Effect of (pp)pGpp on stress responses in *Staphylococcus aureus*

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

Petra Horvatek

aus Stuttgart

Tübingen

2020

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	15.06.2020
Dekan:	Prof. Dr. Wolfgang Rosenstiel
1. Berichterstatter:	Prof. Dr.Christiane Wolz
2. Berichterstatter:	Prof. Dr.Heike Brötz-Oesterhelt

Erklärung: Hiermit erkläre ich, dass ich diese Arbeit selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt und Stellen, die wörtlich oder inhaltlich nach Werken anderer Autoren entnommen sind, als solche gekennzeichnet hab. Eine detaillierte Abgrenzung meiner eigenen Leistungen und Beiträge meiner Kooperationspartner habe ich in „Erklärung zum Anteil gemeinschaftlicher Arbeit im Rahmen der Dissertation“ vorgenommen.

Unterschrift

Tübingen, den……

Meiner Familie und meinen Freunden für die Unterstützung, Geduld, Kraft und Liebe.

Table of contents

1 Summary

The stringent response is a conserved regulatory mechanism among a variety of bacterial species and is characterized by the synthesis of the alarmones pGpp, ppGpp and pppGpp, also collectively called (pp)pGpp. The activating signal of the stringent response differs among species and results in a variety of physiological alterations. In *Staphylococcus aureus* three enzymes regulate the intracellular level of (pp)pGpp and react to different stress signals. The RelA-SpoT homologue RSH from *Staphylococcus aureus* synthesizes (pp)pGpp in response to amino acid deprivation. In contrast, the small alarmone synthetase RelP and RelQ are transcriptionally activated upon cell wall stress. In this thesis, I was interested in whether (pp)pGpp synthesis of the three enzymes results in similar transcriptional changes independent of their activating mechanism. So far, genomic analyses were performed under artificially induced stress conditions. However, side effects cannot be excluded. Therefore, I used transcriptional induction of a truncated RSH, with an inactive hydrolase (RSH-Syn), RelP and RelQ, ensuring results are only (pp)pGpp-mediated. Thereupon, I compared the nucleotide pool of directly inducing (pp)pGpp versus stress mimicking conditions. Indeed, transcriptional induction resulted in similar changes compared to induced stringent response via mupirocin. Typical stringent response related changes of the nucleotide pool are characterized by lowering of the GTP-Pool, increased ATP pool and the detection of pGpp, ppGpp and pppGpp. These changes were detectable for RSH and RelP but minor in RelQ, indicating a limited synthetase activity *in vivo*. Next, RNA-Seq was used to compare global transcriptional changes between RSH and RelQ-mediated (pp)pGpp synthesis. Genes involved in iron storage and oxidative stress response are highly upregulated in response to RSH-induction. Furthermore, phenol soluble modulins (PSMs) α1-4 and β1/2 are upregulated independent of their major regulator Agr. (pp)pGpp-dependent expression of *ftnA*, *dps* and *psms* is independent of the regulators CodY, PerR, Fur and SarA. Furthermore, (pp)pGpp-mediated expression of *psms* results in increased production of reactive oxygen species (ROS). In conclusion, expression of oxidative stress response genes is indispensable for *S. aureus* to be protected of PSM-mediated or exogenous ROS. Hence, in this thesis I identified a new link of a (pp)pGpp-dependent regulation of oxidative stress response and PSMs. The activation of the stringent response and the concomitant expression of *psms* and related oxidative stress genes, likely contribute to the survival within the phagosome.

2 Zusammenfassung

Die stringente Kontrolle ist ein konservierter regulatorischer Mechanismus, welcher in den unterschiedlichsten Bakterien vorkommt und durch die Synthese der Alarmone pGpp, ppGpp und pppGpp charakterisiert ist. Diese Alarmone werden zusammenfassend (pp)pGpp genannt. Die stringente Kontrolle wird durch verschiedene Signale aktiviert und resultiert in unterschiedliche physiologische Veränderungen. Das RelA/SpoT Homolog RSH reagiert auf Aminosäuremangel, wohingegen die “small alarmone” Synthetasen (SAS) RelP und RelQ aufgrund von Zellwandstress transkriptionell aktiviert werden. Trotz unterschiedlicher aktivierender exogener Signale, synthetisieren alle drei Synthetasen (pp)pGpp. Das Ziel meiner Arbeit bestand darin, die transkriptionellen Konsequenzen nach Induktion der einzelnen Enzyme und deren (pp)pGpp-Synthese zu vergleichen und zu bewerten. Frühere genomische Analysen wurden unter Stress-induzierenden Bedingungen durchgeführt. Solche Stress-induzierende Bedingungen wurden durch Antibiotika wie Mupirocin herbeigeführt. Die Verwendung solcher “Stressinduzierer” schließt (pp)pGpp-unabhängige Nebeneffekte nicht aus. Aus diesem Grund wurden RelP, RelQ und eine kurze Variante des RSHs mit inaktiver Hydrolase verwendet, welche mittels Anhydrotetracyclin (ATc) transkriptionell exprimiert wurden. Diese drei Konstrukte wurden in einem (pp)pGpp negativen Stamm analysiert. Somit konnten folgenden Ergebnisse als rein (pp)pGpp-abhängig interpretiert werden. Zunächst wurde der Nukleotidpool zwischen Mupirocin-induzierter und direkter (pp)pGpp-Synthese verglichen. Zum einen konnte ich nachweisen, dass die transkriptionelle Induktion von RSH-Syn, RelP und RelQ zur (pp)pGpp-Synthese führt und zum anderen zum Anstieg von ATP und Abfall von GTP. Diese Veränderungen des Nukleotidpools sind charakteristisch für die stringente Kontrolle. RelQ wies eine geringe (pp)pGpp Synthese auf, was vermutlich auf eine schwache Synthetaseaktivität *in vivo* zurück zu führen ist. Um die (pp)pGpp-abhängigen Folgen, induziert durch RSH und RelQ, zu untersuchen, wurde das Transkriptom mittels RNA-Sequenzierung analysiert. Die Analyse weist eine auffällige Heraufregulierung von Genen auf, welche der oxidativen Stressantwort und Speicherung von Eisen zuzuordnen ist. Darüber hinaus werden “phenol soluble modulins” (PSMs) α1-4 und β1/2 unabhängig von ihrem Hauptregulator Agr stärker exprimiert. Weiter Analysen zeigen, dass die erhöhte Expression von *ftnA*, *dps* und *psms* keine indirekten Effekte der Regulatoren CodY, PerR, Fur und SarA ist.

Die (pp)pGpp-abhängige Expression von *psms* führt zu einem messbaren Anstieg von “reactive oxygen species” (ROS). Schlussfolgernd führt die Synthese von (pp)pGpp zu einem PSM-induzierten Anstieg von endogenen ROS, vor welchem sich *Staphylococcus aureus* durch die simultane Expression von oxidativen Stressgenen schützt. Die Aktivierung der stringenten Kontrolle und die damit einhergehende Expression von *psms* und oxidative Stressgenen, tragen mit sehr hoher Wahrscheinlichkeit zum Überleben innerhalb des Phagosoms bei.

3 List of publications

Publication 1

Steinchen W, Vogt MS, Altegoer F, Giammarinaro PI, **Horvatek P**, Wolz C, Bange G "Structural and mechanistic divergence of the small (p)ppGpp synthetases RelP and RelQ." Scientific Reports 2018 Feb 1;8(1):2195.doi: 10.1038/s41598-018-20634-4

Publication 2

Gratani FL, **Horvatek P**, Geiger T, Borisova M, Mayer C, Grin I, Wagner S, Steinchen W, Bange G, Velic A, Maček B, Wolz C. " Regulation of the opposing (p)ppGpp synthetase and hydrolase activities in a bifunctional RelA/SpoT homologue from Staphylococcus aureus." PLoS Genetics 2018 Jul 9;14(7):e1007514. doi: 10.1371/journal.pgen.1007514. eCollection 2018 Jul.

Manuscript ready for submission

Horvatek P, Hanna AMF, Gratani FL, Keinhörster D, Korn N, Borisova-Mayer, Mayer C, Rejman D, Mäder U, Wolz C. "Increasing the cellular (pp)pGpp level is associated with activation of stress response genes in *Staphylococcus aureus".*

4 Personal contribution to publications

Accepted publications

1.) **Structural and mechanistic divergence of the small (p)ppGpp synthetases RelP and RelQ.**

I contributed scientific ideas and evaluated the role of zinc on interaction with RelP in vivo

2.) **Regulation of the opposing (p)ppGpp synthetase and hydrolase activities in a bifunctional RelA/SpoT homologue from *Staphylococcus aureus***

I contributed to this work by cloning and evaluation of inducible RSH constructs. These results laid the basis for the experimental set-up described in the manuscript. For published experiments I performed growth analyses using a multi-plate reader and Northern blot analyses. I investigated and supported this work with scientific advice and ideas.

Manuscript ready for submission

3.) **Increasing the cellular (pp)pGpp level is associated with activation of stress response genes in *Staphylococcus aureus***

I contributed to the design and contribution of the experiments. I performed or supervised all experiments except for measurement of nucleotides via mass spectrometry (ESI-TOF). I modified and established nucleotide extraction for *Staphylococcus aureus in vivo*. Analysis and data interpretation was performed by me accept for peak separation of GTP and pGpp occurred by bioinformatical support. The paper was written in cooperation with Christiane Wolz.

5 Introduction

5.1 The stringent response

The stringent response is a conserved regulatory mechanism in different bacteria and is characterized by the synthesis of alarmones pGpp, ppGpp and pppGpp, collectively called (pp)pGpp. (Battesti & Bouveret, 2006; Cashel & Kalbacher, 1970; B. Das, Pal, Bag, & Bhadra, 2009; Geiger et al., 2010; Mechold, Murphy, Brown, & Cashel, 2002). (pp)pGpp was described for the first time in 1969 by Michael Cashel who identified two "magic spots" in *E .coli* under amino acid limited conditions (Cashel & Kalbacher, 1970). (pp)pGpp is synthesized by GTP-pyrophosphatases (RelA, SpoT, RSH, RelP/Q/V) via transferring the pyrophosphate group from ATP to either GMP (pGpp), GDP (ppGpp) or GTP (pppGpp). The bifunctional RSH and SpoT can reverse this reaction and hydrolyze (pp)pGpp by cleaving a pyrophosphate group (Fig1). (pp)pGpp is assumed to be synthesized at a basal level during exponential growth in order to fine-tune cellular processes. In stationary growth phase nutrient availability is limited and (pp)pGpp synthesis is increased (Hauryliuk, Atkinson, Murakami, Tenson, & Gerdes, 2015). This results in various physiological changes e.g. inhibition of rRNA synthesis, translation, restricted metabolism and activation of genes necessary to overcome the stringent conditions (Hauryliuk et al., 2015; Potrykus & Cashel, 2008; Steinchen & Bange, 2016; Wolz, Geiger, & Goerke, 2010). In pathogenic bacteria the stringent response triggers gene activation responsible for persister formation, host-pathogen interactions and virulence (Dalebroux, Svensson, Gaynor, & Swanson, 2010; Dalebroux & Swanson, 2012; Hobbs & Boraston, 2019a; Kushwaha, Oyeyemi, & Bhavesh, 2019). Bacteria impaired in (pp)pGpp synthesis were shown to be more sensitive to antibiotics, produce less biofilm and are attenuated in virulence. These features and the fact that the stringent response is a conserved mechanism in many pathogenic bacteria makes it attractive as a novel drug target. Several (pp)pGpp inhibiting compounds were developed (Syal et al., 2017; Wexselblatt et al., 2012) and evaluated (Wexselblatt, Kaspy, Glaser, Katzhendler, & Yavin, 2013) to inhibit bacterial long-term survival.

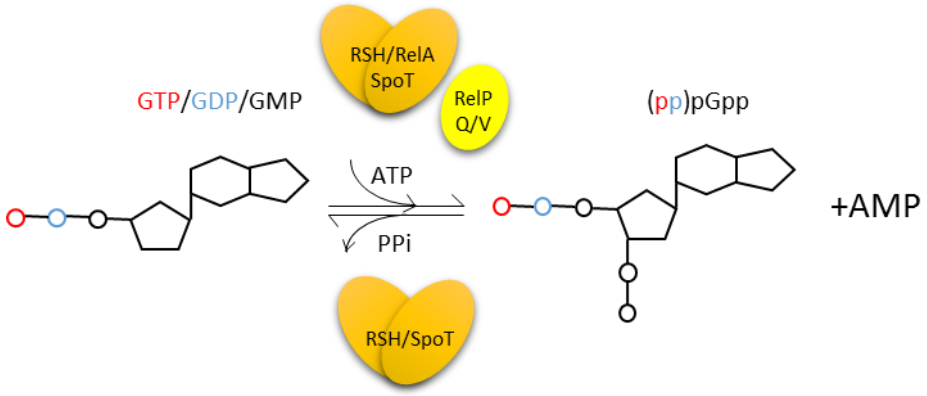

Fig.1 Synthesis and degradation of (pp)pGpp by RSH/RelA/ SpoT and the small alarmone synthetases RelP/Q/V. Under stringent conditions the pyrophosphate group of ATP is transferred on either GMP, GDP or GTP by RSH, RelA, SpoT or RelP/Q/V resulting in pGpp, ppGpp or pppGpp and the by-product AMP. Overcoming stringent conditions (pp)pGpp is hydrolyzed by removing the pyrophosphate group via RSH or SpoT.

5.2 General structure of RelA, SpoT and RelA/SpoT homologues (RSH), the small alarmone synthetases (SAS) RelP/Q/V and small alarmone hydrolase (SAH) RelV

RSH enzymes are conserved among different species like plants, algae and bacteria (Atkinson, Tenson, & Hauryliuk, 2011). “Long” RSH enzymes consist of an enzymatic N-terminal domain with a hydrolase and synthetase domain and a regulatory C-terminus, bearing the TGS, DC and ACT domain. Although all RSH enzymes share the same basic structure, they exhibit differences in their function. In 1989 Metzger proposed RelA and SpoT evolved from a gene duplication of a RSH-like enzyme (Metzger 1989). This gene duplication may explain the different functions and the amino acid substitution event as it may have occurred in the hydrolase domain of RelA (Atkinson et al., 2011; Mittenhuber, 2001). Beside the “long” RSH, a subset of small alarmone synthetase (SAS) and hydrolases (SAH) exist. SAS (RelP/Q/V) and SAH (RelH) are shorter than RSH, do not possess a regulatory domain and exhibit one enzymatic activity (synthesis or degradation) (Fig.2).

The following chapter will explain the function of the single domains of RSH more in detail as exemplified on the *E. coli* RelA enzyme.

