon treatment with these antibiotics, but no difference in MIC between wild-type and *relPQ* mutants could be observed (Geiger et al. 2014). Likewise, mutation of *ileS*, encoding the isoleucyl-tRNA synthetase, leads to Rel-dependent (p)ppGpp synthesis and again increased vancomycin tolerance (Singh et al. 2017). Similar observations were reported for *E. faecalis*. In growth curve experiments with sub-inhibitory concentrations of vancomycin, enhanced tolerance was observed in a *relA* mutant strain that exhibited higher (p)ppGpp levels due to missing hydrolase activity (Abranches et al. 2009). By contrast, a *relQ* mutant strain grew slower than its parental strain and was more rapidly killed in time-kill experiments using a 5x MIC of vancomyin. In *B. subtilis*, chloramphenicol treatment resulted in Rel-dependent (p)ppGpp synthesis and thereby increased tolerance to vancomycin (Yang et al. 2022).

The impact of (p)ppGpp on tolerance towards other antibiotics is less clear. In *E. faecalis*, a (p)ppGpp^0 (*rel*, *relQ*) strain is more sensitive to killing by norfloxacin and ampicillin (Gaca et al. 2013). In methicillin-sensitive *S. aureus* (MSSA), mutations of *rel* that likely lead to higher (p)ppGpp accumulation and an overall stringent-response-like transcription pattern have been linked to ciprofloxacin tolerance (Matsuo et al. 2019). However, Geiger et al. did not observe ciprofloxacin tolerance in *S. aureus* mutants with defective (p)ppGpp synthetases (Geiger et al. 2014). During prolonged daptomycin exposure, *S. aureus* acquired several mutations, one of which was within *rel* (L61F). A *de novo*-generated strain carrying the sole mutation L61F in Rel was less susceptible to daptomycin in time-kill assays (Berti et al. 2018). Of note, it is not clear whether the mutation made the bacteria produce more or less (p)ppGpp. Chemical inhibition of *rel* by the (p)ppGpp analog relacin or transcriptional repression by antisense *rel* RNA reduced tolerance to clindamycin and metronidazole (Pokhrel et al. 2020). Very recently, Yang et al. showed that *B. subtilis* and *E. faecalis* produce (p)ppGpp in response to chloramphenicol treatment through *rel*, which protected the bacteria from antibiotic killing (Yang et al. 2022). The protective effect could be linked to the decreased intracellular GTP pool. Elevated GTP levels in a (p)ppGpp^0 strain promoted the bactericidal effect of chloramphenicol. The widely used antimicrobial triclosan is a bacteriostatic inhibitor of fatty acid synthesis and has been shown to increase tolerance to bactericidal antibiotics in *E. coli* and MRSA (Westfall et al. 2019). Very recently, it has been shown that triclosan treatment induces antibiotic tolerance to ciprofloxacin and vancomycin via the stringent response in *S. aureus* planktonic cultures and biofilms (Walsh et al. 2023).

Biofilm-related antibiotic tolerance

Biofilms are normally more tolerant to high concentrations of antibiotics than planktonic cultures. In *S. aureus*, we could show that vancomycin-induced *relPQ* expression contributes to biofilm formation and biofilm-related antibiotic tolerance. Thus, RelP and RelQ play a major role in biofilm formation and maintenance under cell wall stress conditions (Salzer et al. 2020). A persistent vancomycin-resistant *Enterococcus faecium* (VRE) isolate harboured a *rel* mutation, resulting in elevated (p)ppGpp levels (Honsa et al. 2017). The strain showed tolerance towards linezolid and

daptomycin when growing in biofilm but remained susceptible in MIC testing and during planktonic growth.

In summary, several studies indicate that elevated (p)ppGpp, either through mutations of the Rel hydrolase or via activation of synthetases, can contribute to antibiotic tolerance without necessarily conferring resistance. However, (p)ppGpp is likely not the only or even the main trigger for bacterial tolerance.

(p)ppGpp and formation of persister cells

The molecular mechanisms underlying persister cell formation are highly debated, and the role of (p)ppGpp in this process remains unclear (Balaban et al. 2013, Kaldalu et al. 2016, Kaldalu et al. 2020, Pacios et al. 2020, Song and Wood 2020, Fernandez-Garcia et al. 2022, Verstraete et al. 2022). Persister cells are thought to be 'dormant' subpopulations that can either be carried over from the stationary phase (Type I) or formed by stochastic switching during exponential growth (Type II) (Balaban 2011). The current standard method for persister identification at the population level is a biphasic killing curve. In many studies claiming to analyse persisters such a two-phase kill curve is not demonstrated, hampering the interpretation of many findings. The term 'triggered persistence' indicates a form of persistence that is induced by particular signals. Persistence seems to be an evolvable trait, indicating the existence of active mechanisms that form and maintain the persister phenotype (Wilmaerts et al. 2019, Verstraete et al. 2022). However, it was also assumed that persistence arises as a result of stress and errors (PASH hypothesis-Persistence as stuff happens) rather than 'stochastically' as a form of 'bet hedging' (Levin et al. 2014, Fernandez-Garcia et al. 2022).

(p)ppGpp heterogeneity on the single cell level

Nevertheless, genetic factors can impact persistence levels. (p)ppGpp and toxin/antitoxin systems were discussed as major triggers for dormancy/persistence, at least in Proteobacteria (Korch et al. 2003, Amato et al. 2013, Helaine et al. 2014, Chowdhury et al. 2016, Svenningsen et al. 2019, Pontes and Groisman 2020, Urbaniec et al. 2022, Verstraete et al. 2022). However, the low frequency combined with the transient nature of persister cells makes them very challenging to study. Some models assume that stochastic (p)ppGpp synthesis in a very small number of cells leads to persistence. So far, it has not been possible to measure the levels of (p)ppGpp in single cells, and such assumptions are based on the stochastic expression of stringently regulated marker genes. However, most of the marker genes are also highly regulated independently of (p)ppGpp. The first paper describing such stochastic (p)ppGpp-dependent regulation in *E. coli* has been retracted (Maisonneuve et al. 2018), and the assumption has been challenged by several groups (Ramisetty et al. 2016, Liu et al. 2017, Goormaghtigh et al. 2018). Internalization of *Salmonella* by macrophages induces phenotypic heterogeneity, leading to the formation of non-replicating persisters, which could serve as a reservoir for relapsing infection. Deletion of *relA* and *spoT* resulted in decreased persister numbers, supporting that (p)ppGpp contributes to persistence under these specific conditions. However, in many species, (p)ppGpp mutants are often severely impaired in many processes and often gain compensatory mutations. Thus, these studies were questioned for their ability to conclusively determine the involvement of (p)ppGpp in persister cell formation (Kaldalu et al. 2020, Pontes and Groisman 2020).

It was also proposed that inactivation of ribosomes via (p)ppGpp-dependent dimerization is a general mechanism leading to persistence (Song and Wood 2020, Fernandez-Garcia et al. 2022). In *E. coli*, (p)ppGpp stimulates the expression of hibernation factors to dimerize 70S ribosomes into 100S ribosomes (Song and Wood 2020). Ribosome dimerization protects ribosomal proteins and rRNA from degradation while allowing rapid recovery from stress upon dimer dissociation. For *E. coli*, it was shown that such resuscitation from the dormant state is heterogeneous, and persistence decreases in the absence of (p)ppGpp. However, (p)ppGpp did not affect the resuscitation of single-cell persisters (Chowdhury et al. 2016, Kim et al. 2018).

Is there evidence for a role of (p)ppGpp in persister formation in Firmicutes?

In *S. aureus*, persister formation following ciprofloxacin or gentamycin exposure does not require (p)ppGpp since a (p)ppGpp^0 mutant shows a similar rate of persister formation (Conlon et al. 2016). Hibernation is controlled by one long hibernation-promoting factor (HPF) homologue and two translation factors. For resuscitation, 100S ribosomes are split synergistically by the action of ribosome-recycling factor (RRF), GTPase elongation factor G (EF-G), and HflX (Gohara and Yap 2018). Expression of *hfp* and the recycling factor *hflX* is independent of (p)ppGpp (Horvatek et al. 2020). However, HflX was shown to bind (p)ppGpp (Corrigan et al. 2016). Furthermore, disassembly of the 100S ribosome strictly requires GTP hydrolysis (Basu and Yap 2017, Basu et al. 2020). Thus, the changing nucleotide pools upon stringent response are likely to impact 100S disassembly on the protein level and thus may contribute to rapid growth resumption. In *B. subtilis*, induction of a (p)ppGpp synthetase-encoding gene in a pppGpp^0 strain resulted in 100 S ribosome formation (Tagami et al. 2012), further indicating that also in Firmicutes, (p)ppGpp is involved in hibernation and/or disassembly of 100 S ribosomes. However, whether such processes are stochastic and thus involved in persistent formation remains unclear.

In *B. subtilis*, there is evidence for stochastic (p)ppGpp synthesis. Cultures experiencing nutrient limitation contain distinct subpopulations of cells exhibiting either comparatively high or low protein synthesis activity. This heterogeneity is determined by (p)ppGpp levels (Libby et al. 2019, Diez et al. 2020, Diez et al. 2022). *sasA* encoding the small synthetase SasA/RelP exhibits high levels of extrinsic noise in expression. Rare cells with unusually high levels of *sasA* expression display increased antibiotic tolerance. *sasA* expression depends on the transcription factor WalR, which is in turn regulated via multisite phosphorylation by the Ser/Thr kinase-phosphatase pair PrkC/PrpC and the histidine kinase WalK of the WalKR two-component system (Libby et al. 2019). Furthermore, the absence of any of the (p)ppGpp synthases results in the loss of bimodality. RelA and SasB/RelQ activities are necessary to generate the subpopulation of cells exhibiting low protein synthesis. Interestingly, SasA/RelP generates the subpopulation with high protein synthesis (Diez et al. 2022). This complex interaction is likely due to allosteric regulation of SasB/RelQ by the SasA/RelP product pGpp and the RelA product pppGpp.

It was also shown that in *B. subtilis*, a drop in GTP pools due to induction of the stringent response induces persister formation, and GTP was implicated as the major metabolic effector of (p)ppGpp-mediated persistence (Fung et al. 2020). Altogether, this complex interaction indicates that (p)ppGpp expression is indeed heterogeneous in *B. subtilis* and might thus contribute to the persister phenotype. Whether similar regulation occurs in other Firmicutes is less clear since, albeit highly homologous, the SAS enzymes can show major differences with regard to gene expression and allosteric regulation (Yang et al. 2019). For example, in *S. aureus*, *relP* and *relQ* are under the control of the VraRS

two-component system (Geiger et al. 2014), whereas in *B. subtilis*, at least *relP* is controlled by the WalKR system (Libby et al. 2019). In *S. aureus* and *E. faecalis*, (p)ppGpp contributes to antibiotic tolerance (Abranches et al. 2009, Gaca et al. 2013, Geiger et al. 2014). Whether this indicates a role for (p)ppGpp in triggered persistence is less clear since clear biphasic kill curves were not provided in the above-mentioned studies.

(p)ppGpp and survival in persistent infections

Whereas the role of (p)ppGpp in persister cell formation is still controversial, there is evidence that (p)ppGpp contributes to establish long-term (persistent) infections in diverse host niches. Of note, the term 'persistent infection' has to be clearly distinguished from 'antibiotic persistence' described above. Persistent infection is generally used to describe infections in the host that are not cleared by the host immune system, whereas antibiotic persistence describes a bacterial population that is refractory to antibiotic treatments (Brauner et al. 2016, Balaban et al. 2019). There are several studies indicating that elevated basal (p)ppGpp levels and/or induction of the stringent response *in vivo* contribute to the development of persistent infections.

Increased (p)ppGpp synthesis promotes persistent infections

In *E. faecalis*, it has been observed that during prolonged bloodstream infection in an immunocompromized host, *relA* missense mutations arise that constitutively activate the stringent response. These mutations were associated with a fitness tradeoff but also increased antibiotic tolerance *in vivo* and delayed eradication in an immunocompromized host (Honsa et al. 2017). No change in MIC for several antibiotics, including those that had been administered to the infected patient was observed.

Likewise, after *S. aureus* persistent infection a strain with higher (p)ppGpp levels due to mutations of *rel* (F128Y) was isolated (Gao et al. 2010). Elevated (p)ppGpp levels increased resistance to β-lactam antibiotics and provided a significant fitness advantage in presence of bactericidal antibiotics as a result of (p)ppGpp-mediated reduced growth (longer lag time and/or lower growth rate) (Bryson et al. 2020, Deventer et al. 2022). Moreover, even strains from persistent bacteremia without mutation within *rel*, *relP*, or *relQ* were shown to display significantly higher (p)ppGpp levels compared to MRSA isolates from resolving bacteremia (Li et al. 2020). The persistent strains also showed high expression of the virulence peptides phenol-soluble modulins (PSMs), higher polymorphonucleate (PMN) lysis and survival, and endothelial cell adherence, which were linked to the elevated (p)ppGpp levels. The molecular basis for the upregulation of (p)ppGpp in these strains remains unknown.

Collectively, in *S. aureus* as well as in *E. faecalis* an increase in (p)ppGpp levels has been linked to more chronic persistent infections. The mutations might have been selected due to their immune evasion properties or their higher antibiotic tolerance phenotype.