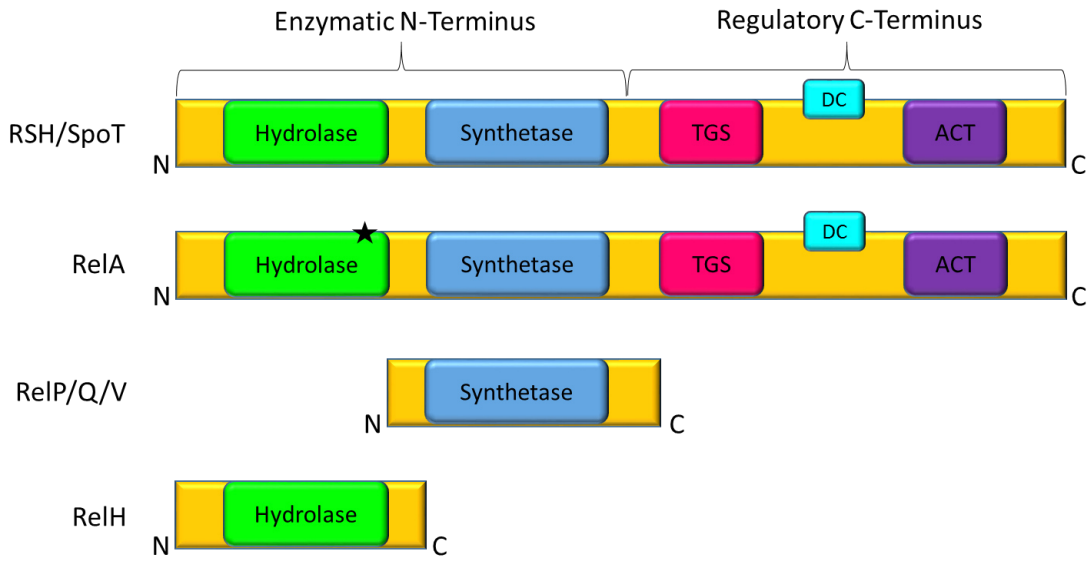

Fig.2 Structural composition of RSH, SpoT, RelA, the small alarmone synthetases RelP/Q/V and small alarmone hydrolases RelH. All three "long" RSH enzymes RSH, SpoT and RelA are divided in an enzymatic N-terminus and a regulatory C-terminus. The N-terminal domain of RSH and SpoT is bifunctional, comprising hydrolase and synthetase activity. In contrast, RelA is a monofunctional enzyme with both domains (hydrolase and synthetase) but exhibits only a synthetase activity due to an amino acid substitution in the hydrolase domain (indicated with a star). The regulatory C-terminus consist of a TGS, ACT and DC domain, which is shared by all three "long" RSH-like enzymes. Unlike the "long" RSH, small alarmone synthetases or hydrolases do not possess a regulatory domain and consist of only one enzymatic activity; either synthetase (RelP/Q/V) or hydrolase (RelH).

5.3 RelA

The first enzyme described was RelA and was named after its "relaxed" phenotype in *E. coli* ($RelA_{Ec}$), meaning non-stringent conditions (Cashel & Kalbacher, 1970). RelA consists of a monofunctional enzymatic N-terminal domain with a non-functional hydrolase and a functional synthetase domain. The defect of the hydrolase probably derives from a mutation of the histidine in motif II or aspartate in motif V (Aravind & Koonin, 1998).

$RelA_{Ec}$ reacts upon amino acid starvation (Cashel & Kalbacher, 1970) and is located at the 70S ribosome under amino acid poor conditions (Haseltine, Block, Gilbert, & Weber, 1972). Upon amino acid depletion, uncharged tRNA enters the A-side of the ribosome and stalls translation. The location of RelA in the process of sensing amino acids is not clear. Two models were postulated namely Hopping model and a subsequently extended version of the same.

5.3.1 Hopping model

The hopping model proposes, that RelA is “floating” around in the cytoplasm searching for stalled ribosomes (Fig.3) (Wendrich, Blaha, Wilson, Marahiel, & Nierhaus, 2002). This is supported by Brown et al suggesting stalled ribosomes recruit RelA (A. Brown, Fernandez, Gordiyenko, & Ramakrishnan, 2016). The C-terminal domain of RelA interacts with the ribosome and senses uncharged tRNAs via TGS (Loveland et al., 2016; Wendrich et al., 2002) resulting in (pp)pGpp synthesis. (pp)pGpp synthesis stimulates the release of RelA from the stalled ribosome and RelA consequently “hops” to the next stalled ribosome repeating this procedure. Therefore this model proposes that the amount of (pp)pGpp is equivalent to the amount of stalled ribosomes (Wendrich et al., 2002).

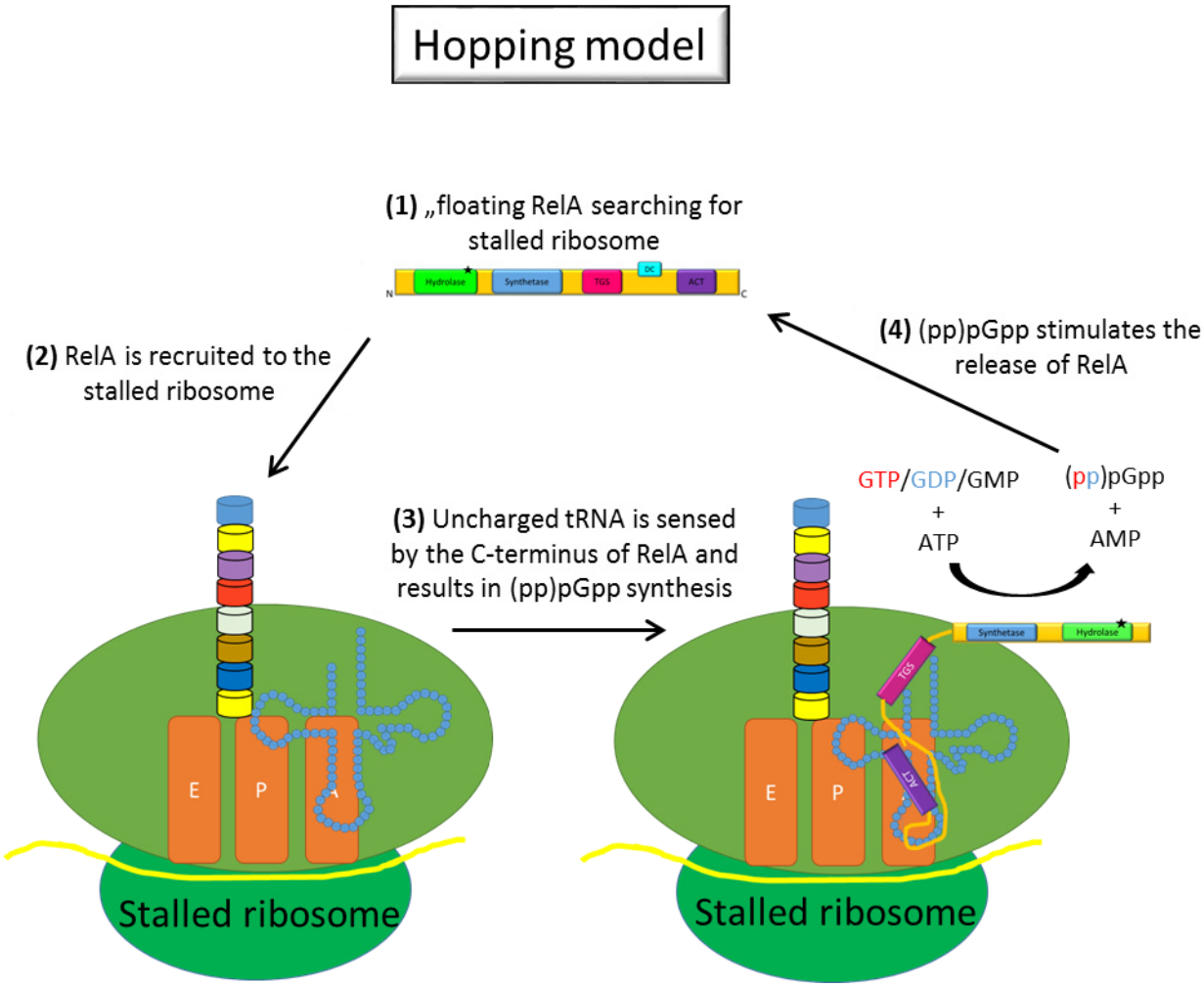

Fig.3 Schematic description of the hopping model. (1) Free RelA wait in the cytoplasm for stalled ribosomes (Wendrich et al., 2002) to be subsequently recruited to the ribosome (A. Brown et al., 2016). (3) Uncharged tRNAs are sensed by the C-terminal domain (Loveland et al., 2016; Wendrich et al., 2002) via TGS (Loveland et al., 2016) and (pp)pGpp is synthesized (Wendrich et al., 2002), (4) which stimulates the dissociation of RelA, followed turning off the synthetase.

5.3.2 Extended Hopping model

In contrast to the hopping model, the extended version suggests, that under “relaxed” conditions RelA is bound to the ribosome waiting for uncharged tRNAs to be sensed (English et al., 2011) and not “floating” around in the cytoplasm (Fig.4). This is supported by recent studies suggesting RelA first binds to the unloaded A-site of the ribosome followed by the recruitment of uncharged tRNAs (Kudrin et al., 2018; Loveland et al., 2016). Consequently, RelA dissociates from the ribosome and synthesizes (pp)pGpp. Additionally, uncharged tRNAs can be recognized by free RelA floating in the cytoplasm leading to a “semi-open” conformation mediating (pp)pGpp synthesis. This RelA-deacetylated-tRNA-complex can then bind to the A-site of the ribosome, leading to an open conformation of RelA and (pp)pGpp synthesis (Arenz et al., 2016). This is supported by recent UV-crosslinking studies suggesting RelA primarily binds uncharged tRNA followed by binding of the RelA-uncharged-tRNAs complex to the A-site of the ribosome, which than results in (pp)pGpp synthesis (Winther, Roghanian, & Gerdes, 2018). In contrast to hopping model, the extended version suggests intracellular level of (pp)pGpp is equivalent to the number of uncharged tRNAs (English et al., 2011). Nevertheless, the majority of these studies suggest, unbound RelA is inactive and (pp)pGpp synthesis is a consequence of RelA bound to the ribosome in the presence of uncharged tRNAs at the A-site (Arenz et al., 2016; A. Brown et al., 2016; Kudrin et al., 2018; Loveland et al., 2016; Wendrich et al., 2002; Winther et al., 2018). However, within these publications it remains unclear, whether RelA first builds a complex with uncharged tRNA which is transported to the ribosome (Arenz et al., 2016; Winther et al., 2018), RelA first binds to the stalled ribosome followed by the binding of uncharged tRNAs (Kudrin et al., 2018; Loveland et al., 2016) or uncharged tRNAs first bind to the A-site of the ribosome recruiting RelA (A. Brown et al., 2016; Wendrich et al., 2002). So far, none of the models and biochemical analyses could clarify whether RelA is permanently bound to the ribosome waiting for uncharged tRNA or “floating” in the cytoplasm waiting to be recruited to the ribosome or “fishing” for unloaded tRNAs followed by binding to the A-site of the ribosome. The discrepancies presumably result from applying a variety of different techniques, such as Cryo-EM (Arenz et al., 2016; A. Brown et al., 2016; Loveland et al., 2016), HPLC (Kudrin et al., 2018; Wendrich et al., 2002), enzymatic and binding assays (Kudrin et al., 2018; Wendrich et al., 2002) and variations within the experimental designs.

Since these studies were conducted independently and both hypothesis are confirmed to a certain extent, RelA can possibly detect amino acid starvation with more than one sensing mechanism. This would be beneficial as it would ensure a rapid response to unfavorable conditions.

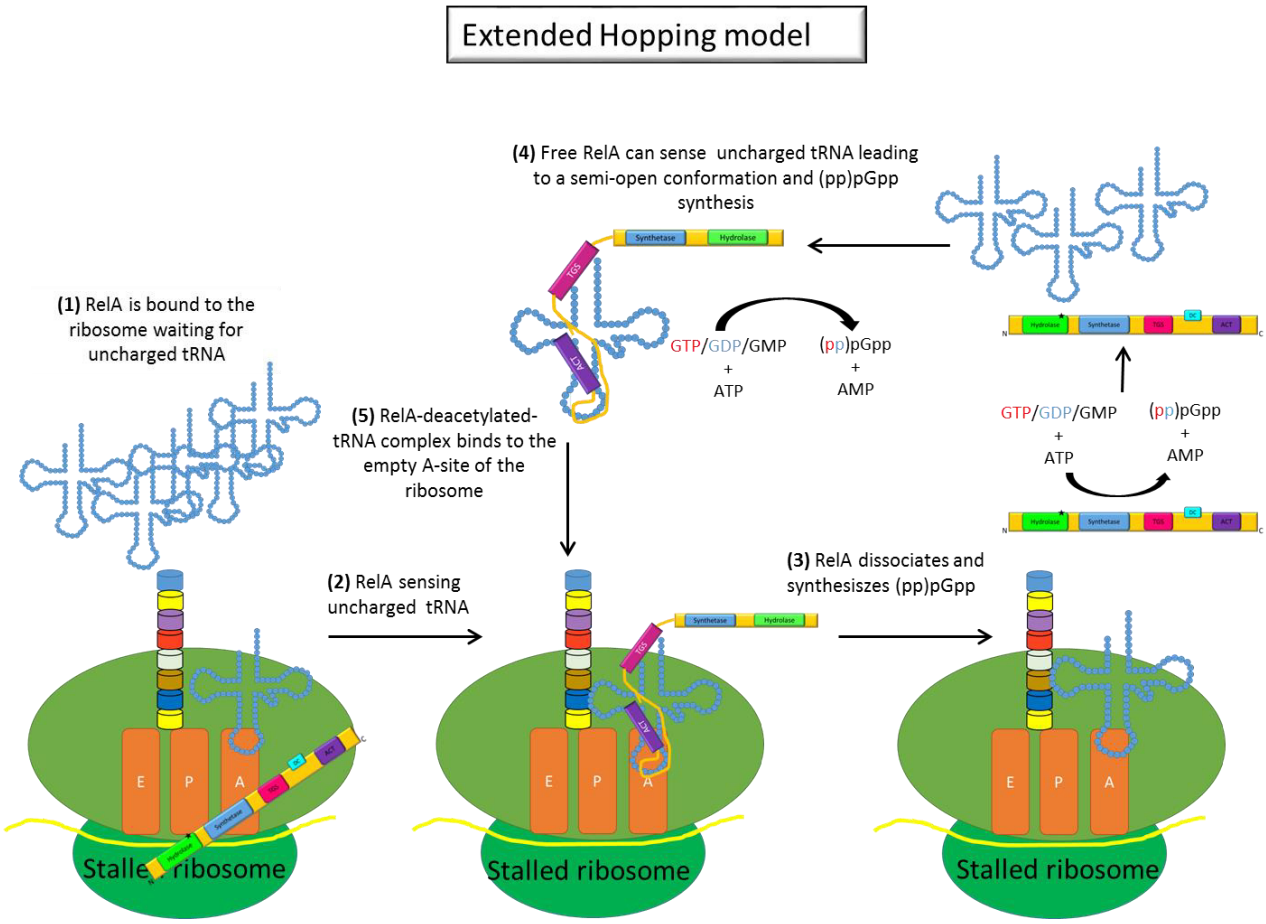

Fig.4 Schematic representation of the extended hopping model. (1) RelA is bound to the A-site of the ribosome waiting to sense (English et al., 2011) or to recruit (Kudrin et al., 2018; Loveland et al., 2016) uncharged tRNA. (2) Uncharged tRNA entering the A-site of the ribosomes are sensed by RelA. (3) Consequently, RelA dissociates and synthesizes (pp)pGpp. (4) Free RelA can recognize intracellular uncharged tRNA leading to a semi-open conformation and subsequent (pp)pGpp synthesis. (5) This RelA-deacetylated-tRNA complex is transported to the empty A-site of the ribosome yielding in an open-conformation and full (pp)pGpp synthesis (Arenz et al., 2016).

5.3.3 The regulatory C-terminal domain

5.3.3.1 TGS

All three enzymes RelA, SpoT and RSH share a common regulatory C-terminal domain (Fig.2). The C-terminal domain carries two major motifs. The TGS is named by the three protein families it was identified in: **t**hreonyl-tRNA synthetase, Obg family of **G**TPases and **S**poT (Battesti & Bouveret, 2006). Recent crystal structures could clarify which domain of $RelA_{Ec}$ senses uncharged tRNA. TGS domain of ribosome-bound $RelA_{Ec}$ senses uncharged tRNAs. TGS can only bind to the CCA sequence-end of deacetylated tRNAs, thereby discriminating between charged or uncharged tRNAs (Fig.5) (Arenz et al., 2016; A. Brown et al., 2016; Kudrin et al., 2018; Loveland et al., 2016). Uncharged tRNA attaches the center of TGS (Fig.5) (Loveland et al., 2016) (A. Brown et al., 2016) to the ribosomal 30S subunit which pushes the synthetase domain closer to the spur of the ribosomal 30S subunit (Loveland et al., 2016).

5.3.3.2 ACT

The ACT domain (**A**spartate kinase, **C**horismate mutase, prephenate dehydrogenase **T**yrA) is a domain which appears in many proteins from different organisms like plants (Hsieh & Goodman, 2002) and bacteria and are often found to be involved in purine and amino acid biosynthesis genes (Chipman & Shaanan, 2001; Hsieh & Goodman, 2002). It contains a GTP and ATP/GTP binding domain (Jin et al., 2004). Aravind and Koonin postulated in 1999 that ACT is a mobile element which integrates in different type of proteins resulting in distinct regulation with specific ligands (Aravind & Koonin, 1998). For example, in mammalian cells the ACT domain of the human phenylalanine hydroxylase (PAH) binds phenylalanine (Ge et al., 2018). In *E. coli* the ACT domain of the 3'phosphoglycerate dehydrogenase binds serine which inhibits its catalytic activity (Hsieh & Goodman, 2002). Cryo-EM studies demonstrate an interaction of the $RelA_{Ec}$ ACT domain with the A-site finger of the 23S rRNA and ribosomal protein L16. Moreover, the linker region between the TGS and ACT wraps around the acceptor stem of tRNAs allowing ACT to bind to the tRNA elbow region (Fig.5). These three independent Cryo-EM studies suggest ACT to be the linker between uncharged tRNAs and the ribosome in *E. coli* (Arenz et al., 2016; A. Brown et al., 2016; Loveland et al., 2016). Recent studies in *Rhodobacter capsulatus* showed ACT of Rel binds valine and isoleucine and probably influences hydrolase activity (Fang & Bauer, 2018).

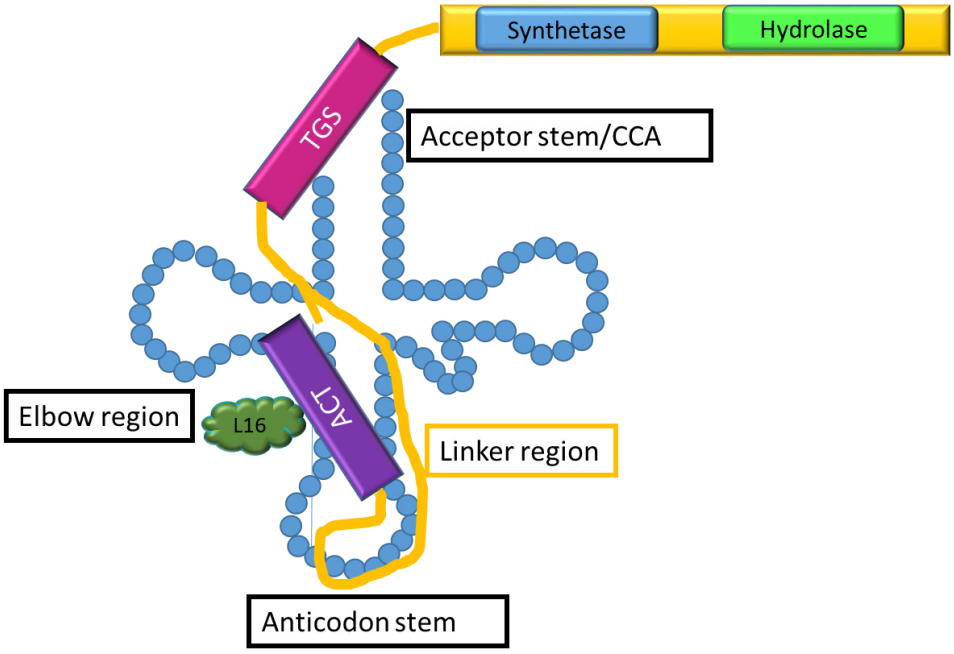

Fig.5: Schematic representation of the interaction of RelA C-terminus with deacetylated tRNA based on (Arenz et al., 2016; A. Brown et al., 2016; Loveland et al., 2016). The TGS domain senses an uncharged tRNA at CCA-end of the acceptor stem. The linker region binds around the tRNA and allows the ACT domain to bind the tRNA-elbow region. Furthermore, the ACT domain interacts with A-site finger of the 23S rRNA and the ribosomal protein L16.

5.3.3.3 The DC domain

The DC domain was characterized by three conserved amino acids. Mutational analysis in *E. coli* showed that these conserved amino acids are essential for a functional C-Terminus. RelA exists in a homooligomeric state and Cys-612, Asp-637 and Cys-638 play a crucial role in oligomerization. Mutation of these amino acids resulted in increased (pp)pGpp synthesis, indicating RelA forms oligomers in the cytoplasm under relaxed conditions and thereby leading to an autoinhibitory state. Consequently, stringent conditions lead to the disassociation into active monomers and (pp)pGpp synthesis (Avarbock et al., 2005; Gropp, Strausz, Gross, & Glaser, 2001; Jain, Saleem-Batcha, & Chatterji, 2007; Yang & Ishiguro, 2001). Furthermore, latest Cryo-EM (A. Brown et al., 2016; Loveland et al., 2016) and biochemical analyses (Kudrin et al., 2018) identified that these amino acids are part of a zinc finger domain (ZFD) and suggest, that the Cys-612 is crucial (A. Brown et al., 2016) for binding of $RelA_{Ec}$ to the ribosome via the A-site finger (ASF) (Loveland et al., 2016) (A. Brown et al., 2016; Kudrin et al., 2018).

Binding of the ZFD to the ribosome and uncharged tRNAs results in activation of the stringent response (Kudrin et al., 2018). Conclusively, the DC or ZFD domain seems to exert two different functions. Under relaxed condition RelA is oligomerized leading to a weak synthetase activity, whereas under stringent conditions the DC/ZFD domain anchors RelA to stalled ribosomes resulting in (pp)pGpp synthesis, presumably via disassociation into active monomers. These data support the idea of the hopping model, which suggest RelA is inactive in a ribosome-unbound state as a consequence of oligomerization and active in a ribosome-bound state resulting in (pp)pGpp synthesis.

5.4 SpoT

Besides RelA, gamma-proteobacteria possess a second enzyme, called SpoT. In contrast to RelA, SpoT is bifunctional, bearing an N-terminus with a strong hydrolase and a weak synthetase activity. Although RelA and SpoT have a very similar structure, SpoT responds to different stringent signals like fatty acid starvation (Battesti & Bouveret, 2006) and carbon depletion (Metzger,Schreiber et al. 1989), since the TGS domain of SpoT senses fatty acid starvation via the acyl carrier protein ACP (Battesti & Bouveret, 2006, 2009).

5.5 RelA/SpoT Homologue Enzymes (RSH)

RSH enzymes are bifunctional enzymes from other bacteria. The nomenclature varies among literature and can be often found as RSH or Rel with corresponding abbreviations of the organism. In this thesis I prefer RSH, since it describes best what it is: a combination of RelA and SpoT.
RSH enzymes are conserved among gamma- and beta-proteobacteria and react to different nutrient limitations resulting in different physiological changes.

5.6 Function of RSH in firmicutes and *Staphylococcus aureus*

In firmicutes such as *Staphylococcus aureus* (*S. aureus*) only one bifunctional enzyme exists which is called RSH (RelA/SpoT homologue). It harbors a functional hydrolase and synthetase comparable to SpoT and a regulatory C-terminal domain which senses amino acid starvation comparable to RelA. Deleting the C-terminus in *Streptococcus equisimilis* exerted in stronger synthetase and weaker hydrolase activity *in vitro* (Mechold et al., 2002).

Crystal structure analysis of the RSH N-terminal enzymatic domain in *Streptococcus equisimilis* revealed two different conformations, which decide whether RSH is in a Hydrolase-ON or OFF state. This conformational change is dependent on the binding of the unusual ligand ppG2:3'p. GDP is bound to the synthetase domain under both conditions. Binding of ppG2:3'p to the hydrolase results in a Hydrolase-ON/Synthetase-OFF conformation. Contrariwise, unbound ppG2:3'p results in an open conformation and a Hydrolase-OFF/Synthetase-ON state (Hogg, Mechold, Malke, Cashel, & Hilgenfeld, 2004). Comparable results have been observed for *S. aureus* (Geiger et al., 2010). Under relaxed conditions RSH_{Sau} is trapped in a Hydrolase-ON/ Synthetase-OFF state (Geiger et al., 2010). At the beginning of this work presented in this thesis, it was not clear, how the C-terminus influences the activity of the hydrolase and/or the synthetase.

5.7 Function of the small alarmone synthetases RelP and RelQ in firmicutes

RSH is not the only enzyme responsible for (pp)pGpp synthesis. In firmicutes, there are small alarmone synthetases, RelP and RelQ (B. Das et al., 2009) which only carry a synthetase domain and sense a variety of stress signals such as cell wall stress (Geiger, Kastle, Gratani, Goerke, & Wolz, 2014) ethanol stress (Pando et al., 2017) and fatty acid/ glucose stress (B. Das et al., 2009).

RelQ from *Bacillus subtilis* ($RelQ_{Bs}$) synthesizes ppGpp more efficiently than pppGpp and its activity is based on forming a homotetramer structure. Furthermore, there is a stimulation of the catalytic activity of RelQ by pppGpp binding into the cleft of the homotetramer thereby enhancing ppGpp synthesis. The physiological function is not yet understood (Steinchen et al., 2015). Further investigations demonstrate RelQ from *Enterococcus faecalis* ($RelQ_{Efa}$) activity is inhibited by single stranded mRNA. This inhibiting mRNA is defined by a specific sequence and length, presumably GAAGAA and a sequence length between 12 and 15 nucleotides (Beljantseva et al., 2017). This inhibition is reversible by ppGpp in RelQ. pppGpp and to a lesser extent ppGpp binding, leads to disassociation of the RelQ:RNA complex and subsequently the activation of RelQ. Under relaxed and stringent conditions RelQ forms homotetramers. Presumably, RelQ acts as a sensor to maintain (p)ppGpp homeostasis (Beljantseva et al., 2017). So far, these results are based on biochemical and structural analyses of purified RelQ. Functional studies *in vivo* are missing.

In *S. aureus*, the two small synthetases RelP and RelQ are activated transcriptionally after induced cell wall stress by either ampicillin or vancomycin (Geiger et al., 2014). In contrast to RelQ, RelP can also be transcriptionally activated after ethanol stress, emphasizing its role in also protecting *S. aureus* from ethanol induced stress (Pando et al., 2017). $RelP_{Sau}$ also forms tetramers (Manav et al., 2018; Steinchen et al., 2018) but, in contrast to RelQ, RelP is inhibited by ppGpp and pppGpp and activated by Zn^{2+} (Manav et al., 2018; Steinchen et al., 2018).

5.8 (pp)pGpp binding targets in firmicutes

Over the last years, more and more (pp)pGpp binding targets have been identified. Binding of (pp)pGpp results in inhibition of the respective target. Such (pp)pGpp binding targets can be categorized in GTP biosynthesis enzymes (HprT, Gmk) (Kriel et al., 2012a; Liu, Bittner, & Wang, 2015), GTPases (RsgA, RbgA, Era, HflX, ObgE and Obg) (Buglino, Shen, Hakimian, & Lima, 2002; Corrigan, Bellows, Wood, & Gründling, 2016) and inhibition of replication by binding to primase thereby preventing binding of RecA, which leads to the stalling of the replication fork (J. D. Wang, Sanders, & Grossman, 2007).

5.9 Effect of (pp)pGpp on the nucleotide pool

Altering the nucleotide pool is one of the major effects of (pp)pGpp resulting in different consequences such as altering gene transcription. (pp)pGpp inhibits enzymes involved in GTP synthesis leading to a drop of the intracellular GTP-pool. The sharp decrease of the GTP-pool provokes the de-repression of the master regulator CodY, which is only present in Gram positive bacteria (Handke, Shivers, & Sonenshein, 2008). CodY is a transcriptional repressor and binds to genes containing a CodY-box (AATTTTCWGAAAATT) (Brinsmade, 2017). Under high GTP level conditions and on abundance of branched chain amino acids, CodY is bound thus preventing RNAP binding. Low GTP-pool and amino acid deprivation changes CodY conformation and thereby its release from DNA. Subsequently, genes for a variety of functions are transcribed (Brinsmade, 2017; Geiger & Wolz, 2014; Handke et al., 2008). In *S.aureus*, (p)ppGpp is synthesized under stringent conditions consequently leading to the de-repression of CodY and to the transcription of genes involved in transport and metabolism of amino acids and virulence genes like *agr* (Geiger & Wolz, 2014; Pohl et al., 2009).

Lowering of the GTP-pool is not sufficient to de-repress CodY and requires additionally a low level of the branched chain amino acids (Pohl et al., 2009). Media lacking branched chain amino acids showed activation of CodY regulated genes and identified isoleucine as the major ligand for CodY besides GTP in *S. aureus* (Geiger et al., 2010). This demonstrates a tight cross talk between the GTP-pool, stringent response and the transcriptional repressor CodY. Therefore, experiments should be performed additionally in a CodY negative background to exclude an indirect effect due to de-repression of CodY.

5.10 Role of (pp)pGpp for biofilm, virulence, antibiotic tolerance and persistence in pathogenic firmicutes

Pathogenic firmicutes are a huge problem in hospitals due to their multiple defense mechanism against antibiotics and the innate immune system. One of the major human pathogen responsible for hospital acquired infections is *Staphylococcus aureus* (T. Das et al., 2019; Santajit & Indrawattana, 2016; Zhang et al., 2019). The preferred habitat of *S. aureus* is the nasal cavity (Krismer et al., 2014; Pynnonen, Stephenson, Schwartz, Hernandez, & Boles, 2011; Sakr, Bregeon, Mege, Rolain, & Blin, 2018). *S. aureus* is challenged by a variety of stress during colonization and infection of the human host. Switching niches from the nasal environment to soft tissue and blood-stream infections (Krismer et al., 2014; Lowy, 1998), is accompanied by a dramatic shift in nutrients and oxygen (S. A. Brown, Palmer, & Whiteley, 2008; George et al., 2019). *S. aureus* has developed different mechanisms to resist antibiotics and escape invasion by the immune system. Within the human host *S. aureus* has to escape the innate immune system. The stringent response supports adaptation by influencing virulence, biofilm formation, antibiotic tolerance and manipulating host immune system.