(p)ppGpp growth control and survival *in vitro*

(p)ppGpp is a major regulator of growth-rate control in *E. coli* (Potrykus et al. 2011, Ahn-Horst et al. 2022). However, the effects of (p)ppGpp on global protein synthesis and survival to starvation seem to be highly dependent on the conditions (N- vs C-limitation) (Iyer et al. 2018). In *S. aureus*, pppGpp^0 mutants are still able to inhibit rRNA synthesis in the later growth phase (Kastle et al. 2015). This indicates that, at least in *S. aureus*, (p)ppGpp-independent mechanisms also account for growth-rate control when nutrients become limited. (p)ppGpp seems to have different roles depending on its abundance in the cell (Fernandez-Coll and Cashel 2020). The role of (p)ppGpp in bacterial survival under non-stressed conditions, i.e. when the levels of (p)ppGpp are low, is less clear. It was proposed that basal levels of (p)ppGpp may have different effects than high level bursts during stressful situations (Fernandez-Coll and Cashel 2020). Several binding partners of (p)ppGpp have been uncovered, displaying dissociation constants from below one micromolar to more than one millimolar. Nucleotide metabolism enzymes are among the first targets to be affected by rising (p)ppGpp, while more fundamental processes such as DNA replication are among the last (Steinchen et al. 2020). (p)ppGpp^0 strains in Firmicutes usually show no growth defect under non-stressed conditions, e.g. in rich medium (Abranches et al. 2009, Geiger et al. 2010, Kriel et al. 2012). However, (p)ppGpp^0 mutants have altered transcriptomes, even under non-stressed conditions (Gaca et al. 2013), and basal levels of (p)ppGpp seem to be important to maintain GTP homeostasis in Firmicutes but not in Proteobacteria (Anderson et al. 2019). After shift to amino acid limiting conditions a sudden raise of (p)ppGpp is necessary to protect the bacteria from death (Geiger et al. 2010, Kriel et al. 2012, Bittner et al. 2014, Kriel et al. 2014). In *B. subtilis*, it was shown that controlling GTP accumulation is required for survival during amino acid starvation (Bittner et al. 2014). Manipulating the GTP levels in pppGpp^0 mutants provided evidence that GTP regulates growth rate in a dose-dependent manner in *B. subtilis*. In the wildtype, (p)ppGpp restricts the upper limit of GTP to ensure optimal growth and survival.

Inhibition of fatty acid synthesis by cerulenin in *B. subtilis* was also found to be lethal only in the absence of (p)ppGpp synthesis (Pulschen et al. 2021). Adaptation to fatty acid starvation in wildtype bacteria requires the activity of the Rel synthetase, although (p)ppGpp levels remain below detection limit. Thus, (p)ppGpp-mediated balancing of the GTP/ATP ratio is essential for bacterial survival at least under certain conditions (fatty acid or amino acid starvation). Under such conditions, the GTP/ATP ratio in pppGpp^0 strains remains high and probably keeps the cell growing, which will eventually perturb membrane function and lead to cell death.

In certain media, the survival of the *S. aureus* (p)ppGpp^0 mutant is diminished in the stationary phase, which has also been linked to uncontrolled GTP accumulation (our unpublished data). In the stationary phase, *S. aureus* acquires tolerance towards oxidative stress by maintenance of redox and iron homeostasis, which is mediated by (p)ppGpp (Fritsch et al. 2020, Horvatek et al. 2020). Consistently, in the (p)ppGpp^0 mutant, the antioxidant stress response genes were induced due to elevated ROS levels in the stationary phase (Fritsch et al. 2020). (p)ppGpp synthesis by the inducible synthetases resulted in the activation of ROS-inducing toxins and the simultaneous expression of detoxifying systems to protect the producer. Thereby, (p)ppGpp prepares the cells for survival to elevated ROS (Horvatek et al. 2020). Thus, in *S. aureus*, (p)ppGpp not only confers tolerance to antibiotics but also to ROS (Fritsch et al. 2020, Horvatek et al. 2020), although oxidative stress *per se* does not seem to induce (p)ppGpp synthesis.

Conclusion and outlook

A tight control of (p)ppGpp level is crucial for bacterial growth and survival. This balance is achieved by the activity of three

(p)ppGpp synthetases (Rel, RelP, and RelQ) and the strong hydrolase activity of Rel. Rel and RelQ-dependent (p)ppGpp synthesis is usually restricted and activated under certain conditions, including amino acid limitation and allosteric activation via (p)ppGpp. Under 'stringent conditions', (p)ppGpp contributes to active adaptation to stressful conditions mainly via up-regulation of certain genes, e.g. amino acid synthesis genes, oxidative stress response genes, and virulence genes. (p)ppGpp acts mainly as an inhibitory molecule (Anderson et al. 2021). High (p)ppGpp levels result in severe growth arrest, which is nevertheless beneficial for the bacteria to survive under certain conditions, e.g. antibiotic stress *in vitro* and during persistent infections *in vivo*. However, (p)ppGpp overproduction comes with a fitness disadvantage. Thus, (p)ppGpp levels are usually controlled to allow optimal growth under non-stressed conditions (basal level) but also to protect from sudden insults (stringent conditions). In Firmicutes, the (p)ppGpp-mediated restriction of GTP accumulation is at least one, if not the only, mechanism for protection. How uncontrolled GTP levels in $pppGpp^0$ strains lead to cell death remains to be elucidated.

In recent years, there has also been growing evidence that the nature of the alarmone (pppGpp, ppGpp, or pGpp) influences the stringent phenotype. The affinity of these molecules towards different targets can differ considerably (Steinchen et al. 2020, Anderson et al. 2021). While ppGpp and pppGpp are well-established alarmones, it has long been reported that bacteria can also produce other nucleotide derivatives, such as pGpp and (p)ppApp (Anderson et al. 2021). The function of pGpp remains elusive since its occurrence and potential activity were only recently appreciated in Firmicutes (Gaca et al. 2015, Horvatek et al. 2020, Yang et al. 2020). pGpp can be directly produced by certain (p)ppGpp synthetases using GMP and ATP as substrates or, alternatively, by enzymatic conversion from (p)ppGpp by the Nudix hydrolase NahA.

In summary, (p)ppGpp is an important molecule that controls bacterial survival and virulence. Of note, there might be major differences also between the different Firmicute species with respect to (p)ppGpp activation pathways as well as down-stream effects, e.g. GTP dysregulation. However, comparative studies have so far been rare, and species-specific properties remain to be further elucidated. It seems likely that the machineries to synthesize and sense (p)ppGpp have evolved in the different species to optimize adaptation to the unique environmental niches.

Common to all species is that high (p)ppGpp levels are growth limiting and thereby contributing to antibiotic tolerance. To what extent (p)ppGpp synthesis facilitates persister formation is still questionable. It was argued that the (p)ppGpp signalling pathway could be a prime target to simultaneously combat bacterial virulence and resistance. Although clinical validation of (p)ppGpp as a new target for antimicrobials is still missing, several approaches towards this end have been devised that show promise (for reviews, see Pulschen et al. 2021).

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(p)ppGpp-mediated GTP homeostasis ensures the survival and antibiotic tolerance of Staphylococcus aureus during starvation by preserving the proton motive force

Andrea Salzer[1], Sophia Ingrassia[1], Lisa Sauer[1], Johanna Rapp[1], Ronja Dobritz[1], Jennifer Müller[1], Hannes Link[1,2], Christiane Wolz[1,2]

[1] Interfaculty Institute of Microbiology and Infection Medicine

[2] Cluster of Excellence EXC 2124 “Controlling Microbes to Fight Infections”, University of Tübingen

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Corresponding author: Phone: +49 7071 2974648 Fax: +49 7071 295165.

E-mail: christiane.wolz@uni-tuebingen.de

Abstract

Upon nutrient limitation bacteria enter a viable nongrowing state and become antibiotic tolerant. Whether and how the messenger molecule (p)ppGpp contributes to transition in this state is debated. Here we show for *Staphylococcus aureus* that (p)ppGpp-dependent restriction of the GTP pool is essential for culturability of starved cells and for antibiotic tolerance. Elevated GTP levels in a starving (p)ppGpp-deficient mutant lead to a division-incompetent, dormant state characterized by low levels of ATP, reduced metabolic activity and alterations in membrane potential. GTP level control of nucleotide-sensitive promoters results in transcriptional downregulation of genes of the TCA cycle and electron transport chain. Increasing transcription of *qoxABCD*, a terminal oxidase of the respiratory chain, through mutation of the transcriptional start site partially restored the culturability of the (p)ppGpp-deficient mutant. Furthermore, we show that the loss of proton motive force (PMF) renders the bacteria susceptible to antibiotics, supporting the idea of applying (p)ppGpp or PMF inhibitors to combat antibiotic tolerant bacteria.

Introduction

Bacteria need to switch between rapid growth under favourable conditions and a nongrowing but stress-tolerant state under limiting conditions. In pathogenic bacteria, such a nongrowing state is often associated with persistent infections and antibiotic tolerance [1-3]. Nongrowing bacteria continue to synthesize macromolecules, have a basal metabolism [4] and are prepared to resume growth. Recent studies have demonstrated that the messenger molecule (p)ppGpp is central to coordinating optimal resource allocation under nutrient-limited conditions [5]. For *Escherichia coli*, (p)ppGpp was postulated to act as the major regulator of growth rate control 6,7. Even subtle fluctuations in levels of (p)ppGpp or basal levels that might be below the detection limit, such as during entry into the stationary growth phase, can modulate cellular processes and growth rate [8]. (p)ppGpp specifically interferes with replication, translation, and transcription [9]. In proteobacteria, (p)ppGpp directly binds to RNA polymerase to regulate transcription. In Firmicutes, however, (p)ppGpp does not interfere with RNA polymerase activity [10]. In these organisms, (p)ppGpp regulate transcription mainly by controlling intracellular GTP levels [11-14]. GTP homeostasis is controlled by specific inhibitors of enzymes involved in GTP synthesis, including the guanylate kinase Gmk, the IMP dehydrogenase GuaB, the hypoxanthine-guanine phosphoribosyltransferase HrpT, and the transcriptional regulator PurR [15]. Thus, (p)ppGpp synthesis correlates inversely with the GTP pool. A decrease in GTP levels during the stringent response may affect transcription via the GTP-sensitive transcriptional regulator CodY [16] or through regulation of initiation nucleotide (iNTP)-sensitive promoters [11,14,17].

The role of (p)ppGpp in stationary phase survival and bacterial tolerance in *Staphylococcus aureus* is controversial [3,18-20]. In this human pathogen and other Firmicutes species, synthesis and degradation of the messenger (p)ppGpp are orchestrated by the bifunctional Rel enzyme with synthetase and hydrolase activity and by the two synthetases SasA/RelP and SasB/RelQ [9,15]. Induction of the stringent response via amino acid limitation (Rel) or cell wall stress (RelPQ) contributes to pathogenicity and antibiotic tolerance [12,20]. However, the role of basal levels of (p)ppGpp in bacterial survival under nonstressed conditions is less clear. Here, we show that under nutrient stress, (p)ppGpp is essential for maintaining GTP homeostasis and thereby preserving electron transport chain activity and the proton motive force (PMF), which ensures culturability and antibiotic tolerance.

Results

(p)ppGpp contributes to culturability in the stationary phase

To study the requirement of (p)ppGpp synthesis for stationary phase survival, we monitored the growth and culturability (determined by CFU/ml enumeration) of *S. aureus* wildtype strain HG001 and its isogenic (p)ppGpp^0 mutant in chemically defined medium (CDM) for 24 h. (p)ppGpp^0 is unable to synthesize (p)ppGpp due to mutations in all three (p)ppGpp synthetases (full deletion of *rel*, synthetase mutations in *relP* and *relQ*) [21]. There was no significant difference in growth measured by optical density (OD_{600}) between the two strains (Fig. 1A). However, after entry into the stationary phase, the culturability of the (p)ppGpp^0 mutant quickly started to decrease until an approximately one log reduction in CFU/ml was observed after 24 h compared to that of wildtype (Figs. 1B & S1A). Reduced culturability of the (p)ppGpp^0 mutant was also observed in the background of the USA300 JE2 or SH1000 strains (the SigB-positive derivative and phage-cured derivative of HG001, respectively) (Fig. 1C). We next induced Rel-dependent (p)ppGpp synthesis in exponentially growing bacteria with a subinhibitory concentration of mupirocin, a tRNA synthetase inhibitor. These conditions mimicked amino acid starvation and resulted in significantly reduced culturability of the (p)ppGpp^0 mutant (Fig. S1B). Furthermore, we monitored expression levels of the known stringent response-activated genes *psmα* and *rsaD* [22,23] during the mid- and late stationary phases (17 h and 24 h) in wildtype and (p)ppGpp^0. We observed a stringent response-like expression pattern for *psmα* and strong derepression of *rsaD* in wildtype, indicating elevated (p)ppGpp levels (Fig. S1C). As expected, in the (p)ppGpp^0 mutant, expression of stringent response genes was not induced, but *rsaD* expression was strongly downregulated, suggesting elevated intracellular GTP levels (Fig. S1C). Individual complementation of (p)ppGpp^0 with rel_{syn}, *relP* or *relQ* (Fig. 1D) confirmed that Rel and RelQ contribute to basal (p)ppGpp levels to ensure survival during the stationary phase. Thus, (p)ppGpp is essential for bacterial culturability in the stationary phase as well as under induced stress conditions in exponentially growing bacteria.

(p)ppGpp-dependent GTP homeostasis is essential for stationary phase culturability independent of CodY

(p)ppGpp has been shown to play an important role in controlling GTP homeostasis [13,14]. Therefore, we assessed the viability of different mutants in which GTP metabolism was disrupted after growth to the late stationary phase. The pleiotropic repressor CodY, when loaded with GTP, inhibits expression of several biosynthesis-related gene clusters and virulence genes. CodY target genes are derepressed under stringent conditions in a (p)ppGpp/lowGTP-dependent manner [20]. We therefore hypothesized that the decreased

survival of the (p)ppGpp^0 mutant might be ascribed to diminished expression of CodY target genes. However, deletion of *codY* did not increase the culturability of the (p)ppGpp^0 mutant (Fig. 2B). PurR is a repressor of the purine operon [24,25], and in *B. subtilis*, it is allosterically activated via (p)ppGpp [15] (Fig. 2A). Thus, deletion of *purR* in the (p)ppGpp^0 mutant will likely lead to increased GTP levels. This mutant was even less culturable in the stationary phase, indicating that elevated GTP levels are detrimental to bacterial culturability (Fig. 2B). Accordingly, lowering the GTP pool should promote bacterial survival. Indeed, deletion of the GTP synthesis operon guaBA rescued the defect in the (p)ppGpp^0 mutant (Fig. 2B). We next manipulated levels of GTP via supplementation with guanine, which feeds into the salvage pathway of GTP synthesis (Fig. 2A). This resulted in a dose-dependent decrease in the culturability of the (p)ppGpp^0 mutant (Fig. 2C).

We used LC–MS/MS analysis to confirm the anticipated nucleotide levels in the (p)ppGpp0 and *ΔguaAB* mutants. As expected, levels of all guanine nucleotides (GMP, GDP and GTP) were significantly elevated in the (p)ppGpp^0 mutant in the late stationary phase (Fig. 2D). Lower levels were observed in wildtype, likely due to (p)ppGpp-dependent inhibition of biosynthetic enzymes, including GuaB (Fig. 2A). Consistently, deletion of *guaBA* also decreased guanine pools in (p)ppGpp^0 (Fig. 2D). Levels of ATP decreased in (p)ppGpp0 independent of deletion of *guaBA* (Fig. S2A & 3E), but levels of the other nucleotides differed only slightly between the strains (Fig. S2A-C). These results confirm a clear correlation between elevated guanine nucleotide levels during stationary phase starvation and decreased culturability.