5.10.1 (pp)pGpp mediated biofilm formation

Biofilm is defined as a microbial community which is attached to a surface or to other cells. Bacteria are coated by extracellular polymeric substances (EPS), which can differ in their composition. Biofilms are critical concerning the medical healthcare system. They stick to medical devices and catheters and are difficult to eradicate, while being responsible for chronic infections and are impervious to antimicrobial peptide (Lister & Horswill, 2014; Romling & Balsalobre, 2012). (pp)pGpp mediated biofilm formation was demonstrated in different bacterial species like *E. faecalis* (Berditsch,

Lux, Babii, Afonin, & Ulrich, 2016; Chávez de Paz, Lemos, Wickström, & Sedgley, 2012; Colomer-Winter, Flores-Mireles, Kundra, Hultgren, & Lemos, 2019; Colomer-Winter, Gaca, Chuang-Smith, Lemos, & Frank, 2018) and *Streptococcus mutans* (Lemos, Brown, & Burne, 2004; Lemos, Nascimento, Lin, Abranches, & Burne, 2008).

5.10.1.1 (pp)pGpp-mediated biofilm formation in *E. faecalis*

In *E. faecalis* biofilm formation and stability of different (pp)pGpp mutants was followed over 72 hours under non-stringent inducing conditions. The (pp)pGpp-deficient *relQ* mutant was capable in developing and sustaining biofilm almost to the same level as the parental strain. The (pp)pGpp^0 mutant *relA*/*relQ* and the (pp)pGpp-deficient *relA* mutant were impaired in establishing a biofilm and were incapable of sustaining biofilm compared to the wild type (WT) (Chávez de Paz et al., 2012). These data show that the intracellular basal level of (pp)pGpp is responsible for formation and maintenance of biofilm rather than activation of the stringent response. Clinical isolates from an immunocompromised leukemia patient were obtained during persistent infection. Each antibiotic therapy failed to eradicate the persistent pathogen. Analyzing these clinical isolates revealed an amino acid substitutions in the RSH enzymes, which led to constitutive (pp)pGpp synthesis, resulting in reduced biofilm. Interestingly, hyperactive RSH mutants remained susceptible to daptomycin and linezolid in liquid culture but were resistant in biofilm. Curing of the patient succeeded after eradication of the biofilm with a ClpP-activator ADEP-4 (Honsa et al., 2017). This is contrast to previous observation, which demonstrated (pp)pGpp contributes to biofilm formation. It remains unclear which role (pp)pGpp plays in biofilm formation in *E. faecalis*.

5.10.1.2 (pp)pGpp-mediated biofilm formation in *S. mutans*

Lemos et al (Lemos et al., 2004)were the first who analyzed behavior of biofilm formation in dependency of (pp)pGpp in *Streptococcus mutans* (*S. mutans*). Interruption of RSH with a kanamycin cassette in the enzymatic N-terminal part in two different positions resulted in decreased (pp)pGpp synthesis after induction of stringent response by serine hydroxamate (SHX). They speculated, the weak (pp)pGpp synthesis derives from an unknown (pp)pGpp synthetase. Both RSH mutants exhibited reduced biofilm formation. This observation contributed to the assumption, RSH-mediated (pp)pGpp synthesis is involved in biofilm formation (Lemos et al., 2004). At this time point the existence of RelP and RelQ was yet unknown.

2007 Lemos et al identified the two small alarmone synthetase RelP and RelQ (Lemos, Lin, Nascimento, Abranches, & Burne, 2007) and repeated biofilm studies in a strain incapable of synthesizing (pp)pGpp. Further analyses performed in (pp)pGpp^0 mutant showed no differences in biofilm formation compared to the wild type (Lemos et al., 2008). These data suggest, (pp)pGpp prevents biofilm formation rather than promoting.

5.10.1.3 (pp)pGpp-mediated biofilm formation in *S. aureus*

A *rsh$_{syn}$* mutant synthesizes less (pp)pGpp and was incapable of forming a proper biofilm compared to the wild type (de la Fuente-Nunez, Reffuveille, Haney, Straus, & Hancock, 2014). Furthermore, a cationic synthetic compound IDR1018 showed to eradicate biofilm in a variety of clinical relevant Gram positive and negative bacteria, including *S. aureus*. ppGpp was suggested being the target for this compound (de la Fuente-Nunez et al., 2014) Subsequently, this assertion was contested by Andresen et al, who showed ppGpp was not the target of IDR1018 and not the reason for eradication of the biofilm (Andresen, Tenson, & Hauryliuk, 2016). In further investigation, several cationic peptide were developed and analyzed. One of these compounds was DJK-5, which inhibits (pp)pGpp and eradicates biofilm in *Pseudomonas putida* and other Gram negative bacteria (de la Fuente-Nunez et al., 2015). Whether this occurs by direct binding to (pp)pGpp or in an indirect manner has to be elucidated. The effect of DJK-5 on biofilm eradication in firmicutes remains to be investigated. At this state of the thesis, no data clearly show a correlation between (pp)pGpp and biofilm formation in *S. aureu*s.

In summary, the role of (pp)pGpp in biofilm formation varies between species. On the one hand, the basal (pp)pGpp level seems to be crucial for building and maintaining biofilm in *E. faecalis* (Chávez de Paz et al., 2012), on the other hand high level of or no (pp)pGpp impairs biofilm formation in *S. mutans* (Lemos et al., 2004; Lemos et al., 2008) and *E. faecalis* (Chávez de Paz et al., 2012; Honsa et al., 2017).

5.10.2 (pp)pGpp-mediated virulence

Virulence in general describes the capability of a pathogenic organism to provoke a disease within the host. The level of virulence depends on the capability of replication within and invading the host, manipulating the host's immune system and the production of toxins (virulence genes).

Several publications implicate a link between the stringent response and virulence in different organisms. The impact of (pp)pGpp on virulence and infection seems to result in the same outcome in many different species. In the following section the consequences of (pp)pGpp on virulence will be described more in detail.

5.10.2.1 (pp)pGpp-mediated virulence in *E. faecalis*

In *E. faecalis* a (pp)pGpp^0 mutants was attenuated in virulence in a *Galleria mellonella* (*G.mellonella*) infection model (Colomer-Winter, Gaca, & Lemos, 2017; Gaca, Abranches, Kajfasz, & Lemos, 2012) and showed less survival in mouse-derived macrophages in comparison to the WT (Gaca et al., 2012) (Colomer-Winter et al., 2019). Δ*relA*$_{sp}$ mutant, which is a short version of RSH bearing only the N-terminal domain, was more virulent compared to the parental strain in *G. mellonella* model. Nearly 100% of Δ*relA*$_{sp}$ infected *G. mellonella* died within 23 hours whereas about 60% survived infection with the WT or Δ*relA* mutant. The absence of the C-terminus assumable leads to a hyper-activation of the synthetase and increase of (pp)pGpp synthesis, which may explain the increased virulence (Yan et al., 2009). In an infective endocarditis model (pp)pGpp^0 mutants failed to colonize porcine heart valve (Colomer-Winter et al., 2018) . Investigating the behavior of (pp)pGpp on catheter associated urinary tract infections (CAUTI) urinary tract of mice were infected with different (p)ppGpp-impaired and (pp)pGpp^0 mutants immediately after catheter implantation. The (pp)ppGpp^0 mutant was inefficient in colonizing catheter and were not detectable in the kidney.

5.10.2.2 (pp)pGpp-mediated virulence in *Streptococcus spp.*

(pp)pGpp also conducts to virulence in *S. pneuomiae*. Microarray data indicated a RSH-dependent upregulation of the toxin pneumolysine *ply*. In a mouse infection model Δ*rsh* strain was attenuated in virulence and infection. Complementation with RSH restored virulence almost to the wild type level (Kazmierczak, Wayne, Rechtsteiner, & Winkler, 2009). More intense studies in relation to virulence were performed with pathogenic zoonotic strain *Streptococcus suis* (J. Zhu et al., 2016). Adherence, invasion and survival in HEp-2 cells of Δ*rsh/relQ* mutant was up to 10 times less compared to the wild type. Furthermore Δ*rsh/relQ* mutant was better phagocytized by THP-1. qRT-PCR confirmed, attenuated virulence is a consequence of down regulation of many virulence genes.

In conclusion, (pp)pGpp contributes to resistance to phagocytosis of macrophages, ability to invade and adhere and survival in blood in *S. suis*. *Streptococcus agalctiae* causes severe infections in new bornes. To elucidate how *Streptococcus agalctiae* (group B *Streptococcus* GBS) switches lifestyle from a commensal to a blood invading, transposon insertion experiments were performed in human blood. Results reveal a high insertion rate into the *relA* genes resulting in impaired (pp)pGpp synthesis. *RelA* mutant produced less hemolysin, which was restorable by complementation or induction of the stringent response by SHX in *relA* mutant or the wild. Activation of the stringent response clearly promotes higher virulence in GBS strains in human blood (Hooven et al., 2018).

5.10.2.3 (pp)pGpp-mediated virulence in *S. aureus*

In *S. aureus* RSH is essential for survival after phagocytosis. A rel_{syn} mutant consist of a functional hydrolase but an inactive synthetase domain leading to lower (pp)pGpp level. In a kidney abscess model of *S. aureus* a rel_{syn} mutant was less virulent in comparison to the WT. No infection and no neutrophil infiltration were detectable in the rel_{syn} mutant, concluding RSH in *S. aureus* contributes to virulence (Geiger et al., 2010). The same mutant was shown to form smaller cutaneous skin abscess lesions in a mouse infection model (Mansour et al., 2016). This effect directly derives from (pp)pGpp. DJK-5 inhibits (pp)pGpp synthesis with a yet unknown mechanism. Co-injection of DJK-5 and *S. aureus* resulted in almost no cutaneous abscesses formation, clearly indicating (pp)pGpp contributes to higher virulence

In summary, the level of (pp)pGpp correlates with the level of virulence. Nevertheless, the mechanism how (pp)pGpp contributes to virulence is not clear and needs to be analyzed more in detail.

5.10.3 How to distinguish between antibiotic tolerance and persistence

Distinguishing between antibiotic tolerance and persistence is not simple. Antibiotic tolerance is defined as the survival of a bacterial population without changing the MIC (minimal inhibitory concentration). The MIC is determined by the lowest antibiotic concentration when growth is not anymore detectable. In contrast to resistance, antibiotic tolerant bacteria cannot grow but survive in presence to antibiotics and do not possess resistance genes which render antibiotic inefficiency or modification of the targets. After the removal of antibiotic, resistant cells can regrow.

Importantly, tolerance only occurs in bacteria exposed to bactericidal antibiotics and not bacteriostatic. Antibiotic tolerance is phenotypically described with slow growth and low metabolic activity. Persistence has basically high similarity to antibiotic tolerance. In contrast to antibiotic tolerant cells, only a subpopulation of a bacterial culture remain tolerant against antibiotics, which could be also named “heterogeneous tolerance”. Similarly, they survive antibiotic treatment without genetically changing and alteration of the MIC. To distinguish between tolerance and persistence characterization of the minimum duration of killing (MDK) is crucial. MDK is defined by the minimal time antibiotics need to kill 99% of a bacterial population. Antibiotic tolerant cells are slower and “lineary” killed and exert a higher MDK compared to the susceptible strain. Persistent cells exert a higher MDK compared to the susceptible strain, too but is characterized by bimodal kill-curve. Initially, a rapid drop of bacterial populations can be observed after exposure to antibiotics but MDK is reached much later in comparison to the susceptible strain (Balaban et al., 2019; Brauner, Fridman, Gefen, & Balaban, 2016). One of this mechanism, which contributes to antibiotic tolerance through slow growth is the stringent response.

5.10.4 (pp)pGpp-mediated antibiotic tolerance

In many organisms activation of the stringent response leads to an increased tolerance against antibiotics with different mode of actions. However, these antibiotics prevent cell growth on different levels. The stringent response provides tolerance against antibiotics targeting exemplary protein biosynthesis (tetracycline (H. Y. Kim, Go, Lee, Oh, & Yoon, 2018) and linezolid (W. Gao et al., 2010)), DNA replication (ciprofloxacin (Corrigan, Bellows, Wood, & Gründling, 2016; M. Matsuo, M. Hiramatsu, et al., 2019)) and cell wall biosynthesis (vancomycin, ampicillin (Gaca et al., 2013; Geiger et al., 2014; Katayama et al., 2017; Matsuo, Yamamoto, Hishinuma, & Hiramatsu, 2019; Singh et al., 2017), oxacillin (Aedo & Tomasz, 2016; Dordel et al., 2014; C. Kim et al., 2013; C. K. Kim, Milheirico, de Lencastre, & Tomasz, 2017)). (pp)pGpp leads to antibiotic tolerance by a) slowing down metabolism, replication and protein biosynthesis and b) activation of genes known to be responsible for increased antibiotic tolerance or resistance. In the following section the emergence of antibiotic tolerance by (pp)pGpp will be explained on the basis of selected species.

5.10.4.1 (pp)pGpp-mediated antibiotic tolerance in *E. faecalis*

A correlation between the stringent response and antibiotic tolerance has been analyzed in *E. faecalis*. A Δ*rsh* mutant was more tolerant towards cell wall active antibiotic vancomycin in consequence of constitutive (pp)pGpp accumulation due to RelQ. This resulted in the conclusion, that low level of (pp)pGpp mediates antibiotic tolerance and the typical induction of stringent response is dispensable. This was supported by a more efficient killing of Δ*relQ* mutant under non stressed condition in comparison to the wild type (Abranches et al., 2009). The essentiality of (pp)pGpp to survive antibiotics was shown by a (pp)pGpp^0 mutant, which was more susceptible to ampicillin compared to the WT or mutants with at least one present synthetase (Gaca et al., 2013). The mechanism of (pp)pGpp-mediated antibiotic tolerance by slow growth was described by Corrigan et al for *S. aureus*. They showed binding of (pp)pGpp to the GTPase RsgA prevented assembly of the 70S ribosome. Unfortunately, the effect on antibiotic tolerance through slow growth was not shown for *E. faecalis*, since only binding assays of (pp)pGpp to RsgA and different GTPases were performed (Corrigan, Bellows, Wood, & Gründling, 2016).

The first clinical relevance of the stringent response in correlation to antibiotic tolerance have been recently reported. Clinical isolated vancomycin-resistant *E. faecalis* (VRE) were isolated from an immunocompromised child. Bacteremia persisted for 26 days and was incurable by any antibiotic therapy. Sequence analyses revealed an exchange of leucine on position 152 to phenylalanine in RSH leading to a constitutive (pp)pGpp synthesis and delicate biofilm formation. Hyperactive RSH remained susceptible to daptomycin and linezolid in liquid culture but was highly tolerant when cultured in a biofilm (Honsa et al., 2017). These data demonstrate, how activation of a mechanism such as the stringent response contribute to antibiotic tolerance and exert an emergent threat for curing persistent infections in patients.

5.10.4.2 (pp)pGpp-mediated antibiotic tolerance in *S. aureus*

As shown for *E. faecalis,* RelP and RelQ, but not RSH, have been implicated in antibiotic tolerance against cell wall antibiotics such as vancomycin and ampicillin. RelQ and RelP are induced on the transcriptional level via VraR/S, wherein RelP induction seems to be highly VraR/S dependent leading to antibiotic tolerance (Geiger et al., 2014).

The VraR/S is a two-component system and rapidly senses cell wall damage (Belcheva & Golemi-Kotra, 2008) to activate the *vra* operon in a feedback loop. The *vra* operon includes genes which positively affect cell wall assembly by regulating synthesis (Kuroda et al., 2003) which in turn leads to antibiotic resistance (McCallum, Meier, Heusser, & Berger-Bächi, 2011).

In subsequent work, the role of the stringent response has been implicated in antibiotic tolerance to vancomycin, without explicitly exploring the mechanism. Vancomycin sensitive *S. aureus* (MSSA) (M. Matsuo, M. Hiramatsu, et al., 2019; Singh et al., 2017) FDA209P and vancomycin-intermediate *S. aureus* (VISA) (Katayama et al., 2017) challenged with either vancomycin (Katayama et al., 2017; Singh et al., 2017) or ciprofloxacin (M. Matsuo, M. Hiramatsu, et al., 2019) developed slow growing tolerant strains. Genetic analyses identified mutations in genes related to an activated stringent response (Katayama et al., 2017; M. Matsuo, M. Hiramatsu, et al., 2019; Singh et al., 2017). Better survival towards vancomycin was provided by mupirocin-induced stringent response (Katayama et al., 2017; Singh et al., 2017). However, further investigations confirmed and unraveled the mechanism of RelQ-mediated antibiotic tolerance. In the highly virulent MRSA strain USA300 a Δ*relQ* mutant is sensitive to β-lactam antibiotics. However, RelQ activation can be bypassed and antibiotic tolerance restored by actively inducing the stringent response with mupirocin via RSH. A *relP* mutant resulted in stronger activation of the *relQ* promoter which was further enhanced by oxacillin-inducing conditions. This leads to a hyperactivation of RelQ and results in a positive influence on *mecA* with increased antibiotic tolerance (Bhawini et al., 2019). The slow growing VISA strain L4 (derivate of V6-5) exhibits decreased MIC to vancomycin and oxacillin. Two mutations were identified by whole genome sequencing. One nonsense mutation was identified in *relQ* and one in an unknown gene, named *ehoM*. Deletion of *relQ* in hVISA strain Mu3 did not affect oxacillin MIC whereas in contrast *ehoM* mutation decreased MIC. Complementation of *relQ* in strain L4 restored MIC but not in the *ehoM* mutant strain Mu3. Furthermore, (pp)pGpp synthesis induced by expressing *relQ* or mupirocin-induced stringent response, increased *ehoM* expression, indicating EhoM contributes to vancomycin tolerance and is transcriptionally regulated via RelQ (M. Matsuo, N. Yamamoto, et al., 2019). These results identify RelQ as the major responder to cell wall acting antibiotics providing antibiotic tolerance through rapid (pp)pGpp synthesis and activation of *ehoM* (Fig.6) (Bhawini et al., 2019; Geiger et al., 2014; M. Matsuo, N. Yamamoto, et al., 2019).

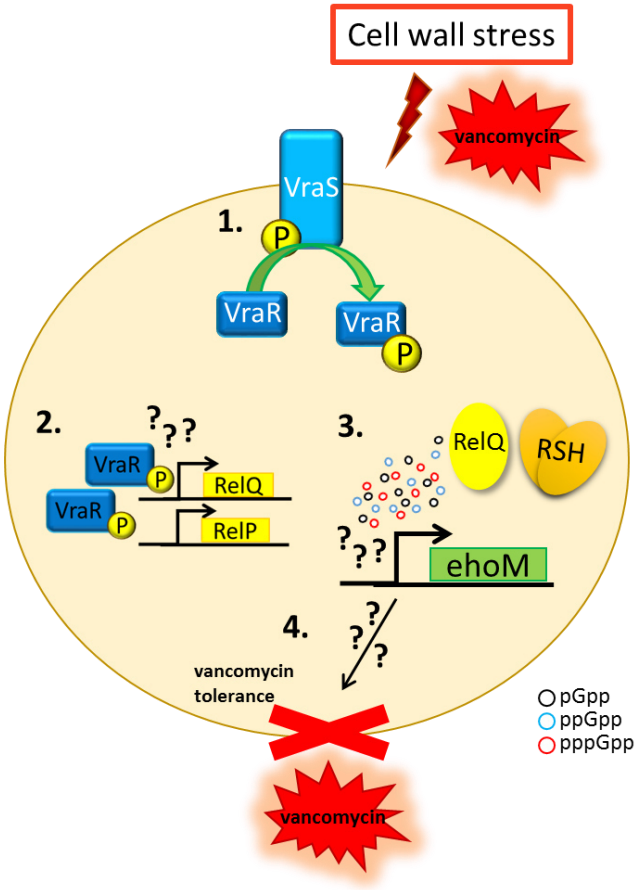

Fig.6 Schematic illustration of cell wall stress induced transcriptional activation of RelP/Q in *S. aureus*. 1.) Cell wall damaging antibiotics are sensed by VraS which phosphorylates the response regulator VraR. (Belcheva & Golemi-Kotra, 2008) 2.) Consequently, RelP and RelQ are activated on a transcriptional level with unknown mechanism (Geiger et al., 2014). 3.) (pp)pGpp transcriptionally actives *ehoM* leading to 4.) tolerance towards vancomycin (M. Matsuo, N. Yamamoto, et al., 2019). The function of EhoM is yet unknown.

So far, we have pointed out the relevance of RelQ rapidly responding to cell wall damaging antibiotics by immediately stalling growth via (pp)pGpp synthesis. As described for *E. faecalis* Corrigan et al (Corrigan, Bellows, Wood, & Grundling, 2016) observed (pp)pGpp binds and inhibits RsgA under stringent conditions which prevents the formation of matured 70S ribosomes and results in slow growth. Consequently, (pp)pGpp-mediated slow growth exhibits higher tolerance against ciprofloxacin and penicillin G. This stringent phenotype could be mimicked by a *rsgA* mutants which resulted in antibiotic tolerance, too (Corrigan, Bellows, Wood, & Gründling, 2016).

Several studies isolated strains from patients with persistent infection. These infections were difficult to treat with appropriate antibiotics. Genetic analyses of the clinical isolates revealed mutations in the RSH enzyme leading to a (pp)pGpp-overproduction and constitutively activated stringent response (W. Gao et al., 2010; Mwangi et al., 2013). Subpopulations of resistant MRSA differed in their resistance to oxacillin. Comparing these heterogeneous resistant strains, whole genome sequencing identified mutations in genes with different functions. Despite their various function, they resulted in activation of the stringent response. Direct activation of the stringent response by sub-MIC concentration of mupirocin, resulted in *mecA*-dependent conversion of heterogeneous to homogeneous high oxacillin resistant MRSA strains as a consequence of increased PBP2 expression. (Aedo & Tomasz, 2016; Dordel et al., 2014; C. Kim et al., 2013; C. K. Kim et al., 2017).

In summary, antibiotic tolerance seems to be a common consequence of an active stringent response in pathogenic firmicutes.

5.10.5 (pp)pGpp-mediated persister formation

Bacterial populations have evolved many different strategies to survive lethal damages by host's immune system or exposure to antibiotics. Beside regulation of virulence, the development of antibiotic resistance and protection by biofilm formation, persister cells have evolved. The persister phenotype in *Staphylococci* was described for the first time in 1944 by Bigger (Bigger, 1944). A population treated with penicillin resulted in nearly complete killing, except for a small subpopulation which survived. When these survivors were repeatedly treated with penicillin, again more than 99% of the bacteria were killed with the exception of a few survivors (Bigger, 1944). In contrast to resistant bacteria, persisters do not grow in presence of antibiotic and stay in a dormancy-like state (Defraine, Fauvart, & Michiels, 2018; Harms, Maisonneuve, & Gerdes, 2016). There are evidences for persister cells presumably being a consequence of an active stringent response because they result in overlapping phenotypes from similar stress signals. In both cases nutrient deprivation and exposure to antibiotics lead to cell growth arrest and antibiotic tolerance with the possibility to resume growth under more favorable conditions (Harms et al., 2016; Hobbs & Boraston, 2019b), but there are also fundamental differences. The switch to a persister cell is not necessarily triggered by an activating signal but most likely happens randomly by a stochastic mechanism in a growing population.

This “mixed” population ensures a fitness advantage by being prepared in case of nutrient deprivation or antibiotic exposure (Hobbs & Boraston, 2019b).
So far, a link between the stringent response and persister formation has mainly been reported in *E. coli* (Amato, Orman, & Brynildsen, 2013; Korch, Henderson, & Hill, 2003; VandenBerg, Ahn, & Visick, 2016) and are linked to the toxin-antitoxin system (Amato et al., 2013; Korch et al., 2003; Tian et al., 2016). In *E. coli*, RelA is stimulated upon amino acid starvation (Cashel & Kalbacher, 1970) and synthesizes (pp)pGpp. A model postulated (pp)pGpp inhibits the polyphosphate hydrolase PPX (p)ppGpp which leads to the accumulation of polyphosphate. This in turn activates the Lon protease which degrades the antitoxin HipB (Germain, Roghanian, Gerdes, & Maisonneuve, 2015; Maisonneuve, Castro-Camargo, & Gerdes, 2013). Unfortunately, this results were retracted because the observed phenotypes were artifacts due to infection with the bacteriophage Φ80. Nevertheless, toxin HipA inactivated the aminoacyl-tRNA-synthetase GltX by phosphorylation (Germain, Castro-Roa, Zenkin, & Gerdes, 2013) provoking activation of the stringent response as consequence of uncharged tRNAs. The molecular mechanism linking (pp)pGpp and persister cell formation remains elusive.

5.10.5.1 (pp)pGpp mediated persistence in *S. aureus*

As described in chapter 5.10.5 persister formation is tightly linked to the induction of the toxin-antitoxin (TA) system in *E. coli*. Conlon et al investigated the relation of TAs and persistence in *S. aureus*. Deleting the 3 TAs modules (*mazEF*, *axe1-txe1* and *axe2-txe2*) did not result in decreased persister formation. A rsh_{syn} and *codY* mutant also did not show any difference in the amount of persisters. Further experiments revealed persister formation occurs by a stochastic mechanism entering the stationary phase and by the drop of ATP (Conlon et al., 2016).
So far, persister formation by (pp)pGpp could not be verified in *S. aureus* and other firmicutes and remains questionable.

In summary, there is no evidence for (pp)pGpp-mediated persistence in firmicutes. While a basal level of (pp)pGpp seems to be necessary for biofilm formation and maintenance, virulence and antibiotic tolerance are consequences of induced stringent response, which might be a consequence of (pp)pGpp overexpression. This is supported by *in vivo* data of clinical isolates which revealed antibiotic tolerance resulted

from mutations in either genes, which activated the stringent response, or mutations within RSH leading to a constitutive (pp)pGpp synthesis.

5.11 Oxidative stress response in *Staphylococcus aureus*

5.11.1 Reactive oxygen species (ROS)

S. aureus has to face different oxygenic conditions. This includes reactive oxygen species (ROS). Oxidative stress can occur endogenous and exogenous. **Endogenous** created ROS occurs when oxygen interacts with flavoproteins and is not completely reduced while the aerobic respiration and superoxide anions (O_2^-) are formed (Massey et al., 1969). Another ROS producing reaction is the Fenton reaction. $HO\cdot$ radicals are formed upon reaction of iron with hydrogen peroxide (H_2O_2) (Imlay, Chin, & Linn, 1988; Kohanski, Dwyer, Hayete, Lawrence, & Collins, 2007). **Exogenous** reactions are reactions which do not occur within the bacteria itself. ROS are formed from external e.g. from macrophages and neutrophils. Neutrophils form O_2^- and H_2O_2 to kill evading bacteria. As consequence, essential molecules such as proteins and DNA are severely damaged (Imlay et al., 1988). Superoxide can oxidize cysteine and methionine (Gaupp, Ledala, & Somerville, 2012) and damage proteins. O_2^- (Kohanski et al., 2007) and H_2O_2 can lead to the release of iron from Fe-S containing proteins (Jang & Imlay, 2007) leading to inactivation and loss of function (Cosgrove et al., 2007). The released positively charged iron can intercalate with the DNA and the Fenton generated $HO\cdot$ radicals will presumably react with the DNA leading to mutations within the DNA (Keyer & Imlay, 1996). To protect from DNA and protein damage (Keyer & Imlay, 1996), bacteria have developed mechanism to protect from ROS-mediated damage which will be explained in the following sections. Iron is a limited trace element because it appears in an insoluble and for bacteria inaccessible Fe^{III} form (Gaupp et al., 2012). Iron is an essential trace element since it is part of a lot of iron containing proteins but at the same time a potential threat due to the potential of damaging DNA and proteins via the Fenton reaction (Horsburgh, Ingham, & Foster, 2001).

5.11.2 Role of iron regulation in *S. aureus*

S. aureus has to challenge an iron depleted environment within the human body. Bacteria in general need a concentration between 0.4µM and 4µM to ensure a proper bacterial growth. These high concentrations are not available in the human body since most of the iron is either bound by iron containing proteins or in an insoluble form (P. Skaar & Schneewind, 2004). *S. aureus* has acquired different strategies to access iron either through extracting iron out of host heme-protein transferrin by staphyloferrin A (Modun, Evans, Joannou, & Williams, 1998) (P. Skaar & Schneewind, 2004) or by importing Fe^{III} via siderophores (Beasley & Heinrichs, 2010) which is reduced to soluble Fe^{II}. Siderophores can also function as iron-storage proteins. On one hand *S. aureus* cannot grow without iron and on the other hand iron is a potential threat due to Fenton reaction. *S. aureus* was killed more efficiently by hydrogen peroxide with increasing concentrations of iron (Repine, Fox, Berger, & Harada, 1981). Therefore regulation of the intracellular iron must be tightly regulated. Iron homeostasis is regulated by the conserved ferric uptake regulator Fur. Under iron rich condition, Fe^{II} is usually bound to Fur repressing genes containing a FUR-Box (ATAATgATTaTcAttat (Horsburgh, Ingham, et al., 2001)) (table 1). At iron-low conditions Fe^{II} is not bound to Fur leading to de-repression from DNA and genes for iron transport and storage are activated (Gaupp et al., 2012; Sheldon & Heinrichs, 2012) (Fig.7B).