(p)ppGpp-deficient cells enter a division-incompetent but viable state during starvation

To further investigate the cause and mechanism of the decreased culturability of the (p)ppGpp^0 mutant, we used different viability assays based on membrane integrity or metabolic activity. Despite the large decrease in CFUs during the stationary phase (Fig. 1B & S1A), only a small percentage of (p)ppGpp^0 mutant cells were stained with the membrane-impermeable dye propidium iodide, and there was no significant difference between wildtype and the (p)ppGpp^0 mutant (Fig. 3A). Analysis of the metabolic activity of late stationary phase cells by RedoxSensor™ Green staining, which monitors bacterial reductase activity, revealed slightly reduced metabolic activity in the (p)ppGpp^0 mutant (Fig. 3B). However, the decrease in metabolic activity was less than expected from the difference in CFU counts between wildtype and (p)ppGpp^0 during the late stationary phase (only approximately 10% culturable (p)ppGpp0 mutant). Resazurin conversion (Fig. 3C), an indicator of cellular redox potential, also revealed decreased respiratory activity in the (p)ppGpp^0 mutant compared to wildtype and the *guaBA*-negative strain. To further confirm the alterations in the redox state of the mutant cells, we

directly measured the NADH/NAD^+ ratio (Fig. 3D) and ATP level (Fig. 3E). Both parameters were significantly decreased in the (p)ppGpp^0 mutant. The effect was less prominent in the *guaBA* mutant background, indicating that the change in redox state is mainly a consequence of changes in the GTP pool.

The discrepancy between the decreased CFU counts and low propidium iodide staining, together with the decreased metabolic activity in the late stationary phase, suggest that the (p)ppGpp^0 cells did not immediately lose viability. It is more likely that the cells entered a dormant state, slowing their metabolic activity and halting cell division, resulting in the observed large decrease in CFU.

The division-incompetent state of (p)ppGpp^0 cells is not the result of increased translation during the stationary phase

(p)ppGpp is known to efficiently inhibit protein synthesis [9,26] and thereby may prevent accumulation of protein aggregates [27]. We measured protein synthesis at different time points during growth by incorporating the puromycin analogue O-propargyl-puromycin (OPP) into newly synthesized proteins. As expected, in the (p)ppGpp^0 mutant, translation was significantly greater in the late exponential and early stationary growth phases (7 h and 9 h) (Fig. 4A). However, in the late stationary growth phase (24 h), complete shutdown of protein synthesis was observed, indicating that this occurred independently of (p)ppGpp. Delayed or absent translation inhibition can lead to formation of protein aggregates [27]. However, only a slight increase in protein aggregates was observed in the (p)ppGpp^0 mutant, as assessed by densitometric quantification of Coomassie-stained protein aggregates isolated from wildtype and (p)ppGpp^0 late stationary phase cultures (Fig. S3A&B).

To further analyse whether increased translation in (p)ppGpp^0 might contribute to entry into a division-incompetent state, we inhibited translation with 100x the MIC of the bacteriostatic antibiotic chloramphenicol. Chloramphenicol treatment almost completely inhibited protein synthesis in both wildtype and (p)ppGpp^0 cells (Fig. 4B). However, chloramphenicol-induced inhibition of translation did not increase the culturability of (p)ppGpp^0 cells (Fig. 4C).

Thus, translation inhibition appears to be delayed in the (p)ppGpp0 mutant, leading to slightly increased protein aggregation during the stationary phase. However, this delay in translation inhibition does not seem to be the cause of the nonculturable state of (p)ppGpp^0 cells during stationary-phase starvation.

Division-incompetent cell state of (p)ppGpp^0 cells is not the result of oxidative stress during the stationary phase

In *S. aureus*, (p)ppGpp was shown to contribute to tolerance to oxidative stress [28], suggesting that elevated oxidative stress is a potential cause of the "dormant" state of (p)ppGpp^0. To test this hypothesis, we analysed culturability under anaerobic and oxygen-limited, static (biofilm) conditions. Under these conditions, the culturability of the (p)ppGpp^0 mutant was not compromised (Fig. 5A). However, treatment of aerobically grown cultures with the ROS scavenger N-acetyl cysteine (NAC) did not have any impact on culturability in the late stationary phase (Fig. 5B). Furthermore, no notable difference in endogenous ROS formation was detected between wildtype and the (p)ppGpp^0 mutant during the stationary growth phase (Fig. 5C). Thus, (p)ppGpp-deficient cells were impaired only in culturability under aerobic conditions. However, this phenotype could not be linked to elevated ROS levels. Thus, we investigated the contributions of metabolic cues such as aerobic respiration and proton motive force (PMF).

Membrane function and architecture are impaired in (p)ppGpp^0 cells

Changes in membrane potential can impact cell vitality by perturbing ATP synthesis and ion transport. We measured membrane potential by staining with the carbocyanine dye $DiOC_2(3)$. During the exponential growth phase, there was no difference in membrane potential between wildtype, (p)ppGpp^0 and *guaBA*-negative cells (Fig. 6A). However, during the late stationary phase, the membrane potential of the (p)ppGpp^0 mutant was notably lower than that of wildtype. In contrast, there was no significant difference in the level of the *guaBA*-negative strain, indicating that high levels of GTP disturb the membrane potential. The PMF is composed of the electrical potential $\Delta\Psi$ and the transmembrane proton gradient ΔpH. The intracellular pH was similar between wildtype and the (p)ppGpp^0 mutant (Fig. 6B), indicating that membrane potential $\Delta\Psi$ mainly reduced in cells with high GTP levels. Analysis of the membrane and cell wall architecture by co-staining with the dye FM4-64X and the BODIPY-vancomycin conjugate revealed irregular staining of the (p)ppGpp^0 cell membrane during starvation in the stationary phase, which was not observed in wildtype cells (Fig. 6C). In contrast, no abnormalities were detected in the cell wall of (p)ppGpp^0 cells during stationary phase starvation compared to wildtype cells (Fig. 6C). These irregularities in the cell membrane of the (p)ppGpp^0 strain, together with increased membrane fluidity, as measured by DPH fluorescence polarization (Fig. 6D), are indicative of membrane patches with increased fluidity, also known as regions of increased fluidity (RIFs).

Taken together, the results show that in the stationary phase, high levels of GTP, as found in the (p)ppGpp^0 mutant, led to decreased membrane potential, changes in cell membrane architecture and increased membrane fluidity. Thus, the decreased culturability of the (p)ppGpp^0 mutant is likely due to loss of membrane potential.

Impact of a lack of (p)ppGpp biosynthesis and GTP imbalance on the transcriptome during stationary phase starvation

To better understand how changes in GTP levels might alter membrane potential, we analysed changes in gene expression in wildtype (GTP low), (p)ppGpp^0 mutant (GTP high) and *ΔguaAB* mutant (GTP low) cells after 24 h of growth (Supplementary Tables S1 und S2). Genes with at least a log2-fold change of ≥ 1 and ≤ -1 and a p value ≤ 0.001 were defined as differentially regulated. In total, 235 and 288 genes were significantly upregulated or downregulated, respectively, in the (p)ppGpp^0 mutant compared to wildtype. Such (p)ppGpp-dependent and GTP-independent differences were much less prominent in the *guaBA* background (only 50 and 65 genes up- or downregulated, respectively). To assess (p)ppGpp-independent and GTP-dependent changes in gene expression, the (p)ppGpp^0 *ΔguaBA* mutant was compared to the (p)ppGpp^0 mutant, and 514 and 628 genes were found to be significantly up- or downregulated, demonstrating that under our experimental conditions, the majority of transcriptomic changes are mediated by differences in GTP levels, as opposed to being direct effects of (p)ppGpp.

Many of the previously identified stringent response genes identified after challenge with amino acid limitation [12] or transcriptional induction of (p)ppGpp synthetases [22] were differentially regulated during stationary phase starvation (see Fig. S4 and S1C). Most CodY target genes were downregulated in the (p)ppGpp^0 mutant [e.g., *rsaD* (-185.8-fold), the *cap* operon (-5,2-fold for *capA*) and *aur* (-65,7-fold)]. Likewise, genes involved in amino acid synthesis and transport, such as *brnQ2* (-6.1-fold), *gltBD* (-14.8-fold and -12.2-fold), *dapABDL* (-12.5-fold for *dapA*), the *opp-3ABCDEF* operon (-20.1-fold for *opp-3B*) and *ilvD* (-7.2-fold), were also downregulated. Furthermore, several genes of the SigB regulon were downregulated in (p)ppGpp^0. For instance, *krtA*, which encodes a potassium transporter, was downregulated 2.1-fold. Similarly, *clpL*, which is involved in protein metabolism, was downregulated 6.1-fold. The *mnh* operon, which is hypothesized to encode a redox-energized complex involved in PMF maintenance [29], was also downregulated 5.1-fold. Furthermore, *groEL* (2.4-fold) and *groES* (4.9-fold), which are part of the CtsR/HrcA regulon, were found to be upregulated, indicative of proteotoxic stress. Both protein complexes were upregulated during proteotoxic stress, which leads to protein misfolding and aggregation [30]. These findings are consistent with the slightly elevated protein aggregates detected in the (p)ppGpp^0 mutant during stationary phase starvation (Fig. S4F). Interestingly, phage-encoded genes were upregulated under GTP-high conditions, indicating that phages φ11, φ12 and φ13 are induced in (p)ppGpp^0 during the stationary phase. These observations are in agreement with previous studies that found the ΦSa3 phage to be downregulated under guanine-limited conditions [31]. Notably, the transcriptome further revealed strong downregulation of TCA cycle (*citB* -3.1-fold, *sucA* -2.3-

fold and *sucB* -2.6-fold) and aerobic respiration (*qoxABCD* -7.6-fold for *qoxA*, *ctaA* -3.4-fold and ATP synthase e.g. *atpD* -2,3-fold) genes (Fig. 7), which was GTP dependent, as both the TCA cycle and respiration were mostly unaffected in the *guaBA* negative background (Fig. 7). Downregulation of the TCA cycle and respiration may account for the reduced membrane potential observed during stationary phase starvation in the (p)ppGpp^0 mutant (Fig. 6A).

The sensitivity of *qoxABCD* expression to GTP determines the activity of the electron transport chain and PMF

Since the transcriptome analysis of the (p)ppGpp^0 mutant revealed not only downregulation of the TCA cycle but also strongly decreased expression of the terminal oxidase gene *qoxABCD*, a component of the electron transport chain (ETC), we hypothesized that these transcriptional changes lead to decreased PMF.

We verified the RNAseq data by RT–qPCR and confirmed decreased *qoxA* expression in (p)ppGpp^0 but increased expression in the *guaBA*-negative strain compared to wildtype (Fig. 8B). Expression of rRNA is controlled by the concentration of the transcription-initiating nucleotide (iNTP) within the promoter [11]. Analysis of potential transcriptional start sites upstream of *qoxABCD* predict an ATP as the transcription-initiating nucleotide (based on data from [32]) (Fig. 7). Thus, we mutated the TSS+1 to a GTP in wildtype and (p)ppGpp^0 (named WT PqoxABCD$_{mut}$ and (p)ppGpp^0 PqoxABCD$_{mut}$) (Fig. 8A). This mutated TSS+1 increased *qoxA* expression in the late stationary phase in (p)ppGpp^0, indicating that this promoter is nucleotide sensitive (Fig. 8B). Alteration of the iATP to iGTP also resulted in greater CFU counts than those of (p)ppGpp^0 (Fig. 8C). However, culturability was still lower compared to wildtype, showing that additional factors are likely to contribute to entry into dormancy.

To further demonstrate the importance of the PMF for stationary phase survival, we treated wildtype and (p)ppGpp^0 cultures with subinhibitory concentrations of valinomycin during the late stationary phase and evaluated survival after 24 h. Valinomycin is an ionophore that disrupts membrane potential by selectively translocating potassium ions across the membrane. Upon valinomycin treatment, wildtype and (p)ppGpp^0 showed similar reductions in CFU counts compared to untreated cultures (Fig. 8D). Thus, disruption of membrane potential indeed compromises culturability and survival.

Together, these findings illustrate the significance of preserving PMF, particularly $\Delta\Psi$, throughout stationary phase starvation to sustain culturability.

Preservation of PMF during starvation is crucial for antibiotic tolerance

It has been demonstrated that maintenance of PMF is essential for the survival of starvation-induced tolerant bacteria [33,34]. We sought to determine whether the decreased membrane potential in the (p)ppGpp^0 mutant would also result in a reduction of starvation-induced antibiotic tolerance. Therefore, we cultured wildtype and (p)ppGpp^0 cells to exponential or stationary growth phase and exposed them to either 100x MIC oxacillin or ciprofloxacin. No significant difference in antibiotic survival between wildtype and (p)ppGpp^0 cells was observed when treated during exponential growth (Fig. 8E). However, wildtype cells were barely killed by the antibiotics during the stationary phase, confirming the higher antibiotic tolerance of non-growing bacteria [3]. However, both antibiotics efficiently decreased the survival of the (p)ppGpp^0 mutant during the stationary phase (Fig. 8F).

Discussion

Here, we show that bacterial culturability in the stationary phase is dependent on (p)ppGpp-driven GTP depletion. Uncontrolled, high levels of GTP in the (p)ppGpp^0 mutant decrease membrane potential, at least in part, via transcriptional downregulation of nucleotide-sensitive genes of the TCA cycle and the respiratory chain. Maintenance of respiratory chain activity by (p)ppGpp/low GTP is also responsible for the tolerance of stationary phase cells to bactericidal antibiotics (Fig. 9).

(p)ppGpp-dependent GTP homeostasis determines long-term bacterial culturability

The defect in culturability of the (p)ppGpp^0 mutant is clearly linked to an uncontrolled increase in GTP levels upon entry into the stationary phase. Culturability correlated inversely with changes in the GTP level induced by deletion of *purR* or *guaAB* or by guanine feeding. Mutation of *guaAB* rescued the defect in the (p)ppGpp^0 mutant. A high guanine concentration did not interfere with the survival of wildtype, indicating that (p)ppGpp can also control GTP levels provided by the salvage pathway. Direct (p)ppGpp-dependent alterations in translation or replication machinery [9,15] are likely to also be less prominent in a *guaAB*-negative background. Thus, we conclude that the level of GTP homeostasis is the main determinant of bacterial culturability in stationary phase cells.

Control of guanine metabolism by (p)ppGpp is conserved in several Firmicutes species, such as *B. subtilis*[13], *E. faecalis* [35] and *S. aureus* [14]. Recently, reduced long-term survival of the *S. aureus* USA300 (p)ppGpp^0 mutant [18] was linked to disrupted GTP homeostasis since suppressor mutants with alterations in *gmk* were selected during long-term culture. In *B. subtilis*, low GTP levels reduce the growth rate but promote survival during amino acid starvation [13,36] and cell viability during fatty acid starvation [37]. Elevated GTP levels in a (p)ppGpp^0 mutant of *B. subtilis* promoted the bactericidal effect of otherwise bacteriostatic antibiotics [38].

Disturbance of GTP homeostasis during nutrient stress leads to induction of a VBNC state

Previously, it was proposed that (p)ppGpp deletion in *B. subtilis* results in “death-by-GTP” and that this cell death might be due to unrestricted translation [13]. However, in *S. aureus*, uncontrolled translation could not be linked to diminished bacterial survival since translation inhibitors did not restore culturability of the (p)ppGpp^0 mutant. Furthermore, our results do not support the hypothesis that high GTP levels trigger a classical “cell death” program. The (p)ppGpp^0 mutant of *S. aureus* was nonculturable but negative for propidium iodide uptake

and maintained basal metabolic activity and transcription, albeit with significantly reduced ATP levels. This is indicative of the previously defined state of VBNC (viable but nonculturable) or a deep dormant-like state [39]. It was proposed that ATP depletion is involved mainly in formation of VBNCs [27,40], in which intracellular energy (ATP) is the main factor maintaining the culturability of bacteria on solid media. In some gram-negative bacteria, (p)ppGpp was shown to prevent VBNC state formation in response to certain stress conditions [41,42]. In (p)ppGpp^0 mutants of *B. subtilis*, fatty acid starvation leads to membrane rupture and, consequently, cell death [37]. The authors proposed a model whereby during fatty acid starvation, a (p)ppGpp-dependent decrease in GTP levels halts growth, preventing collapse of membrane potential and loss of membrane integrity. However, nonculturable (p)ppGpp^0 *B. subtilis* mutants died, as indicated by propidium iodide staining [37,38]. Thus, there might be major differences between GTP-dependent cell death pathways in *S. aureus* and *B. subtilis*.

How do elevated GTP levels lead to VBNC-state formation?

Our RNAseq analysis revealed that TCA cycle genes and genes coding for major components of the electron transport chain, including the terminal oxidase (*qoxABCD*) and the F0F1 ATP synthase, were significantly downregulated in the (p)ppGpp^0 mutant during the stationary phase. Accordingly, ATP levels (Fig. 3E), the NADH/NAD$^+$ ratio and membrane potential (Fig. 6A) were decreased in the (p)ppGpp^0 mutant. In Firmicutes, (p)ppGpp-dependent transcriptional changes are regulated mainly by the GTP-dependent transcriptional repressor CodY [20] or by the availability of the NTP substrate for transcription initiation [11,14,43]. We could exclude any influence of CodY (Fig. 2B) on induction of the VBNC state. We mutated the predicted initiation of NTP transcription in the promoter region of *qoxABCD*, the most prominently downregulated component of the electron transport chain. By using this approach, we were able to restore wildtype levels of *qoxA* expression in the (p)ppGpp^0 mutant (Fig. 8B). Furthermore, the culturability of the (p)ppGpp^0 mutant was partially restored by this single nucleotide exchange. Expression of other genes involved in respiration and generation of membrane potential was downregulated in the (p)ppGpp^0 mutant, which was also initiated with iATP (Fig. 7); these genes may further contribute to the observed phenotype.

PMF maintenance during starvation is crucial for antibiotic tolerance

We showed that a (p)ppGpp^0 mutant grown to the stationary phase lost antibiotic tolerance to the β-lactam oxacillin and the fluoroquinolone antibiotic ciprofloxacin. Nongrowing wildtype bacteria are tolerant to these antibiotics, as shown previously [3]. However, Conlon et al. did not find evidence that (p)ppGpp is involved in antibiotic persistence or tolerance. The authors proposed that ATP depletion is important for the observed tolerance of stationary phase cells 3. This seems to contrast our findings that (p)ppGpp-dependent maintenance of low GTP/high

ATP levels protected the cells. This discrepancy might be explained by the experimental design. The growth medium used by Conlon et al. [3] likely does not induce a stringent response and/or might contain low amounts of guanine. Thus, under these conditions, the intracellular GTP pool might not be elevated in the (p)ppGpp^0 mutant. In agreement with our results, previous studies have shown that PMF is essential for starvation-induced tolerance in both gram-negative and gram-positive bacteria, including *S. aureus* [33,34,44]. Hence, these findings support the idea of applying PMF inhibitors to combat antibiotic-tolerant bacteria, including *S. aureus* [45,46].

Materials and Methods

Growth conditions and CFU determination

The strains and plasmids used in this study are listed in the Supplementary Information, Table S1 and S2. If not indicated elsewhere, S. aureus strains were grown in LB-Miller, tryptic soy broth (TSB) or chemically defined medium (CDM) 47 at 37 °C and 200 rpm.

For CFU experiments, bacteria from an overnight culture grown in LB-Miller were diluted to an initial optical density at 600 nm (OD600) of 0.05 in fresh medium without antibiotics and grown with shaking (200 rpm) at 37 °C to the desired growth phase. LB-Miller medium was used overnight culture since the (p)ppGpp^0 mutant in this medium showed no defect in survival. For oxygen-limited growth (biofilm conditions), bacteria were inoculated into 1 ml of fresh medium in a 24-well plate and statically grown at 37 °C. For anaerobic growth conditions, 14 ml of fresh medium was inoculated into glass tubes, which were closed with a rubber stopper to minimize the oxygen supply and subsequently grown at 37 °C and 200 rpm. For certain CFU experiments, 400 µg/ml chloramphenicol (100x MIC of HG001 wildtype and (p)ppGpp0), 12.5 mM N-acetylcysteine or 20 µM valinomycin was added at the indicated timepoints during growth. For CFU/ml determination, a 10-fold dilution series was prepared in phosphate-buffered saline (PBS), 10 µl of each dilution was spotted onto TSB agar, and CFU counts were determined after 16–18 h of incubation at 37 °C.

Generation of *S. aureus* mutant strains

Generation of SH1000 (p)ppGpp^0 – The mutagenesis strategy described by Geiger et al. was used for generation of the SH1000 (p)ppGpp^0 mutant (SH1000-229-230-263) [20]. Lysates were prepared from RN4220 strains containing the mutagenesis vectors pCG229, pCG230 and pCG263. After plasmid transduction, mutagenesis was performed as previously described [48]. The mutations were verified by PCR and sequencing using the oligonucleotides listed in Table S3.

Transduction of purR mutants – Φ11 lysates were generated from the transposon mutant NE1237 (purR) of the NARSA transposon library 49 to transduce S. aureus HG001 wildtype and (p)ppGpp0 cells. The mutations were verified via PCR using the oligonucleotides listed in Table S3.

Mutagenesis strategy for WT-PqoxABCD$_{mut}$ and (p)ppGpp0-PqoxABCD$_{mut}$ – The plasmid pCG919mut was constructed for base substitution at the position +1 of the transcriptional start site of the PqoxABCD promoter. The complete promoter region with approximately 1000 bp of the left and right flanking regions was amplified in two PCRs using the primers pCG919gibfor

and pCG919-2mutfor and pCG919-2mutrev and pCG919gibrev. The PCR products were subsequently cloned and inserted into the Bam HI-digested pIMAY-Z vector via Gibson cloning and transformed into *E. coli* IM08B. After verification of the base substitution (A → G) by sequencing, the plasmid was transformed into *S. aureus* HG001 wildtype and (p)ppGpp^0 cells by electroporation. The mutation was introduced into the genome by pIMAY mutagenesis [50,51] and verified by sequencing. All oligonucleotides used are listed in Supplementary Table S3.

RNA isolation, qRT–PCR and RNAseq

RNA isolation and qRT–PCR were performed as described previously 22. Briefly, bacteria were pelleted, resuspended in 1 ml of TRIzol (Thermo Fisher Scientific) and lysed using zirconia/silica beads (0.1 mm diameter) and a high-speed homogenizer. RNA was isolated following the procedure recommended by the TRIzol manufacturer.

Relative quantification of *psmα, rsaD, rpsL* and *qoxA* transcripts by qRT–PCR was performed with QuantiFast SYBR Green RT–PCR Kit (Qiagen) using the Quantstudio3 system (Applied Biosystems). Briefly, 5 µg of total RNA was DNase-treated and diluted 1:10 for qRT–PCR. Relative expression of transcripts was calculated with the ΔΔCT method and normalized to *gyrB* gene expression. The sequences of the oligonucleotides used for qRT–PCR are listed in Table S3.

For RNA-seq analysis, RNA isolated from the aqueous phase was further purified with an Amp Tech ExpressArt RNA Reading Kit. For each sample, a total of 100 ng of RNA was subjected to rRNA depletion, followed by cDNA library construction via IlluminaTM Stranded Total RNA Prep Ligation with a Ribo Zero Plus Kit according to the manufacturer's instructions. Libraries were sequenced as single reads (100 bp read length) using the NextSeq platform (Illumina) and NextSeq 500 Mid Output Kit v2.5. The sequences were demultiplexed with bcl2fastq (v2.19.0.316), quality checked with fastq (v0.20.1) and visualized with MultiQC (v1.7). Analysis of the RNAseq results was performed using CLC Genomic Workbench 23.0.1 (Qiagen). Reads were mapped to the reference genome of HG001 (NZ_CP018205.1). Differential gene expression was performed comparing (p)ppGpp^0 vs. wildtype, (p)ppGpp^0 *ΔguaBA* vs. *ΔguaBA* (GTP independent) and (p)ppGpp^0 vs. (p)ppGpp^0 *ΔguaBA* ((p)ppGpp independent). Genes with at least log2 fold change of >1 or <-1 and a p-value <0.001 were defined as differentially regulated. Annotation of genes was performed according to the recent "Aureowiki" annotation of strain 8325 (http://aureowiki.med.unigreifswald.de/Main_Page) 52.

Metabolite extraction and LC–MS/MS quantification

For metabolite extraction, 3 ml of bacterial culture was harvested by vacuum filtration, washed twice with 0.6% NaCl solution, transferred directly into 5 ml of quenching solution (acetonitrile:methanol:water, 40%:40%:20%, (v/v/v)) and shock frozen in liquid nitrogen. Afterwards, 1 ml of the cell suspension was lysed using zirconia/silica beads (0.1 mm diameter) and a high-speed homogenizer. The lysate was centrifuged at -9 °C at 20 000 × g for 15 minutes, and the clear supernatant was stored at -80 °C for LC–MS/MS analysis. Relative concentrations of the nucleotides in the cell extracts were measured via isotope ratio LC–MS/MS as described previously [53]. For evaluation, the 12C/13C ratios were normalized to the OD600 of the bacterial culture, and the relative abundances of each metabolite are shown.

LIVE/DEAD and viability staining using microscopy and flow cytometry

Bacterial cultures were concentrated 10-fold and washed in 0.85% NaCl prior to staining for bacterial viability, as recommended by the manufacturer (Live/Dead BacLightTM Bacterial Viability Kit). All cultures were further diluted 5- to 10-fold to an OD_{600} of 0.2-0.4 before staining with the Syto 9 and propidium iodide dye mixture (1:1 dye ratio). Fluorescence microscopy was performed with a Leica DM5500B fluorescence microscope using the following filter sets (excitation and emission filters): Syto 9: BP470 40-nm and BP525 50-nm; propidium iodide: BP535 50-nm and BP610 75-nm. Images were captured using a Leica DFC360FX monochrome camera with the following exposure times: 40 ms for Syto 9, 150 ms for propidium iodide and 6 ms for phase contrast channels. Images were viewed with in-built Leica ASF software. Alternatively, the percentage of propidium iodide-stained cells was analysed via flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson).

RedoxSensor Green assay

To monitor bacterial reductase activity as an indicator of electron transport chain function, cells from the stationary phase were stained with RedoxSensor™ Green reagent (BacLight™ RedoxSensor™ Green Vitality Kit) and analysed by flow cytometry according to the manufacturer's instructions.

AlamarBlue measurement

To measure cell viability via resazurin conversion, a stationary phase culture was tenfold diluted in PBS and mixed according to the manufacturer's instructions with alamarBlue™ reagent at a 1:10 ratio. After incubating at 37 °C, resofurin fluorescence (excitation: 540 nm, emission: 600 nm) was measured every 5 min. The increase in resofurin fluorescence over time was analysed via simple linear regression in GraphPad Prism 10.

ATP and NADH/NAD^+ ratio determination

ATP levels and the NADH/NAD^+ ratio were determined using the BacTiter-Glo™ Microbial cell Viability assay or the NAD/NADH-Glo™ assay (Promega) following the manufacturer's instructions. Luminescence was measured in a white 96-well plate using the Tecan Spark luminescence module.

Click-it OPP protein synthesis labelling

Protein synthesis was quantified with a Click-it™ Plus OPP Alexa Fluor™ 488 protein synthesis labelling kit according to the manufacturer's instructions. Briefly, growing bacterial cells were incubated with 20 µM O-propargyl puromycin (OPP) for 30 min. Afterwards, the cells were harvested by centrifugation, fixed with formaldehyde and permeabilized with 70% ethanol (v:v) and lysostaphin. Then, OPP was conjugated to Alexa Fluor™ 488 using click chemistry according to the manufacturer's protocol. The stained cells were analysed using flow cytometry (Beckton Dickinson FACS Calibur).