Table 1: Summery of genes with putative Fur or/and PerR box

Gene name	Fur box	PerR box	Gene function	Reference
fhuCBD	Yes	No	Putative ferrichrome siderophore uptake	(Horsburgh, Ingham, et al., 2001)
fhuD2	Yes	No	fhuD homologue	(Horsburgh, Ingham, et al., 2001)
sstABCD	Yes	No	Putative siderophore transporter	(Horsburgh, Ingham, et al., 2001)
sirABC	Yes	No	Putative siderophore transporter	(Horsburgh, Ingham, et al., 2001)
feoB1/2	Yes	No	Putative Fe^{II} transporter	(Horsburgh, Ingham, et al., 2001)
yfiY	Yes	No	Putative Fe^{III} dicitrate transporter	(Horsburgh, Ingham, et al., 2001)
orf4	Yes	No	Putative transporter	(Horsburgh, Ingham, et al., 2001)
ycgT	Yes	No	Putative thioredoxin reductase	(Horsburgh, Ingham, et al., 2001)
katA	No	Yes	Catalase	(Horsburgh, Clements, Crossley, Ingham, & Foster, 2001)
ahpCF	No	Yes	Alkyl hydroxyperoxide reductase	(Horsburgh, Clements, et al., 2001)
mrgA	No	Yes	Ferritin-like dps	(Horsburgh, Clements, et al., 2001)
perR	No	Yes	Peroxide regulon regulator	(Horsburgh, Clements, et al., 2001)
Fur	No	Yes	Ferric uptake regulator	(Horsburgh, Clements, et al., 2001)
ftnA	Yes	Yes	Ferritin	(Horsburgh, Clements, et al., 2001; Morrissey, Cockayne, Brummell, & Williams, 2004)
trxB	No	Yes	Thioredoxin reductase	(Horsburgh, Clements, et al., 2001)
Bcp, pdh	No	Yes	Bacterioferritin comigratory protein, 3-phosphoglycerate dehydrogenase	(Horsburgh, Clements, et al., 2001)

5.11.3 Role of the peroxide regulon regulator PerR in *S. aureus*

S. aureus possesses different regulators for controlling ROS and iron storage. One of the major regulators is PerR (peroxide sensing protein). PerR functions as a transcriptional repressor. In manganese rich conditions and low iron, PerR is bound to a specific DNA sequence (PerR-box) (AAGTATTATTTATTATTATTA) and transcription of PerR regulated genes (*katA*, *ahpC*, *dps* and *ftnA*) (table 1) (Horsburgh, Clements, et al., 2001; Morrissey et al., 2004) cannot occur (Fig.7A).

Regulation of both, detoxification of oxygen and iron homeostasis has to be balanced. In the presence of hydrogen peroxide and iron rich conditions PerR reacts with iron leading to the oxidation of histidine residues (Ji et al., 2015) and the release of hydroxyl radicals ($HO^{\cdot}$) and oxidation of iron. PerR cannot bind to the DNA and oxidative stress genes can be transcribed (Fig.4A) (Gaupp et al., 2012; Ji et al., 2015; Morrissey et al., 2004).

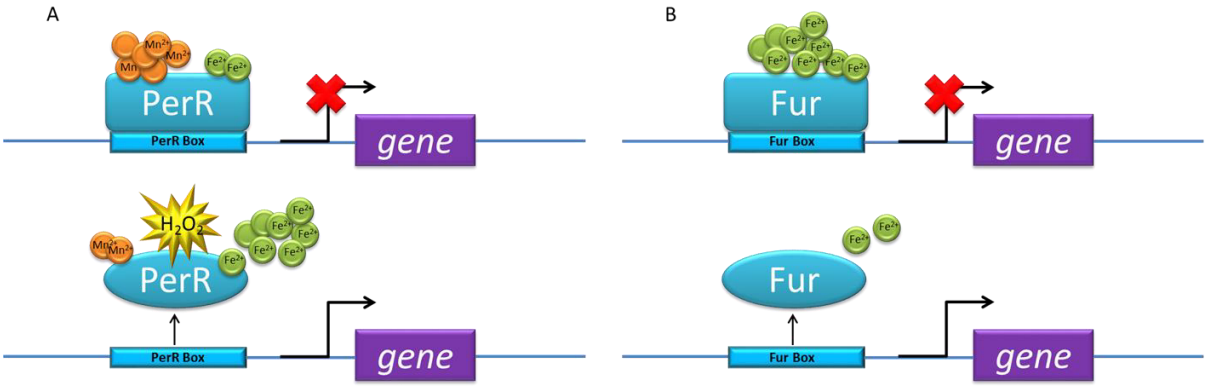

Fig.7 PerR and Fur regulation in *S. aureus*. A) Under iron low and manganese rich condition PerR is bound to the PerR box represses transcription of PerR regulated genes. In the presence of H_2O_2 and a high iron level histidine is oxidized leading to the de-repression of PerR and transcription of PerR regulated genes. B) Fur is bound to the Fur box under iron rich condition thereby repressing transcription of Fur regulated genes. Low iron level leads to the de-repression of Fur and transcription of Fur regulated genes.

One of these genes is *katA* which encodes for catalase. *KatA* is the major scavenger for H_2O_2 (Horsburgh, Clements, et al., 2001) and converts it to H_2O and O_2 (Beavers & Skaar, 2016; Gaupp et al., 2012; Ji et al., 2015). Possibly it is indirectly regulated by a small regulatory RNA (Cosgrove et al., 2007). It was shown, that *S. aureus* strains with a high catalase activity were more virulent and therefore protected of the hydrogen peroxide produced by PMNs (Mandell, 1975).

The alkyl hydro peroxide reductase *ahpC* is induced upon hydrogen peroxide stress (Cosgrove et al., 2007; Gaupp et al., 2012). In contrast to KatA, AhpC is not only active against H_2O_2, but additionally provides resistance to organic peroxides and peroxinitrite (Cosgrove et al., 2007; Hussain, Abdullah, & Amom, 2016). Although AhpC contributes to H_2O_2 resistance, the activity of AhpC in a *katA* mutant is weaker. Vice versa an *ahpC* mutant results in increased H_2O_2 resistance as a consequence of increased *katA* expression (Cosgrove et al., 2007). Moreover, a *katA ahpC* double mutant cannot detoxify H_2O_2 (Cosgrove et al., 2007). Another protein which can be seen as a linker between iron homeostasis and oxidative stress response is MrgA. It is a homologue to *dps* (DNA-binding protein from starved cells) from *E. coli* and both belong to the ferritin super family. MrgA functions as an iron chelator protein and is induced upon hydrogen peroxide stress (Morikawa et al., 2006). In a *perR* mutant *mrgA is* overexpressed leading to a condensed nucleoid independent of H_2O_2, confirming a negative regulation by PerR (Horsburgh, Clements, et al., 2001; Morikawa et al., 2006). In summary, H_2O_2 leads to the de-repression of PerR and the transcription of *mrgA* scavenging iron to protect DNA from toxic Fenton reaction. There is a tight link between the oxidative stress regulation and the iron homeostasis which can be additionally demonstrated by *ftnA*, which is regulated by PerR but also by Fur (Morrissey et al., 2004).

6 Aim of this thesis

The stringent response is a conserved mechanism among a variety of bacterial species. Its activation is triggered by different nutrient signals leading to the synthesis of the alarmones (pp)pGpp. The presence of the three enzymes RSH, RelP and RelQ determine and regulate the intracellular (pp)pGpp level in *S. aureus*. While RSH responds to branched-chain amino acid starvation, RelP and RelQ are transcriptionally activated by cell wall damage. Interestingly, RSH and RelP/Q react to different stress signals, but commonly synthesize (pp)pGpp and result in similar physiological changes such as slow growth, reduced metabolism and reprogramming of the transcriptome. The physiological consequence of (pp)pGpp synthesis range from survival of nutrient depletion, increased virulence and antibiotic tolerance. Therefore, I aimed to decipher whether RSH and RelP/Q, in response to different stimuli, leads to significant differences in the nucleotide pool and selective regulation of specific genes. So far, (pp)pGpp-mediated transcriptional changes were analyzed by actively inducing the stringent response by antibiotics, such as mupirocin and serine hydroxamate. Nevertheless, these experimental designs do not exclude side-effects which derive direct from antibiotics and not from (pp)pGpp. In this thesis, I was interested in the downstream effects, which are directly caused by (pp)pGpp to exclude any side-effects and the decipherment of differences between RSH and RelP/Q induced (pp)pGpp synthesis *in vivo* and *in vitro*. Therefore I used ATc-inducible truncated and hydrolase mutated RSH, RelP and RelQ which were introduced into a (pp)pGpp^0 mutant. This method ensured later results are strictly (pp)pGpp-dependent. Distinguishing between RSH and RelP/Q effects included (pp)pGpp measurements *in vivo* and *in vitro* and investigation of the transcriptome. For this purpose, pGpp, ppGpp and pppGpp pool were quantified via HPLC-MS after transcriptional induction or purified RSH, RelP and RelQ. To investigate the global effect of (pp)pGpp on the re-programming of the transcriptome, I performed RNA-Seq analyses. Statistical relevant and differential regulated genes were used for further approaches such as Northern Blot analyses, growth curves, survival assays, ROS measurement and MIC determination.

Different experiments have been performed in *Streptococcus equisimilis in vitro* indicating, that deletion of the C-terminus results in stronger synthetase activity (Mechold et al., 2002) and hydrolase activity is dependent on the state of conformation (Hogg et al., 2004).

Nevertheless, no *in vivo* and *in vitro* data are available for *S. aureus*. In this thesis, the influence of the C-terminus of RSH and the single domains TGS, ACT and DC on the synthetase and hydrolase activity were analyzed, using several analytic approaches. Therefore, different ATc-inducible full length ("long" RSH) and C-terminal deletion ("short" RSH) w/wo hydrolase were expressed in (pp)pGpp^0 deletion mutant. The impact of the C-terminus on either synthetase or hydrolase was analyzed by growth curves analyses and Norther blot under relaxed conditions. The influence of the C-terminus was also performed *in vitro* purifying "long" and "short" RSH w/wo hydrolase and enzymatic activity evaluated by measuring (pp)pGpp and AMP via HPLC. Next, the impact of the single C-terminal domains TGS, ACT and DC were analyzed. Therefore, (pp)pGpp^0 mutants were complemented with different "long" RSH enzymes, harboring a mutation in one of the three domains expression of stringent response genes were analyzed via Northern blot and growth analyses. Finally, we wanted to elucidate how TGS, ACT and DC influence synthetase activity via possible interaction partners. There RSH enzymes with harboring mutations in TGS, ACT or DC were purified and Co-immunoprecipitation was performed.

.

7 Results

7.1 Manuscript ready for submission

Unpublished manuscript. This manuscript is ready for submission and can differ from published exemplar.

Increasing the cellular (pp)pGpp level is associated with activation of stress response genes in *Staphylococcus aureus*

Petra Horvatek[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[2], 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 Genomics and Functional Genomics, University Medicine Greifswald, Greifswald

#**Address correspondence:** Christiane Wolz, Interfaculty Institute of Microbiology and Infection Medicine, Universität Tübingen, Elfriede-Aulhorn-Strasse 6, 72076 Tübingen, Germany. Phone: 49-7071-2980187. Fax: 49-7071-295165. E-mail: christiane.wolz@med.uni-tuebingen.de

Short Title: (pp)pGpp in *S. aureus*

Key words: Stringent response, (p)ppGpp, RSH, oxidative stress, PSM, *Staphylococcus aureus*

Abstract

The stringent response is characterized by the synthesis of the messenger molecules pppGpp, ppGpp or pGpp (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 were 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-homologue (RSH_{Sau}) upon amino-acid limitation or via one of the two small (p)ppGpp synthetases RelP or RelQ upon cell-wall stress. We used RNA-seq to compare global effects in response to transcriptional induction of the synthetase domain of RSH (RSH-Syn), RelP or RelQ without the need to apply additional stress conditions. Changes in the nucleotide pool were similar to induction of the stringent response via mupirocin, namely lowering of the GTP pool, increase of the ATP pool and synthesis of pppGpp, ppGpp and pGpp. On the transcriptional level, all three enzymes resulted in similar changes. However, RelQ was less active compared to RSH-Syn and RelP indicating strong restriction of its (pp)pGpp synthesis activity *in vivo*. Genes involved in iron storage (e.g. *ftnA, dps*), stress response (e.g. *lexA, katA, sod*A) and the *psmα1-4 and psmß1-2* operons coding for toxic, phenole soluble modulins (PSMs) are highly up-regulated upon (pp)pGpp induction. Analyses of *ftnA*, *dps*, and *psm* in different regulatory mutants revealed that their (pp)pGpp dependent regulation is 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. (pp)Gpp mediated up-regulation of *psm* is accompanied by increased production of endogenous reactive oxygen species (ROS). The expression of central genes of the oxygen stress response in turn protects the bacteria from anticipated ROS stress derived from PSMs or exogenous sources. Thus, we identified a new link between stringent response and oxidative stress in *S. aureus* which is likely crucial for survival upon phagocytosis.

Significance

Most bacteria make use of the second messenger (pp)pGpp to reprogram the bacterial physiology under nutrient scare conditions. In the human pathogen *Staphylococcus aureus,* (pp)pGpp plays important role in virulence (Geiger et al., 2010), phagosomal escape (Geiger et al., 2012) and antibiotic tolerance. Here, we analyzed the immediate consequences of (pp)pGpp synthesis upon transcriptional induction of the (pp)pGpp producing enzymes RSH, RelP or RelQ. RelQ enzyme showed low activity under the tested conditions. (pp)pGpp synthesis via RSH resulted in immediate changes in the nucleotide pool and severely impacts transcription of more than thousands genes. A newly identified 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 by granulocytes.

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 (Potrykus and Cashel, 2008; Wolz et al., 2010; Hauryliuk et al., 2015)(Dozot et al., 2006; Gaca, Colomer-Winter, & Lemos, 2015; Hobbs & Boraston, 2019b; Irving & Corrigan, 2018; Liu et al., 2015; Steinchen & Bange, 2016; Wu & Xie, 2009; M. Zhu, Pan, & Dai, 2019). However, the phenotypic consequences resulting from (pp)pGpp accumulation vary among species and can be mediated by different underlying mechanisms. 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 (Dalebroux et al., 2010; Hobbs & Boraston, 2019b). It is now clear that there are fundamental differences between the stringent response initially characterized in *E. coli* and the response in Firmicutes (Liu et al., 2015; Wolz et al., 2010). Differences are seen in the enzymes involved in synthesis and degradation of the messengers and in the downstream effects of (pp)pGpp.

(pp)pGpp is synthesized by long RelA-SpoT-homologs (RSH) or small alarmone synthetases (SAS) by transferring pyrophosphate originating from ATP to the 3´ OH group of GTP, GDP or GMP. RSH enzymes are present in nearly all bacteria and show a conserved molecular architecture composed of a C-terminal sensory domain and an N-terminus with distinct (pp)pGpp hydrolase and synthetase domains (Atkinson et al., 2011). Firmicutes, such as *Staphylococcus aureus* possess one bifunctional RSH enzyme and one or two SAS enzymes, RelP and RelQ. Of note, bifunctional RSH enzymes are also named Rel or RelA. Amino-acid limitation is the only condition known resulting in a RSH mediated stringent response phenotype (Geiger et al., 2010). Under non-inducing conditions, RSH_{Sau} is primarily in a hydrolase-On/synthetaseOff conformation even when the C-terminal sensory domain is deleted (Gratani, Horvatek et al. 2018). The strong hydrolase activity makes RSH an essential molecule required to detoxify (pp)pGpp produced by RelP or RelQ (Geiger et al., 2010).

The small SAS enzymes in *S. aureus* are part of the cell-wall stress regulon and are transcriptionally induced e.g. after vancomycin treatment. Thereby, they contribute to tolerance towards cell-wall active antibiotics such as ampicillin or vancomycin (Geiger et al., 2014). Recently, structural and mechanistic characterization revealed that RelQ from *Bacillus subtilis* and *Enterococcus faecalis* form tetramers (Beljantseva et al., 2017; Steinchen et al., 2015). RelQ activity is strongly inhibited through binding of single stranded RNA. pppGpp binding leads to disassociation of the RelQ:RNA complex and its activation (Beljantseva et al., 2017). In contrast, RelP activity is inhibited by both pppGpp and ppGpp, activated by Zn^{2+} and is insensitive to inhibition by RNA (Manav et al., 2018; Steinchen et al., 2018). Thus, although very similar, RelP and RelQ seem to full-fill different functions within the cell. One can assume that different post-translational regulatory mechanism are in play to fine-tune (pp)pGpp synthesis during different growth conditions.