Detection and quantification of protein aggregation

Protein aggregates within cells were isolated according to a described protocol [54,55]. Briefly, protein aggregates were isolated from 5 ml of culture. First, bacterial cells were harvested by centrifugation and washed twice with PBS. Then, the cells were resuspended in buffer A (50 mM Tris, 150 mM NaCl, pH 8) and lysed using zirconia/silica beads (0.1 mm diameter) and a high-speed homogenizer at 6 m/s three times for 30 seconds each. The crude extract was centrifuged at 18 000 × *g* for 30 minutes, after which the pellet was washed twice with buffer A containing 0.5% Triton X-100. The protein aggregates were then solubilized in rehydration buffer (7 M urea, 2 M thiourea, 4% (wt/vol) CHAPS, 100 mM DDT) and loaded on a 12% SDS–PAGE gel. Afterwards, the SDS–PAGE mixture was stained with InstantBlue™ Coomassie blue-based staining solution (expedeon). Protein aggregates were densitometrically quantified from Coomassie blue-stained gels using the GelAnalyzer plugin of ImageJ software.

ROS measurement

Endogenous ROS levels were measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH2-DA) dye based on a previous protocol 56. DCFH2-DA is deacetylated by alkaline hydrolysis to generate DCFH2, which is oxidized by ROS to the fluorescent dye 2′,7′-dichlorofluorescein (DCF). Briefly, *S. aureus* HG001 wildtype and (p)ppGpp^0 cells were cultivated in CDM for the indicated durations and harvested at an OD600 equivalent of 3 × 108 cells by centrifugation. The cell pellets were incubated with DCFH2 for 40 min as previously described [75]. Relative DCF fluorescence was measured using a plate reader (Tecan Spark),

with an excitation wavelength of 480 nm with a bandwidth of 20 nm and an emission wavelength of 530 nm with a bandwidth of 20 nm.

Membrane potential measurement

Membrane potential was measured using a BacLight Membrane Potential Kit (Thermo Fisher). Cells were grown in CDM to the desired growth phase. The optical density was then adjusted to an OD600 of 0.3 in PBS. $DiOC_2(3)$ was added to a final concentration of 30 µM, and the bacteria were incubated at 37 °C and gently agitated for 30 min. For plate reader measurements, 200 µl of stained cells was transferred to a Greiner Sensoplate (96-well, black, clear bottom). Using a Tecan Spark plate reader, red and green fluorescence was detected (excitation 488 nm, emission 535 nm and 600 nm), and the red:green fluorescence ratio was calculated. For flow cytometry, stained bacteria were pelleted by centrifugation, resuspended at $1x10^7$ cells/ml in PBS and analysed using a BD FACSCalibur FL-1H and FL-3H.

Intracellular pH determination

For intracellular pH determination, the fluorogenic probe pHRodo™ Green AM was used. *S. aureus* bacterial cells were grown to the stationary phase, and 0.6 ODU of the bacterial culture was harvested and washed with PBS. The cells were resuspended in 100 µl of pHRodo™ Green AM staining solution (final concentration of 10 µM) and incubated at 37 °C for 30 min. Afterwards, the cells were washed in 300 µl of PBS. Fluorescence was detected in a 96-well Sensoplate (Greiner) using a Tecan Spark plate reader (excitation: 500 nm, emission: 545 nm) or by flow cytometry using a BD FACS Calibur. For calibration of pHRodo Green AM, the "Intracellular pH Calibration Kit" was used according to the manufacturer's instructions.

Cell membrane and cell wall costaining

Formaldehyde-fixed *S. aureus* cells were stained with the fixable cell membrane dye FM4-64X (final concentration: 4 µg/ml) and the cell wall dye Bodipy™ Vancomycin-FL conjugate (final concentration: 1 µg/ml) for 10 min on ice. Fluorescence microscopy was performed using a Leica DM5500B fluorescence microscope and the following filter sets (excitation and emission filters): Bodipy™ Vancomycin-FL conjugate: 40-nm BP470 and 50-nm BP525; and FM4-64X: 50-nm BP535 and 75-nm BP610.

Membrane fluidity measurement

Membrane fluidity was determined via DPH fluorescence polarization. Briefly, one ODU of bacterial cells was harvested by centrifugation, resuspended in prewarmed PBS containing 20 µM DPH (1,6-diphenyl-hexa-1,3,5-triene) and incubated at 37 °C for 15 min. Fluorescence

polarization was measured with a BMG CLARIOStar plate reader in a 96-well Sensoplate (Greiner) (excitation: 360 nm-20 nm, emission: 450 nm-10 nm, dichroic filter: LP 410) at 37 °C.

Antibiotic tolerance assay

To assess the antibiotic tolerance of *S. aureus* HG001 wildtype and (p)ppGpp^0 cells, either mid-exponential bacteria or late stationary phase bacteria were treated with 100 µg/ml oxacillin (100x MIC of HG001 wildtype and (p)ppGpp^0) or 50 µg/ml ciprofloxacin (100x MIC of HG001 wildtype and (p)ppGpp^0). Minimal inhibitory concentrations (MICs) were determined beforehand by the broth microdilution method or E-Test strips in accordance with EUCAST guidelines [57]. At the indicated time points, CFU counts were performed (as described under "CFU determination" and survival was calculated by comparison to t_0 ($t_{treated}/t_0$).

Statistical analysis

Data were analysed using GraphPad Prism 10 or R. Relevant information regarding statistical tests was added to each figure caption where appropriate. For ANOVA, assumptions of homoscedasticity and normality of the residuals were checked visually by comparing the fitted values with the residuals and comparing the predicted values with the actual residuals. Post hoc tests (posttests) were performed to conduct and correct for multiple comparisons. All tests for which this test was applied were two-tailed. A significance level cut-off of alpha = 0.05 was used. For visual purposes, asterisks denote significance levels as defined in figure captions.

Data Availability

All data are available from the GEO database under the accession number GSE254567 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE254567).

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Sequencing was performed by the Institute for Medical Microbiology (part of the NGS Competence Center NCCT (Tübingen, Germany), while data management, including data storage of the raw data for this project, was performed by the Quantitative Biology Center (QBiC), University of Tübingen, Germany. Mutants from the Nebraska library were obtained through the Network on Antimicrobial Resistance in Staphylococcus aureus (NARSA) program. Figs. 2A, 8A and 9 were created with BioRender.com.

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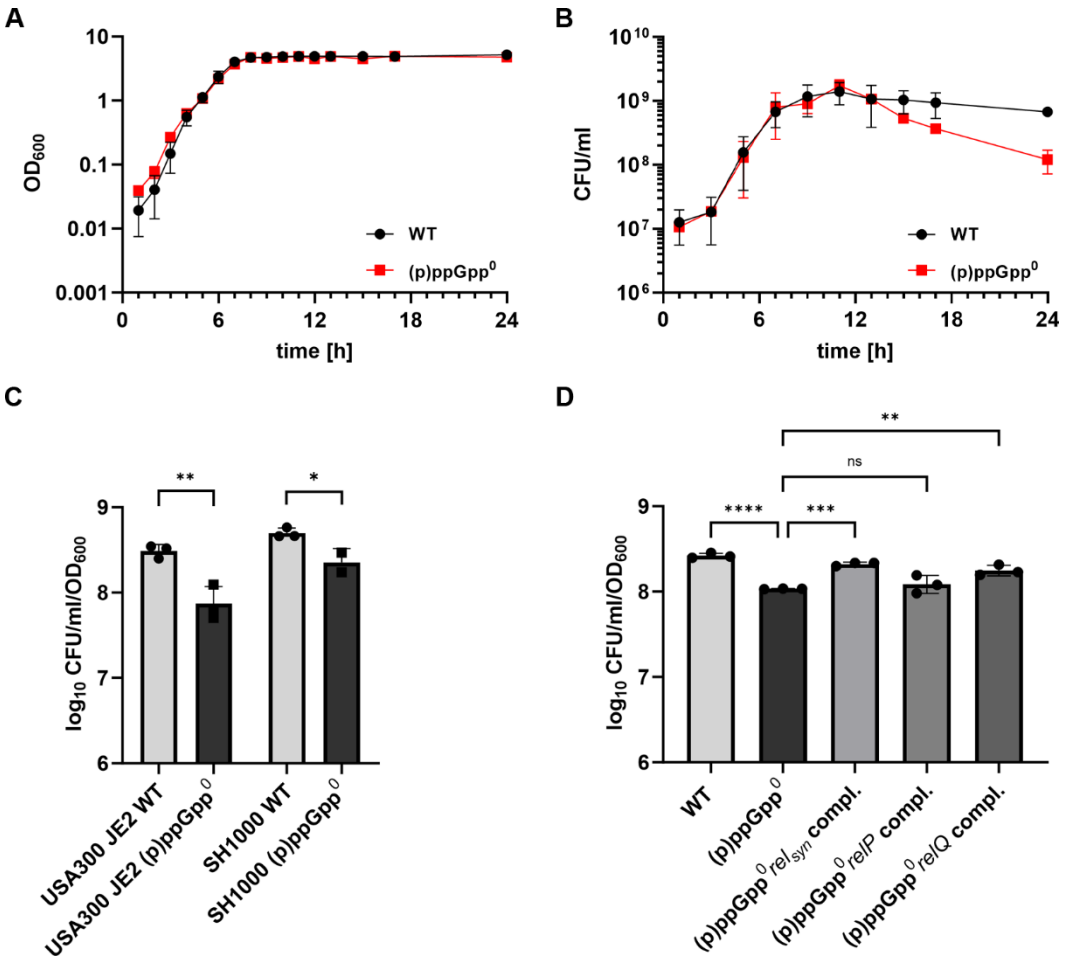

Fig. 1 Culturability of the (p)ppGpp^0 mutant decreases during the stationary growth phase.

(A) *S. aureus* HG001 wildtype (WT) and the isogenic (p)ppGpp^0 strain were cultured in CDM **[56]** and growth was monitored by optical density OD_{600} and (B) CFU/ml determination. Data are shown as mean ± SD (n=3 biological replicates). (C) USA300 JE2 and SH1000 and their isogenic (p)ppGpp^0 mutants were grown in CDM for 24h and culturability was determined by CFU/ml enumeration normalized to OD_{600}. Data are shown as mean ± SD (n=3 biological replicates). Statistical significance was determined by two-tailed unpaired t-tests performed on log_{10} transformed data (**p-value <0.01, *p-value <0.05). (D) Strains were grown for 24h in CDM and culturability was determined by CFU/ml enumeration normalized to OD_{600}. Data shown are mean ± SD (n=3 biological replicates). Statistical significance was determined by one-way analysis of variance (ANOVA) with a Šidák's post-test on log_{10} transformed data (****p-value <0.0001, ***p-value <0.001, **p-value <0.01, ns >0.05).

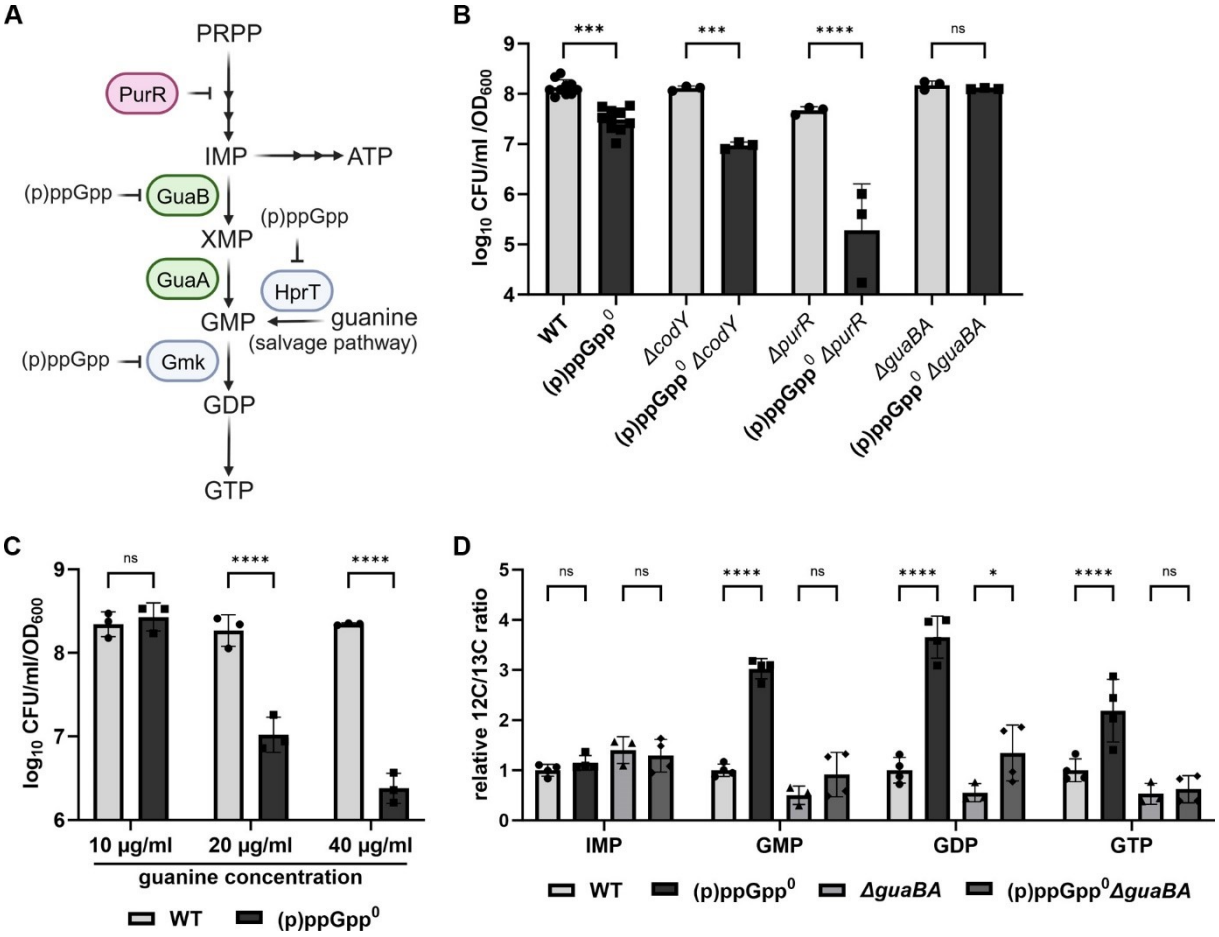

Fig. 2 (p)ppGpp is essential for GTP homeostasis during stationary phase starvation.

(A) The purine biosynthesis pathway is regulated by PurR repression and by (p)ppGpp repressing the biosynthetic enzymes GuaB, HrpT and Gmk. (B) Strains were grown in CDM for 24h and survival was determined by CFU/ml enumeration normalized to OD_{600}. Data shown are mean ± SD (n≥3). Statistical significance was determined by one-way analysis of variance (ANOVA) with a Tukey's post-test on log_{10} transformed data (****p-value <0.0001, ***p-value <0.001, ns >0.05). (C) HG001 wildtype (WT) and (p)ppGpp^0 were grown in CDM with varying concentrations of guanine for 24h and culturability was determined by CFU/ml enumeration normalized to OD_{600}. Data shown are mean ± SD (n=3). Statistical significance was determined by two-way way analysis of variance (ANOVA) with a Šidák post-test (****p-value <0.0001, ns >0.05). (D) Relative nucleotide levels were determined from late stationary phase cells by LC-MS/MS and normalized to OD_{600}. Data shown are mean ± SD (n=4). Statistical significance was determined by two-way analysis of variance (ANOVA) with a Tukey's post-test (****p-value <0.0001, ***p-value <0.001, **p-value <0.01, ns >0.05).

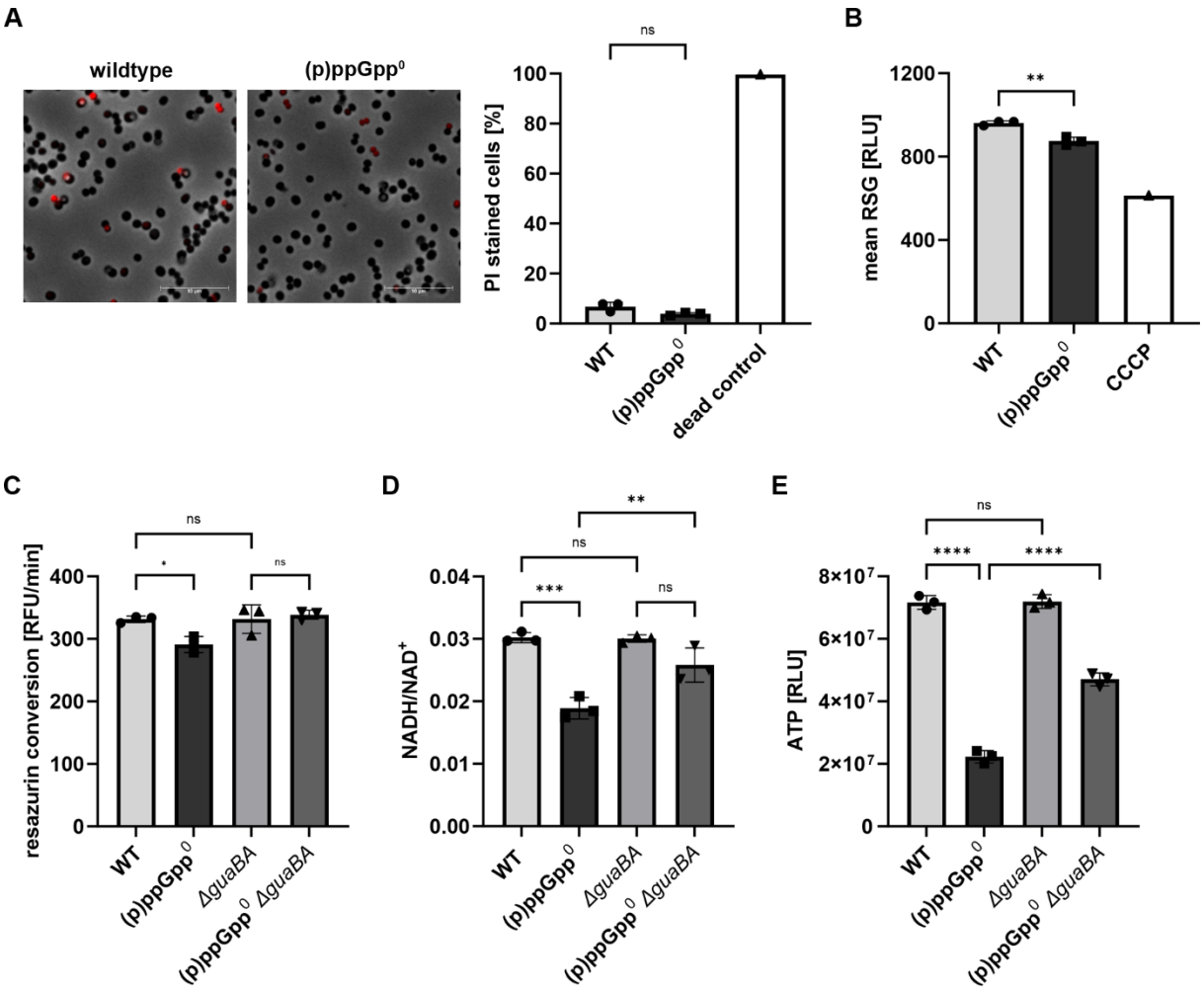

Fig. 3 The (p)ppGpp^0 mutant maintains membrane integrity but displays decreased metabolic activity

Strains were grown to late stationary phase (24h) in CDM. (A) Representative fluorescence microscopy images of propidium iodide (PI)-stained HG001 wildtype (WT) and (p)ppGpp^0 are shown. The percentage of propidium iodide stained cells was measured by flow cytometry. For dead control bacteria were permeabilized with 70% EtOH. Data shown are mean ± SD (n=3 biological replicates, n=1 for dead control). Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey's post-test (ns >0.05). Scale bar: 10µm (B) Bacteria were stained with RedoxSensor™ Green reagent and analysed by flow cytometry to monitor metabolic activity. Data shown are mean of RSG fluorescence ± SD (n=3 biological replicates, n=1 for CCCP control). Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey's post-test (**p-value <0.01). (C) Redox potential was accessed by resazurin conversion over time in HG001 wildtype, (p)ppGpp^0, *ΔguaBA* and (p)ppGpp^0 *ΔguaBA*. Data shown are mean ± SD (n=3 biological replicates). Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey's post-test (*p-value <0.05, ns >0.05). (D) NADH/NAD$^+$ ratio and (E) relative ATP levels were determined. Data shown are

mean of ± SD (n=3 biological replicates). Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey's post-test (****p-value <0.0001, ***p-value <0.001, **p-value <0.01, ns >0.05).

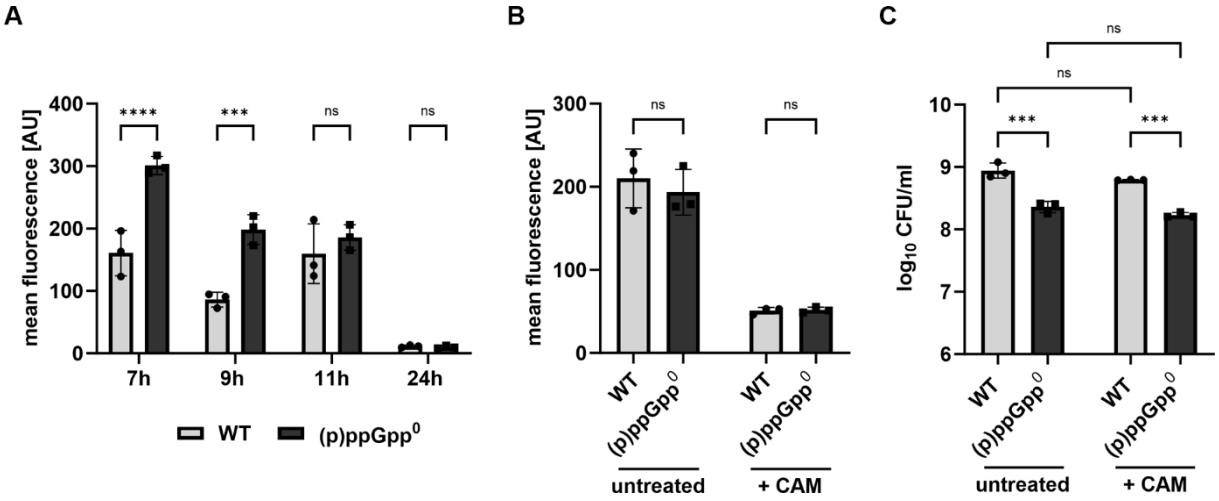

Fig. 4 Increased translation of (p)ppGpp^0 does not contribute to entrance into dormant state

(A) Protein synthesis was monitored at different timepoints during growth in wildtype (WT) and (p)ppGpp^0 cells by labelling with the puromycin analogue OPP and subsequent flow cytometry analysis. Data shown are mean of ± SD (n=3 biological replicates). Statistical significance was determined by two-way analysis of variance (ANOVA) with Šidák's post-test (****p-value <0.0001, ***p-value <0.001, ns >0.05). (B) Wildtype and (p)ppGpp^0 cells grown to transition phase were treated with 100x MIC chloramphenicol (+ CAM), and protein synthesis by OPP incorporation and (C) culturability were assessed by CFU/ml enumeration. Data shown are mean of ± SD (n=3 biological replicates). Statistical significance was determined by two-way analysis of variance (ANOVA) with Šidák's post-test (on $\log_{10}$ transformed data for (C), ***p-value <0.001, ns >0.05).

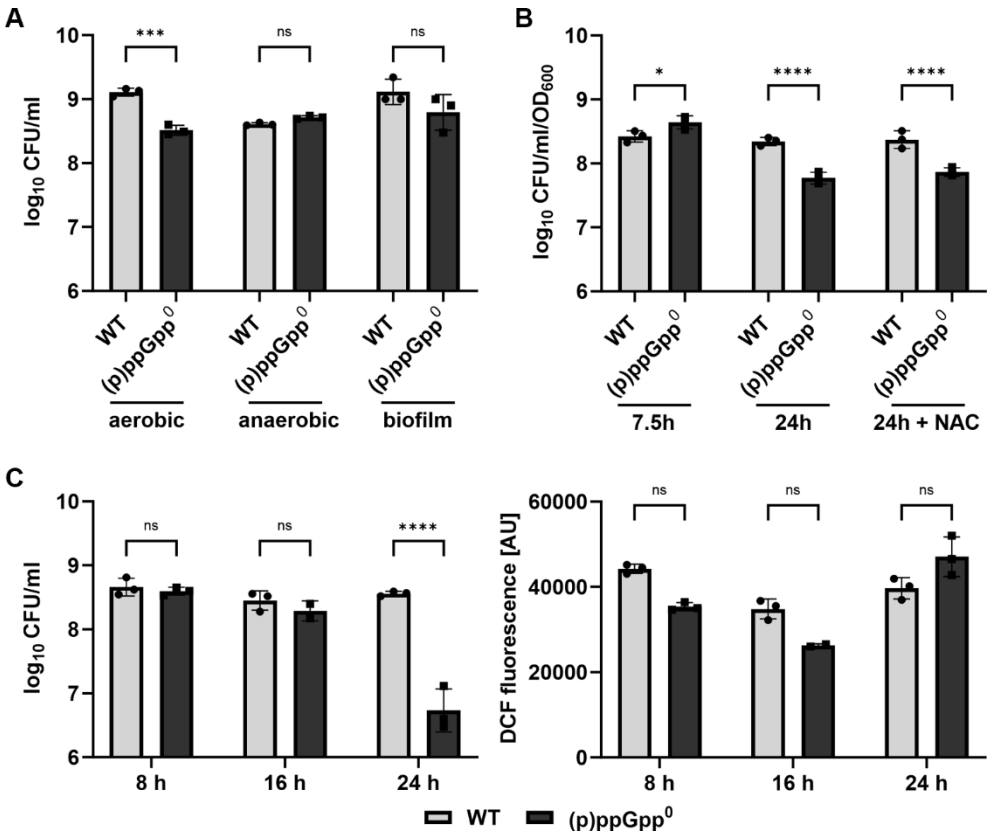

Fig. 5 Oxidative stress in (p)ppGpp^0 does not contribute to entrance into dormant state

(A) HG001 wildtype (WT) and (p)ppGpp^0 strain were grown under aerobic, anaerobic or oxygen-limited, static (biofilm) conditions for 24h and culturability was accessed by CFU/ml enumeration. Data shown are mean ± SD (n=3 biological replicates). Statistical significance was determined by two-way analysis of variance (ANOVA) with Šidák's post-test on $\log_{10}$ transformed data (***p-value <0.001, ns >0.05). (B) Wildtype and (p)ppGpp^0 cells were treated with 12.5 mM N-acetylcysteine (+ NAC) during transition to stationary phase (7.5h) and CFU/ml counts normalized to OD_{600} were determined before and after treatment. Data shown are mean of ± SD (n=3 biological replicates). Statistical significance was determined by two-way analysis of variance (ANOVA) with Šidák's post-test (****p-value <0.0001, * <0.05). (C) Monitoring of culturability, as determined by CFU/ml enumeration, and intracellular ROS levels, as measured by using the DCFH2-DA dye, which is oxidized to DCF by ROS, from early to late stationary phase. Data are shown as mean ± SD (n=3 biological replicates). Statistical significance was determined by two-way analysis of variance (ANOVA) with Šidák's post-test (****p-value <0.0001, ns >0.05).