In *S. aureus* the stringent response plays important roles in virulence (Geiger et al., 2010), phagosomal escape (Geiger et al., 2012) and antibiotic tolerance (Corrigan, Bellows, Wood, & Grundling, 2016; Dordel et al., 2014; W. Gao et al., 2010; Geiger et al., 2014; Hobbs & Boraston, 2019b; Katayama et al., 2017; Miki Matsuo et al., 2019). 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 (Corrigan, Bellows, Wood, & Grundling, 2016; Kriel et al., 2012a)(Wang et al., 2007). (pp)pGpp binding results in inhibition of these proteins resulting in lowering of the GTP pool, inhibition of the translation apparatus and replication, respectively. ppGpp, pppGpp and pGpp might exert different activities. In *E. coli* for instance, ppGpp seems to be more potent than pppGpp with regard to growth rate regulation and shutdown of growth-associated processes in the course of the stringent response (Mechold, Potrykus, Murphy, Murakami, & Cashel, 2013). Of note, in contrast to *E. coli,* (pp)pGpp from firmicutes does not interfere with RNA polymerase activity (Hauryliuk et al., 2015). Instead, in these organisms, (pp)pGpp regulates transcription via an indirect mechanism that strongly relies on the lowering of intracellular GTP pool (Geiger et al., 2012; Krasny, Tiserova, Jonak, Rejman, & Sanderova, 2008; Kriel et al., 2012b). A decrease in the GTP level leads to the repression of nucleotide sensitive, GTP-initiating promoters, e.g. those of stable RNA genes (Kastle et al., 2015; Krasny & Gourse, 2004). 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 (Majerczyk et al., 2010; Pohl et al., 2009).

Global transcriptional effects of (pp)pGpp have been examined previously in several Firmicutes such as *B. subtilis* (Eymann, Homuth, Scharf, & Hecker, 2002), *Streptococcus pneumoniae* (Kazmierczak et al., 2009), *Enterococcus faecalis* (Gaca et al., 2012), *Streptococcus mutans* (Nascimento, Lemos, Abranches, Lin, & Burne, 2008) and *S. aureus* (Geiger et al., 2012). These studies are based on the comparison of wild type and RSH mutants under conditions mimicking amino acid starvation. Of note, these stress conditions are accompanied with profound physiological changes, which are only in part mediated by (pp)pGpp. For instance amino acid limitation leads to stabilization of many transcripts independent of (pp)pGpp (Geiger et al., 2010)(unpublished observation). Thus, from these analyses it is hard 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* gaining major new insights (Sanchez-Vazquez, Dewey, Kitten, Ross, & Gourse, 2019).

Here, we aimed to compare RSH, RelQ and RelP mediated effects on nucleotide pools, transcription and functional consequences without imposing nutrient starvation. Therefore, the synthetase domain of RSH (RSH-Syn), RelP and RelQ were expressed from an anhydrotetracyline (ATc) inducible promoter in a (pp)pGpp^0 strain in which the enzymatic domains of all three synthetases were deleted. Through RNA-Seq analyses we identified new (pp)pGpp regulated genes many of which are involved in oxidative stress response, iron storage and synthesis of phenol-soluble modulins (PSMs). Thus, (pp)pGpp synthesis contributes to PSM derived ROS production but also to protection from these toxic molecules.

Results

Changes of the nucleotide pools after transcriptional induction of RSH-Syn and RelQ

We first compared stringent response imposed by mupirocin (tRNA syntethase inhibitor) in wild type bacteria and transcriptional induction of (pp)pGpp synthetases in a (pp)pGpp^0 strain. (pp)pGpp^0 carries mutations in all three (pp)pGpp synthesis

enzymes (full deletion of *rsh*, synthetase mutation in *relP* and *relQ*) and thus is unable to synthesize (pp)pGpp. RelQ or RSH-Syn (N-terminal part of RSH in which the hydrolase domain was mutated) were expressed using an ATc inducible expression system. Strains were grown to early exponential growth phase and gene expression was induced for 30 min. Consistent with previous results (Kastle et al., 2015), treatment of the (pp)pGpp^0 strain with mupirocin resulted in a significant increase of the GTP pool. Induction of the stringent response in the wild type by either mupirocin or transcriptional induction of RSH-Syn resulted in similar changes of the nucleotide pools: immediate increase of the ATP pool, decrease of the GTP pool and synthesis of all three alarmones pppGpp, ppGpp, and pGpp (Fig. 1). After induction of RelQ only minor changes of the nucleotide pool were detectable. Thus, the effect on the nucleotide pools elicited by RelQ was significantly lower compared to RSH-Syn.

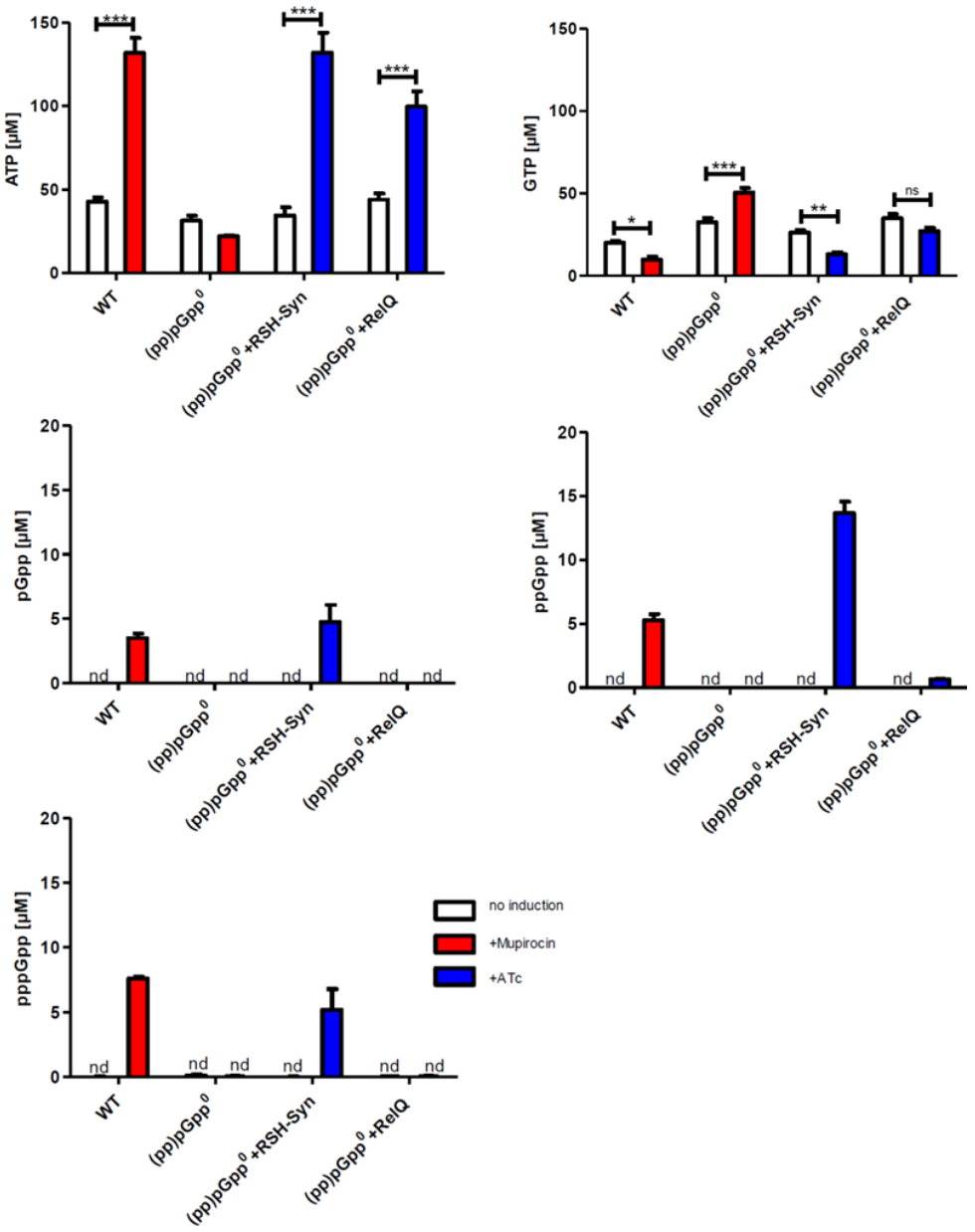

Fig.1 Changes in the nucleotide pool after transcriptional induction of RSH-Syn or RelQ. Strain HG001 and derivatives were grown to OD_{600}=0.3 and treated for 30 min with or without 0.125µg/ml mupirocin for HG001 wild type and (pp)pGpp^0 mutant or 0.1µg/ml ATc for (pp)pGpp0 or (pp)pGpp^0/*codY* mutants with inducible RSH-Syn or RelQ. Nucleotide analyses were performed using mass spectrometry (ESI-TOF) in negative ion mode. Error bars represent SEM (n=3) from three biological replicates. Statistical significance determined by two-way ANOVA with Tukey's posttest, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001

Impact of (pp)pGpp synthesis on the transcriptome

We next analyzed the impact of (pp)pGpp synthesis after induction of RSH-Syn or RelQ on mRNA abundance. RNA-Seq data revealed that 1449 genes and sRNAs were significantly affected by either RSH-Syn (total: 1388, 717 up, 671 down) or RelQ (total: 352, up: 223, down 129). Most of the RelQ affected genes were also changed in their expression in response to RSH-Syn induction (Fig. 2A. Suppl. Table 1). However, consistent with the nucleotide measurements (Fig. 1) the effect of RelQ induction was less prominent (Supl. Table 1). We compared the data with previous microarray analyses after induction of stringent response imposed by amino acid limitation (Geiger et al., 2012). Most of the previously identified stringent genes were verified by the RNA-seq analysis (Fig. 2A). Of note, in the present analysis only genes with at least three fold differences and significance level of $p< 0.001$ are included in the analysis shown in Fig. 2 and Supl.Table 1. Therefore, some of the previously detected genes are excluded although most of them show the same tendency (see Supl. Table 1). Despite the higher stringency in the analysis, the present analysis revealed far more (pp)pGpp regulated genes and additionally also sRNAs. Genes were classified in functional categories using the SEED annotation (http://pubseed.theseed.org). (pp)pGpp induction resulted in down-regulation of many metabolic genes involved in protein, RNA and DNA metabolism consistent with previous results that the stringent response mainly leads to the shutdown of translation and replication (Geiger et al., 2012) (Fig. 2B). More than 500 RNAs were significantly upregulated upon RSH-Syn induction (Supl. Table 1). Most of them are sRNAs or code for hypothetical proteins with unknown function. Amino acid biosynthesis gene clusters were also found up-regulated. Most of them are part of the CodY regulon and thus likely regulated via lowering of the GTP pool. Phage encoded genes were also found up-regulated indicating phage inducing conditions. This is in line with the up-regulation of *recA* and *lexA*. When sorting for genes which are most affected by RSH-Syn induction (Fig. 2C, Supl. Table 1), it became evident that many of them were assigned to iron acquisition/metabolism (up-regulation of genes involved in iron storage*;* down-regulation of genes involved in siderophore biosynthesis and iron transport), stress response (*dps, sodA, katA ahpC. uspA1/2, asp23, ptpA, msrA2),* and virulence (up-regulation of *psmsα/ß,* down-regulation of *agr*). For further analysis we used *ftnA*, *dps, agr* and *psm*α as read-out for (pp)pGpp mediated activities under various conditions.

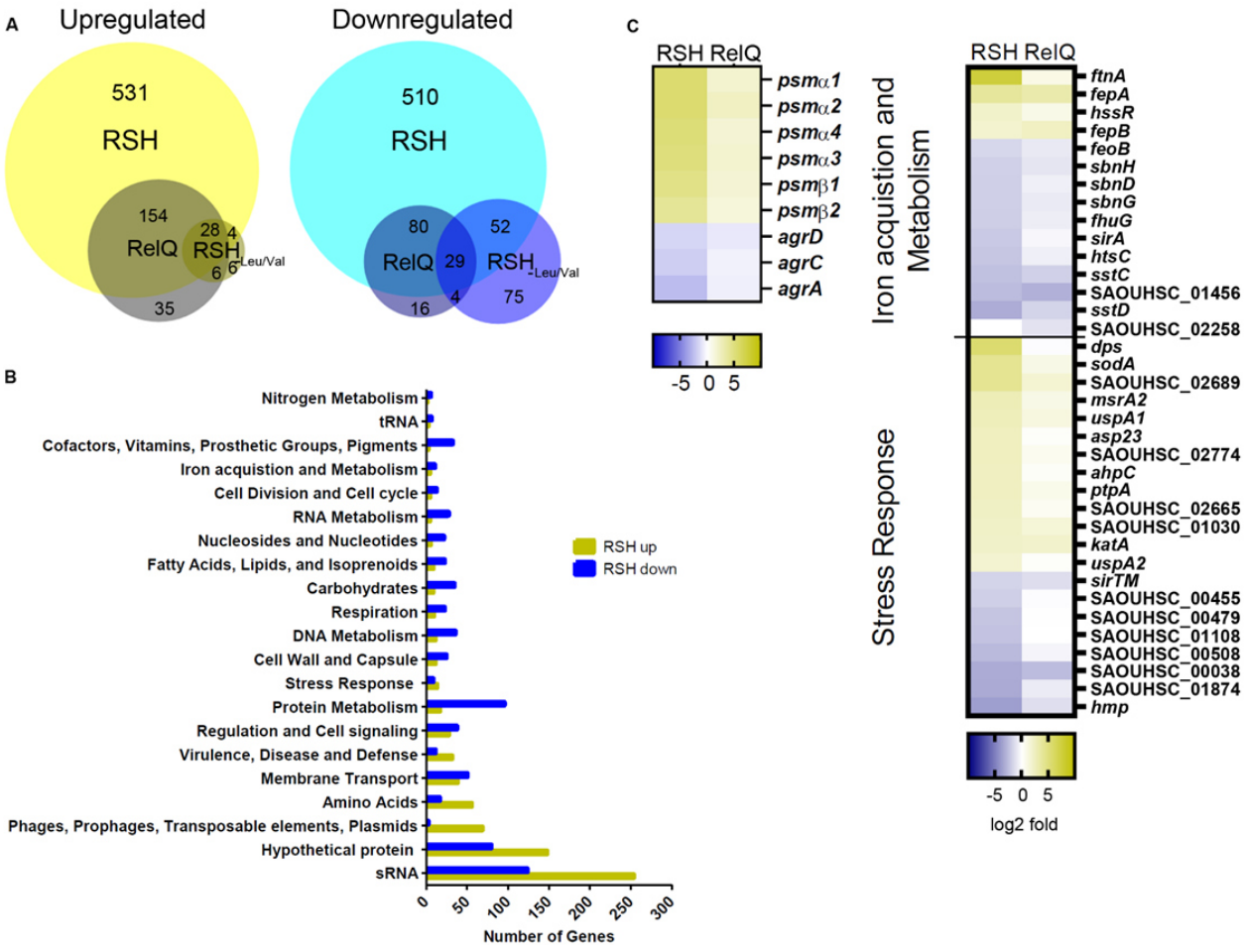

Fig.2 Global changes in gene expression upon transcriptional induction of RSH-Syn or RelQ. A. Number of genes or sRNAs upregulated (yellow) or down-regulated (blue) after induction of RSH-Syn or RelQ in comparison to uninduced cultures (< 3 fold difference, p< 0.001). Previously described stringent genes (Geiger et al., 2012) are indicated as $RSH_{leu/val}$. **B**. Genes with significant changes after induction of RSH-Syn (< 3 fold difference, p< 0.001) according to role categories. **C.** Heatmap representing RSH-Syn dependent up- and down regulated genes assigned to role categories iron acquisition metabolism and stress response and agr independent upregulation of *psms*.