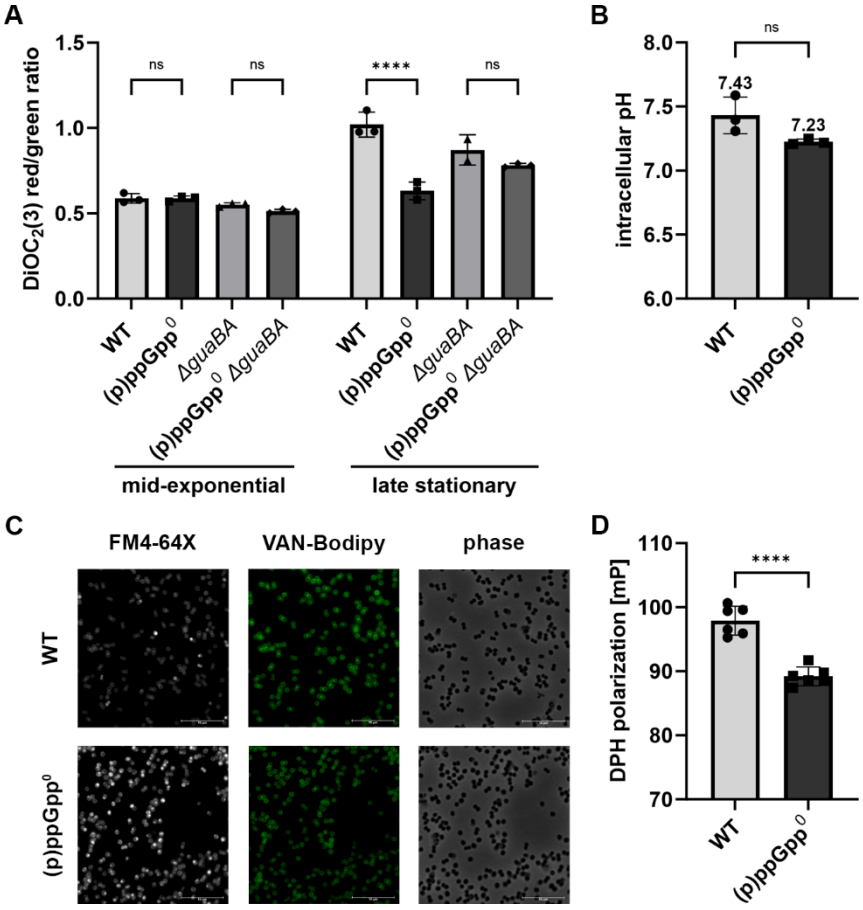

Fig. 6 Membrane function and architecture is altered in the (p)ppGpp^0 mutant

(A) Membrane potential was measured during mid-exponential and late stationary phase by staining with the carbocyanine dye $DiOC_2(3)$. Data shown are mean of ± SD (n=3 biological replicates). Statistical significance was determined by two-way analysis of variance (ANOVA) with Šidák's post-test (****p-value <0.0001, ns >0.05) (B) Intracellular pH was determined by pHRodo Green AM staining of late-stationary phase bacteria. Data shown are mean ± SD (n=3 biological replicates). Statistical significance was determined by a two-tailed, unpaired t-test (ns > 0.05). (C) Representative microscopic images of FM4-64X (cell membrane) and BODIPY-vancomycin conjugate (cell wall) double-stained *S. aureus* from late stationary phase (24h). Scale bar: 10µm (D) Membrane fluidity was measured by DPH fluorescence polarization in late stationary phase (24h). Data shown are mean ± SD (n=6 biological replicates). Statistical significance was determined by a two-tailed, unpaired t-test (****p-value <0.0001).

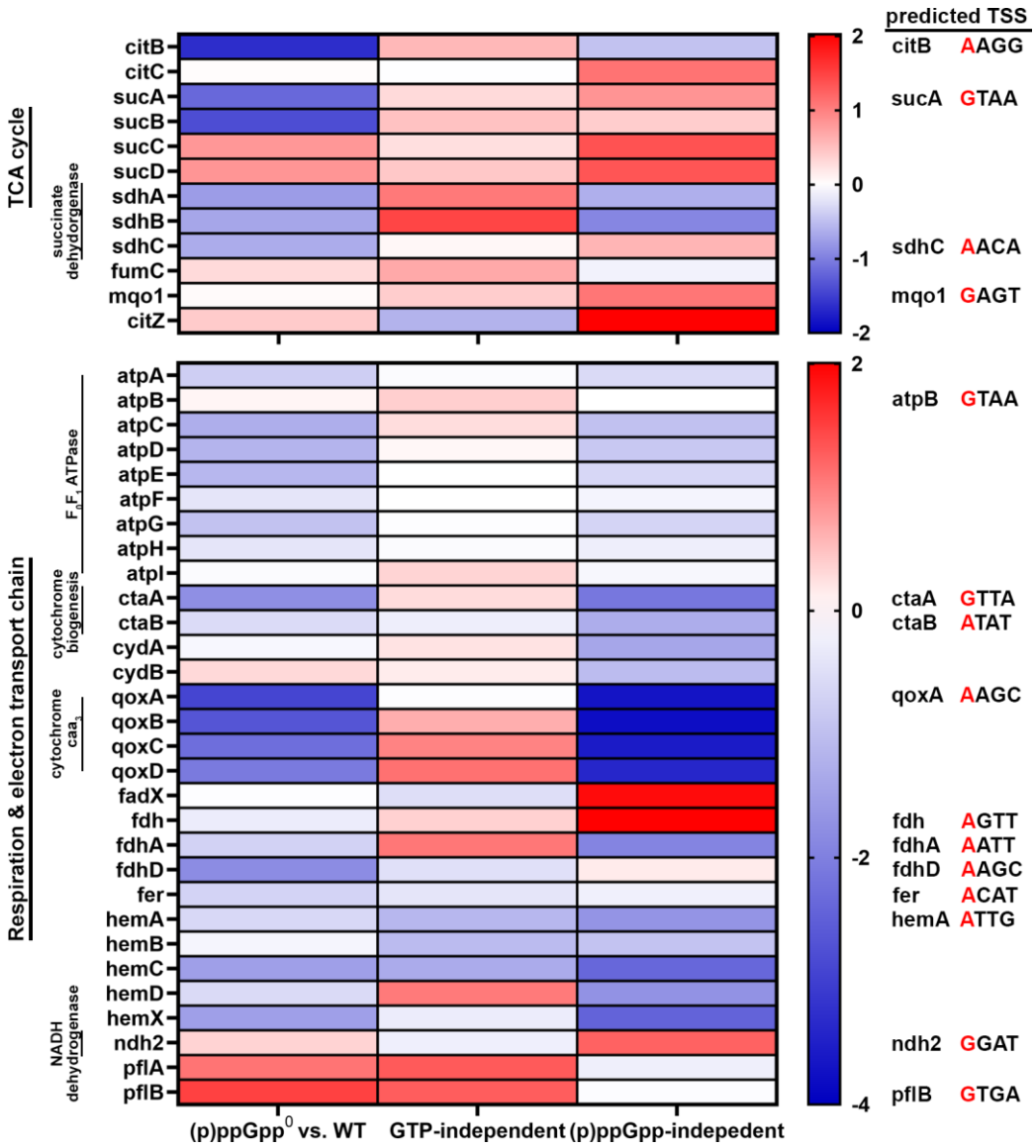

Fig. 7 Transcriptomic changes in TCA cycle and respiration in dormant (p)ppGpp^0 cells.

Heatmaps displaying the RNA-seq fold change of selected genes involved in the TCA cycle and electron transport chain, and related to respiration (SEED annotation). Log_2 fold changes in relative transcript abundances are colour-coded with red and blue, indicating up- and downregulation, respectively. “GTP-independent” compares the expression levels of (p)ppGpp^0 *ΔguaBA* vs. *ΔguaBA,* while “(p)ppGpp-independent” compares the expression levels of (p)ppGpp^0 vs. (p)ppGpp^0 *ΔguaBA*. The predicted TSS position between nucleotide +1 and +4 is depicted, while the TSS +1 is indicated in red colour. The RNA-seq data shown are from three individual biological replicates (n=3). Full RNA-seq data are available in Data S1 and S2 in the supplementary material.

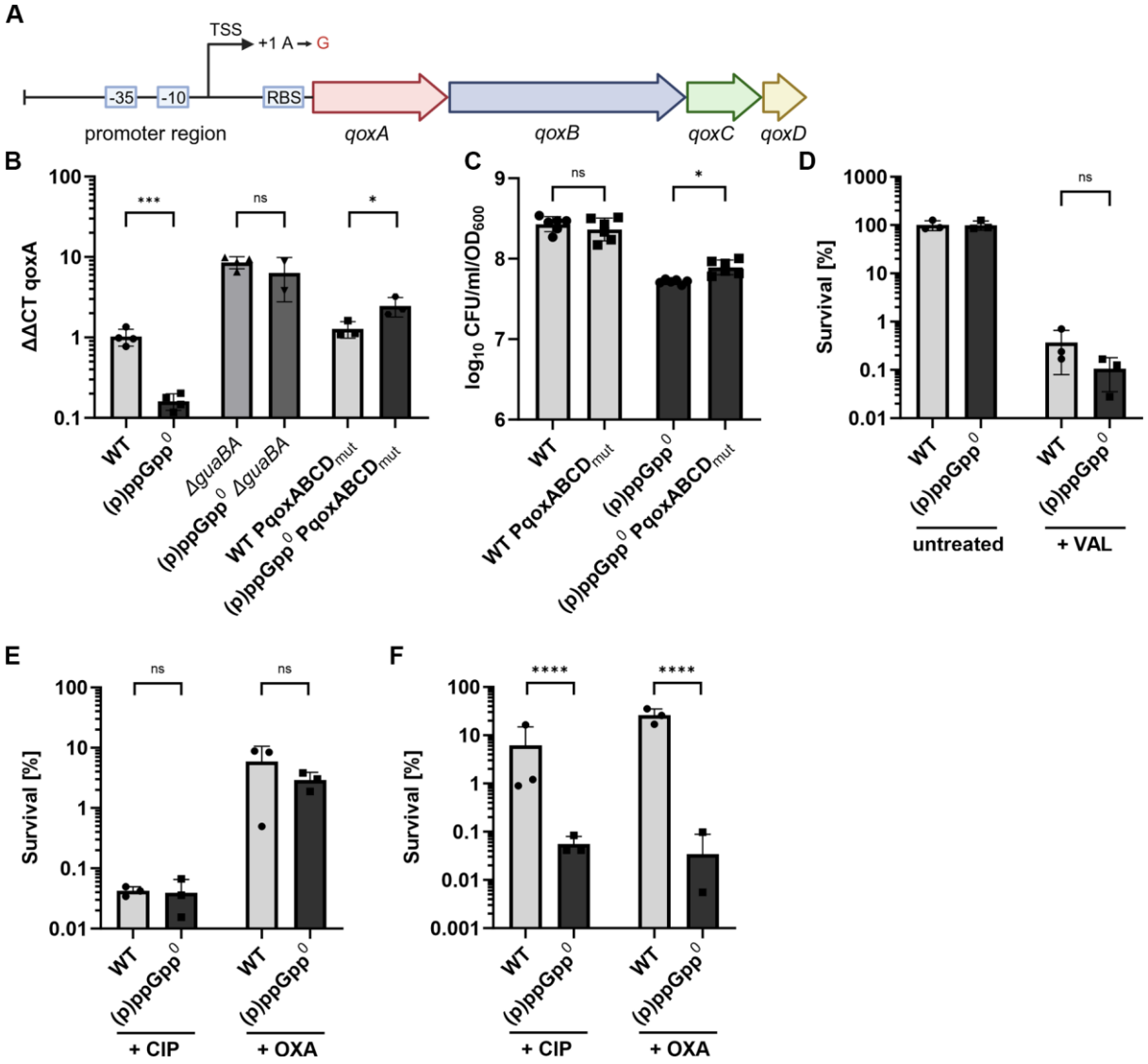

Fig. 8 Decrease in ETC activity contributes to entrance into dormant state and decreased antibiotic tolerance

(A) *qoxABCD* gene operon with promoter region. The predicted initiation nucleotide (TSS +1) iATP was mutated to iGTP. (B) Bacterial cells were harvested during the late stationary growth phase and total RNA was isolated. *qoxA* transcript levels were determined by RT-qPCR and normalized to *gyrB* expression by ΔΔCt method. Data are shown as mean ± SD (n=3 biological replicates). Statistical significance was determined by two-tailed unpaired t-tests (***p-value <0.001, *p-value <0.05, ns >0.05). (C) Culturability during late stationary phase (24h) was determined by CFU enumeration and normalized to OD_{600}. Data shown are mean of ± SD (n=6 biological replicates from two individual experiments). Statistical significance was determined by a one-way analysis of variance (ANOVA) with Tukey's post-test (*p-value <0.05). (D) Wildtype (WT) and (p)ppGpp^0 cells were treated with a sub-inhibitory concentration of valinomycin (20 µM) during the late stationary growth phase (24h) and viability was assessed by CFU enumeration after 24h of treatment. Survival was calculated in comparison to

untreated samples. Data shown are mean of ± SD (n=3 biological replicates). Statistical significance was determined by a two-way analysis of variance (ANOVA) with Šidák's post-test (ns >0.05, based). (E) Mid-exponential phase or stationary phase (F) cells were treated with 100x MIC ciprofloxacin (+ CIP) or oxacillin (+ OXA) and survival was calculated after 3h (E) or 24h (F) of treatment in comparison to untreated cells. Data shown are mean of ± SD (n=3 biological replicates). Statistical significance was determined by a two-way analysis of variance (ANOVA) with Šidák's post-test (****p-value <0.0001, ns >0.05).

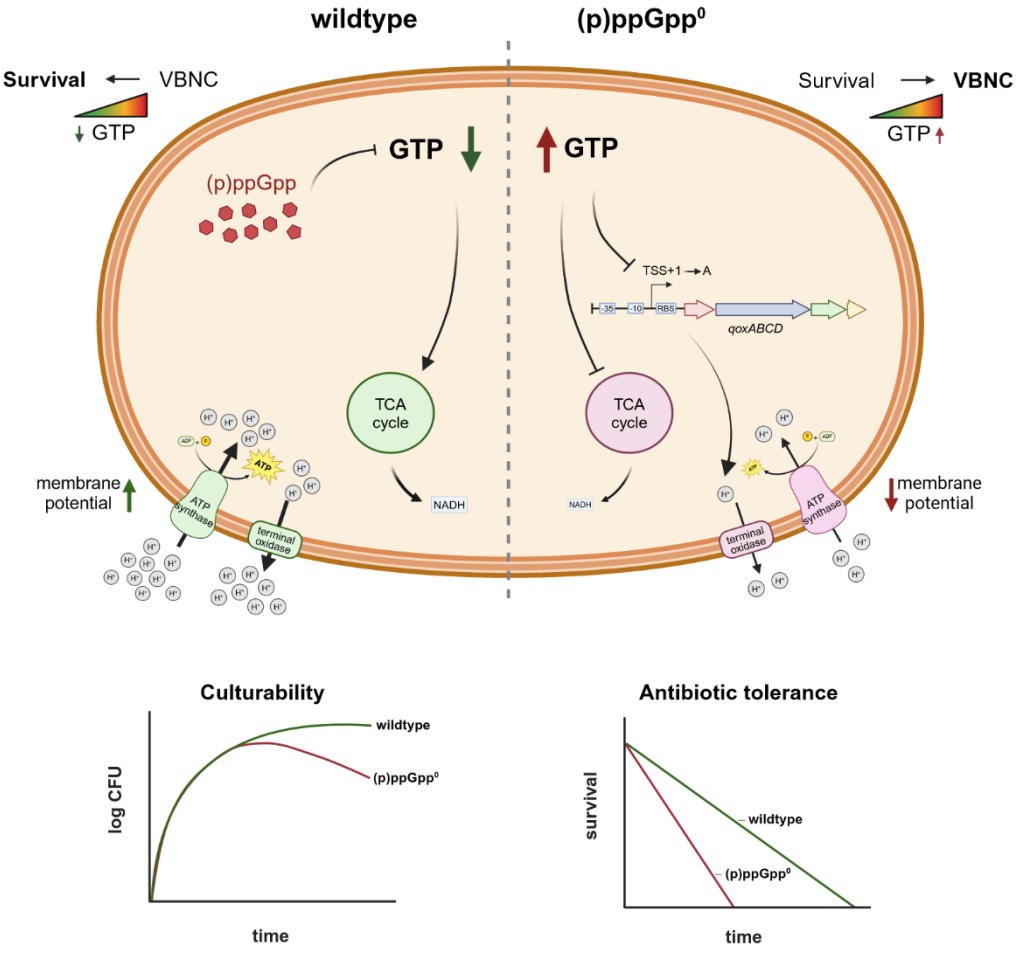

Fig. 9 (p)ppGpp-dependent regulation of GTP homeostasis enables maintenance of culturability.

During stationary phase starvation, (p)ppGpp synthesis contributes to regulation of intracellular GTP levels and thereby maintains membrane potential and ensures culturability and survival during antibiotic treatment. In a (p)ppGpp-deficient mutant, GTP homeostasis is disturbed leading to decreased expression of the TCA cycle and electron transport chain (ETC) genes including the terminal oxidase *qoxABCD*. This in turn leads to a decreased membrane potential which causes entrance into a division-incompetent but viable (VBNC) state, accompanied by reduced antibiotic tolerance.

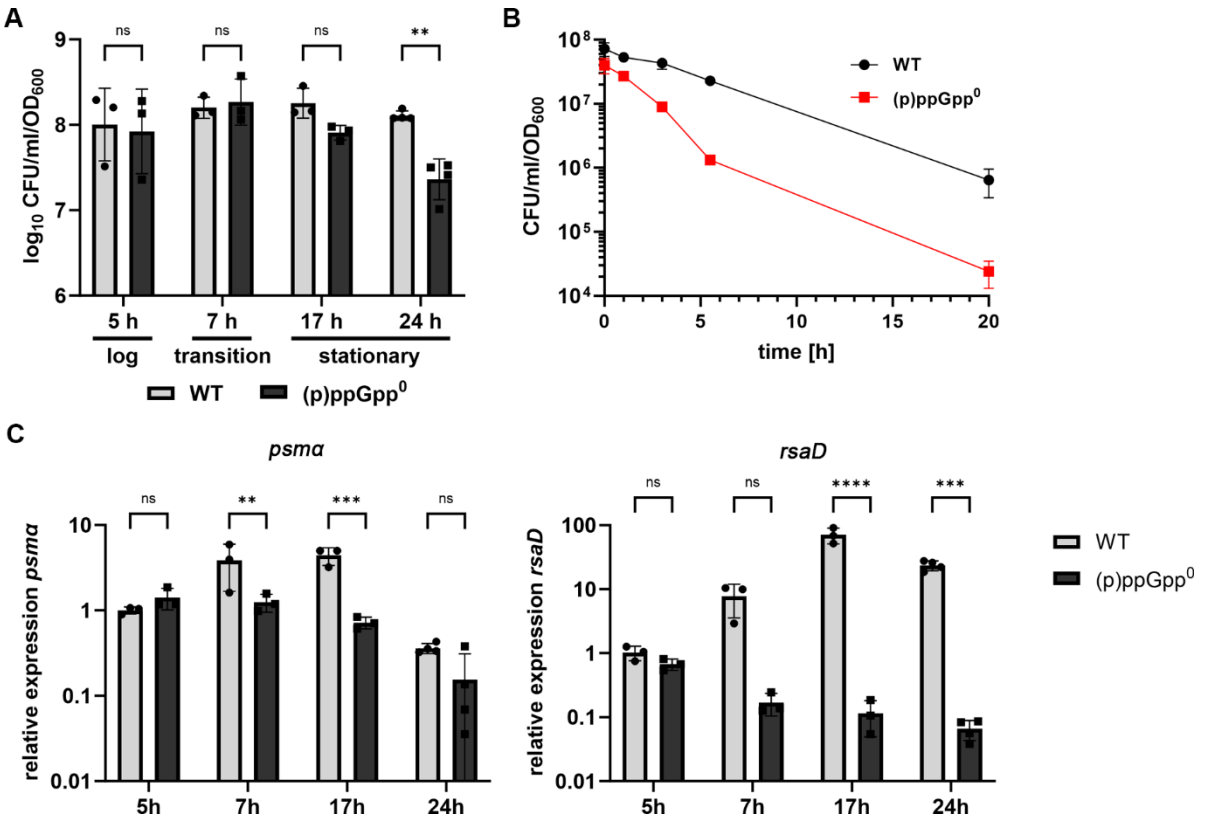

Fig. S1 Decreasing cultuarbility of (p)ppGpp^0 cells during stationary phase and stringent response induction

(A) Culturability of HG001 wildtype (WT) and (p)ppGpp^0 mutant was evaluated during different growth stages by CFU/ml enumeration normalized to OD_{600}. Data shown are mean of ± SD (n=3 biological replicates). Statistical significance was determined by a two-way analysis of variance (ANOVA) with Šidák's post-test performed on log_{10} transformed data (**p-value <0.01, ns >0.05). (B) Bacteria grown to exponential growth phase were treated with sub-inhibitory concentrations of mupirocin (0,125 µg/ml) and culturability was determined by CFU/ml enumeration normalized to OD_{600}. Data shown are mean of ± SD (n=3 biological replicates). (C) Bacterial cells were harvested during different growth phases and total RNA was isolated. *psmα and rsaD* transcript levels were determined by RT-qPCR and normalized to *gyrB* expression by ΔΔCt method. Data are shown as mean ± SD (n=3 biological replicates). Statistical significance was determined by two-way analysis of variance (ANOVA) with Šidák's post-test (***p-value <0.001, **p-value <0.01, *p-value <0.05, ns >0.05).

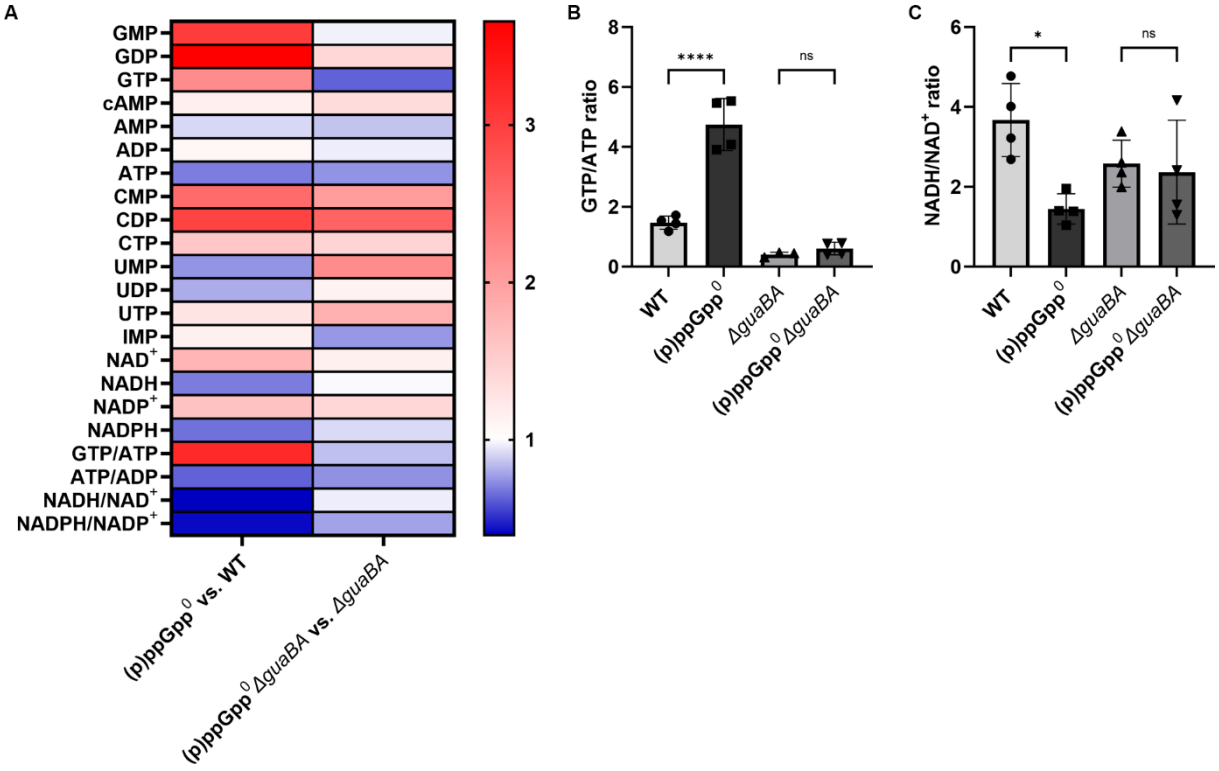

Fig. S2 Determination of nucleotide levels in late stationary phase cells.

(A) Metabolites were extracted and nucleotides were measured and quantified via LC-MS/MS from cells grown to late stationary growth phase (24h). Shwon are relative nucleotide levels as determined by comparing (p)ppGpp^0 vs. WT and (p)ppGpp^0 *ΔguaBA* vs. *ΔguaBA*. (B) The GTP/ATP ratio and (C) the NADH/NAD$^+$ ratio were calculated from the relative nucleotide levels. Data shown are mean of ± SD (n=4 biological replicates). Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey's post-test (****p-value <0.0001, *p-value <0.05, ns >0.05).

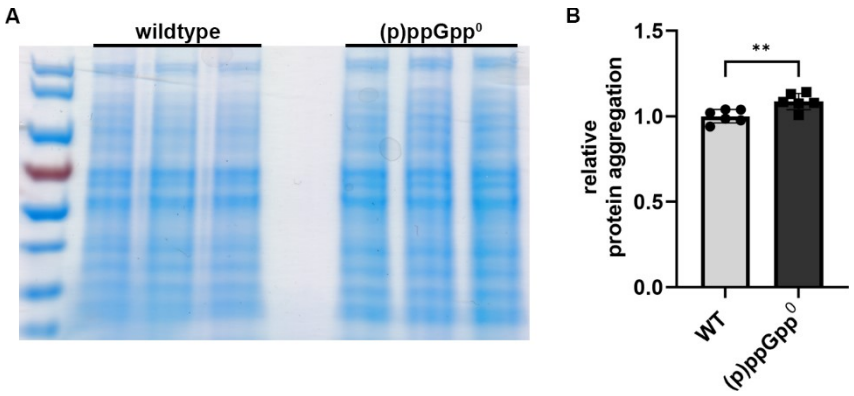

Fig. S3 Quantification of protein aggreagates.

(A) Protein aggregates were isolated from wildtype and (p)ppGpp^0 cells grown to late stationary phase and anaylsed by SDS-PAGE and Coomassie staining. For each strain, protein

aggregates were isolated from three individual biological replicates (n=3). (B) Protein aggregates were quantified by densiometric analysis. Data shown are mean of ± SD (n=6 biological replicates from two individual experiments). Statistical significance was determined by a two-tailed, unpaired t-test (**p-value <0.01).

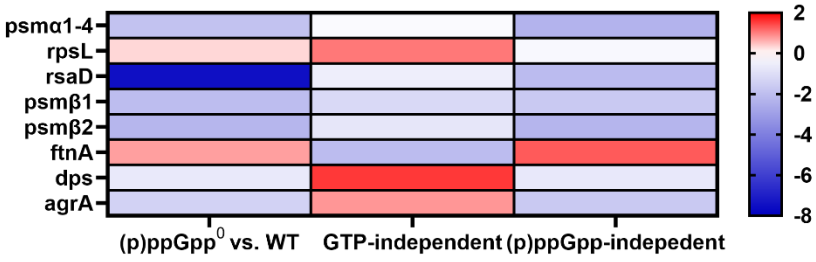

Fig. S4 RNA-seq analysis of stringent response genes

Heatmaps displaying the RNA-seq fold change of selected stringent response regulated genes. Log_2 fold changes in relative transcript abundances are colour-coded with red and blue, indicating up- and downregulation, respectively. “GTP-independent” compares the expression levels of (p)ppGpp^0 *ΔguaBA* vs. *ΔguaBA,* while “(p)ppGpp-independent” compares the expression levels of (p)ppGpp^0 vs. (p)ppGpp^0 *ΔguaBA*. The RNA-seq data shown are from three individual biological replicates (n=3). Full RNA-seq data are available in Table S1 and S2 in the supplementary material.

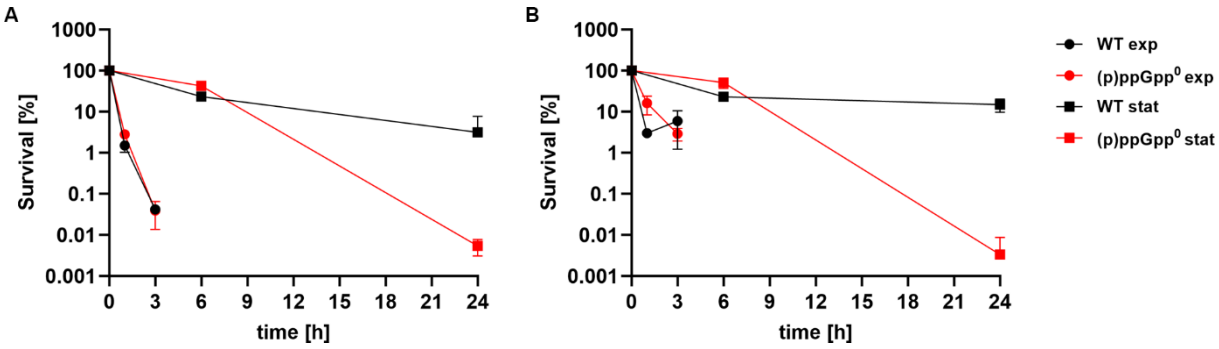

Fig. S5 Antibiotic tolerance in exponential and stationary phase.

(A) Stationary phase (■) or mid-exponential phase (●) bacteria were treated with 100x MIC ciprofloxacin (A) or oxacillin (B) and survival was calculated in comparison to untreated cells at different time points after treatment. Data shown are mean of ± SD (n=3 biological replicates).

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