Comparison of mupirocin induced stringent response and transcriptional RSH-Syn induction

We compared expression of the selected genes after induction of stringent response via mupirocin with the effect of transcriptional RSH-Syn induction. We verified the upregulation of *ftnA, dps* and *psm* under both conditions (Fig. 3A). Mupirocin resulted also in *fntA* and *dps* activation in the (pp)pGpp^0 strain, although to a lesser extent, indicating additional (pp)pGpp independent effects of mupirocin on expression of these genes. (pp)pGpp activating effect on *psm* expression is clearly not correlated to *agr* expression. Agr is the main know activator required for *psms* expression (Queck et al., 2008). However, expression of the *agr* operon was even lower upon (p)ppGpp synthesis (Suppl. Table S1 and Fig. 3A). Consistent with the RNA-seq analysis, induction of RelQ showed no (*ftnA*, *dps*) or only minor (*agrA*, *psm*) effects.

CodY independent activation of gene expression by RSH-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 RSH-Syn upregulated genes belong to the CodY regulon (Supl. Table 1). However, none of our selected marker genes are known to be regulated via CodY. To exclude CodY-dependent regulation, we compared their expression in a *codY* negative mutant. Induction of RSH-Syn or RelQ resulted in similar expression pattern in *codY* positive and negative background (Fig. 3B). This shows that (pp)pGpp impact the expression of these genes independent of CodY.

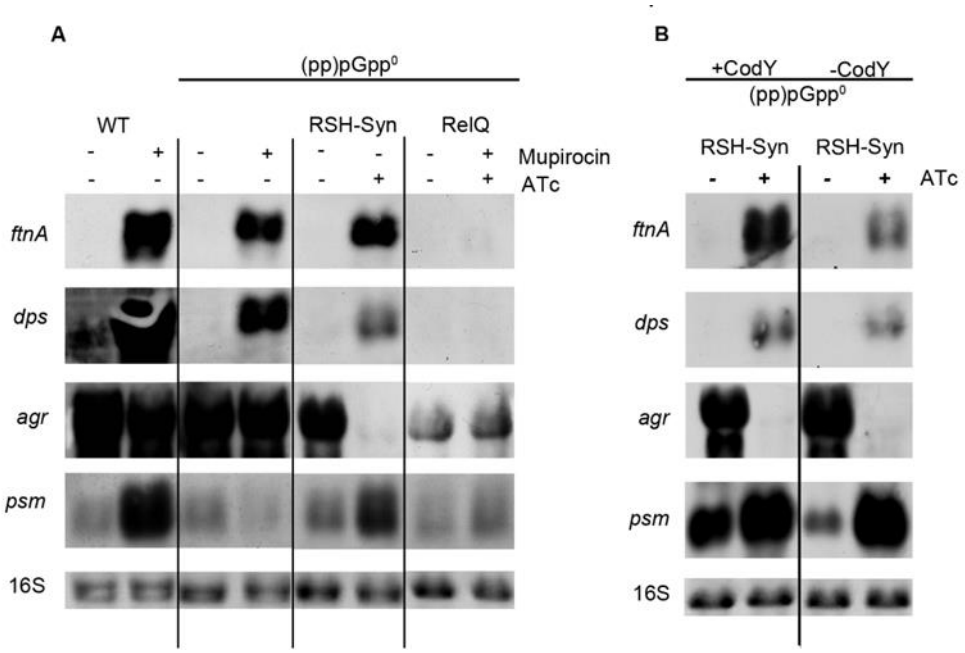

Fig.3. Correlation of mupirocin induced stringent response and transcriptional RSH-Syn of selected CodY independent genes. Strain HG001 and derivatives were grown to OD_{600}=0.3 and treated for 30 min with or without 0.125µg/ml mupirocin for HG001 wild type and (pp)pGpp0 mutant or 0.1µg/ml ATc for (pp)pGpp^0 or (pp)pGpp0/codY mutants with inducible Rsh-Syn or RelQ. For Northern analysis RNA was hybridized with digoxigenin-labelled probes specific for *ftnA*, *dps*, *psmα* or *agrA.* The 16S rRNA detected in the ethidium bromide-stained gels is indicated as loading control in the bottom lane.

RSH-Syn induction influences 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 (Gaupp et al., 2012). *ftnA*, *dps, ahpC* and *katA* (Supl. Table 1) are likely controlled via PerR binding to a conserved PerR-binding motif based on the public databases RegPrecise (Novichkov et al., 2013) and Aureowiki (Fuchs et al., 2018)). We speculated that (pp)pGpp induction of these genes may somehow be mediated via PerR activity. Therefore, we induced RSH-Syn in a *perR/*(pp)pGpp^0 background (Fig. 4A). As expected *ftnA* and *dps* are both up-reguated in the *perR* mutants. Inducing RSH-Syn shows an even higher upregulation of *ftnA*, indicating that (pp)pGpp acts in addition and independent of PerR. For *dps* the *perR* mutation alone resulted in high expression which was not further increased by (pp)pGpp indicating that *dps* is expressed at its maximum in the *perR* mutant. *PerR* deletion resulted in slight decrease in *psm* expression, which was compensated by

RSH-Syn induction. Thus, (pp)pGpp affects gene expression also in a *perR* negative background.

We found that many of the RSH-Syn effected genes are involved in iron-homeostasis indicative for iron overload condition (up regulation of *ftnA, dps*). We asked whether the iron-responsive regulator Fur is involved in the regulation of these genes. Therefore, we induced RSH-Syn in a *fur*/(pp)pGpp^0 background under low and high iron conditions (Fig. 4B). Independent of the availability of iron, *ftnA, dps* and *psm* are upregulated and *agr* down-regulated after RSH-Syn induction also in the *fur* negative background.

SarA was shown to activate transcription of the *agr* operon (Heinrichs, Bayer, & Cheung, 1996; Zielinska et al., 2011) and proposed to be involved in oxidative-stress sensing via a single Cys9 residue (Ballal & Manna, 2010; Grosser, Weiss, Shaw, & Richardson, 2016; Sun et al., 2012). *sarA* was found to be significantly up-regulated by RSH-Syn (Sup. Table 1). To analyze whether SarA is involved in (pp)pGpp regulation we induced RSH-Syn in a *sarA*/(pp)pGpp^0 background (Fig. 4C). *ftnA* and *dps* expression was not influenced by *sarA* mutation. *agr* and *psm* expression was down-regulated in the *sarA* mutant, consistent with the proposed activation of the *agr* system by SarA (Heinrichs et al., 1996). Inducing RSH-Syn again showed the typical induction of *ftnA*, *dps* and *psm* and repression of *agr* also in the *sarA* mutant. Of note, *psm* induction was less pronounced in the *sarA* mutant compared to the *sarA* positive strains.

All together our results show, that (pp)pGpp regulates the selected genes independent of main transcriptional regulators (CodY, PerR, Fur, SarA) known to control the expression of some of them.

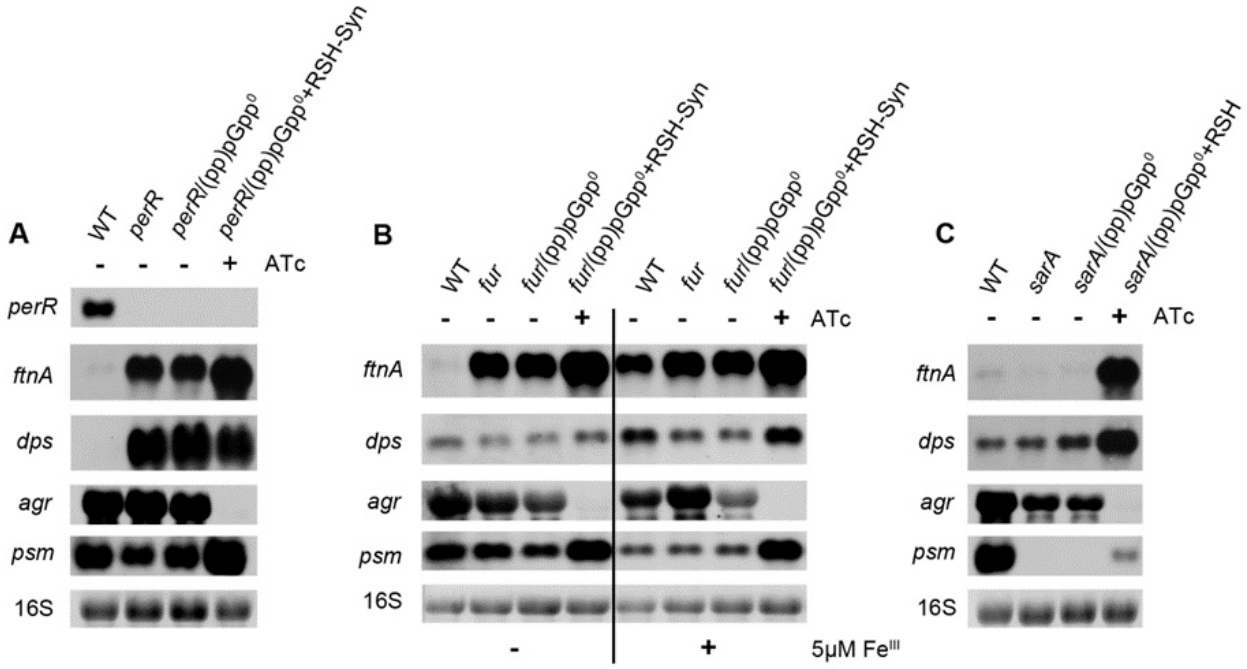

Fig.4 (pp)pGpp dependent transcriptional changes are independent of PerR, Fur or SarA. Strain HG001 and derivatives were grown to OD_{600}=0.3 and treated for 30 min without or with 0.1µg/ml ATc (mutant strains with inducible RSH-Syn). For Northern analysis RNA was hybridized with digoxigenin-labelled probes specific *for ftnA, dps, psmα* or *agrA*. The 16S rRNA detected in the ethidium bromide-stained gels is indicated as loading control in the bottom lane.

Effects of RSH-Syn induction in strain USA300

So far we concentrated our analysis on the effects of RSH-Syn induction in strain HG001. We also constructed a (pp)pGpp^0 mutant in strain USA300 and analyzed gene expression after RSH-Syn induction (Fig. 5A). As in HG001, induction of RSH-Syn resulted in the typical induction of *ftnA*, *dps* and *psm* and down-regulation of *agr* in strain USA300.

RelP induction is similar to RSH-Syn induction.

The analyses revealed that induction of RelQ showed only a minor effect on the target genes compared to induction of RSH-Syn, although RelQ was highly expressed after ATc treatment (Sup. Tabl.1). We analyzed whether this was also true in strain USA300 and whether induction of the homolog enzyme RelP would be similar to RelQ induction. We verified that RelQ induction has only minor effects on marker gene expression

(Fig. 5). However, induction of RelP was highly effective resulting in an expression pattern comparable to RSH-Syn induction.

Since we observed a severe difference between RelP and RelQ induction *in vivo* we wondered whether RelP is just a more active enzyme. Therefore, we analyzed (pp)pGpp synthesis of recombinant RelP and RelQ *in vitro* (Fig. 5B). 0.2 µM of RelP or RelQ were incubated with ATP and an equal molar mixture of the potential substrates GTP, GDP and GMP. RelQ was even more active compared to RelP indicated by increased levels of generated AMP. However, RelP preferentially synthesizes pppGpp, whereas RelQ preferentially synthesizes ppGpp and pGpp.

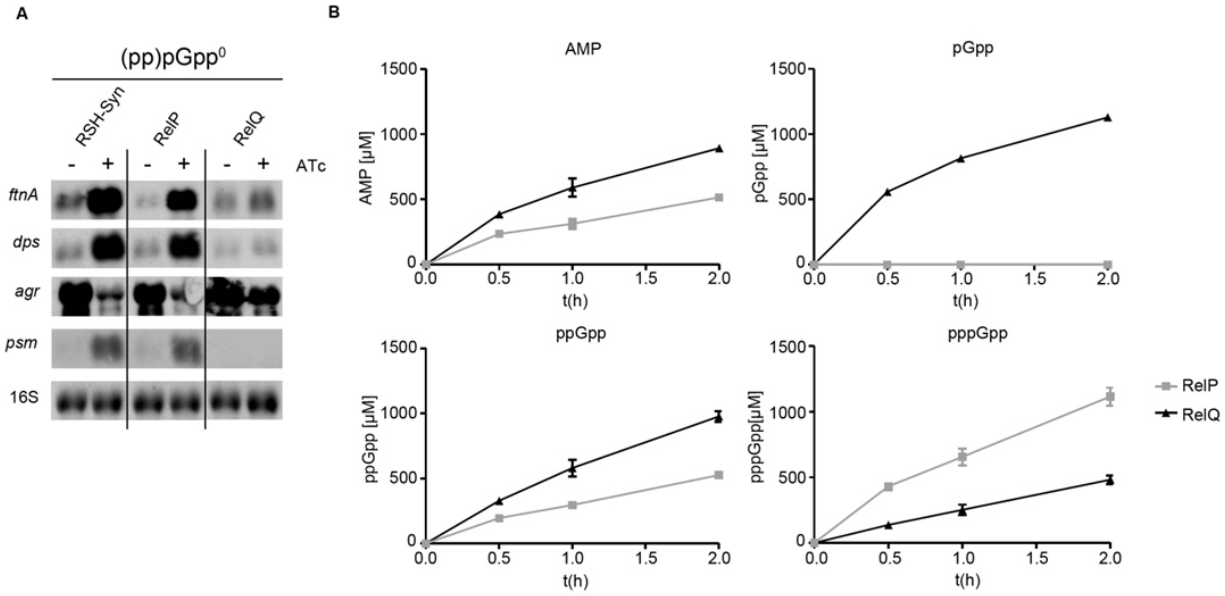

Fig.5 RelP activity *in vitro* and *in vivo*. A. Strain USA300 and derivatives were grown until an OD_{600}=0.3 and treated for 30 min without or with 0.1µg/ml ATc (mutant strains with inducible RSH-Syn or RelP). For Northern analysis RNA was hybridized with digoxigenin-labelled probes specific for ftnA, dps, psmα or agrA. The 16S rRNA detected in the ethidium bromide-stained gels is indicated as loading control in the bottom lane. **B**. 0.2µM of purified RelP and RelQ were incubated with 1mM of ATP, GMP, GDP and GTP at 37°C for 30min, 1h and 2h. The products AMP and (pp)pGpp were quantified by mass spectrometry.

(pp)pGpp is involved in oxidative stress resistance

PSMs were shown to result in intracellular production of reactive oxygen species (ROS) (George et al., 2019). Thus, it is likely that under stringent conditions (pp)pGpp mediated PSM synthesis further increases ROS formation. Indeed, the wild type strain produces significantly more ROS compared to the (pp)pGpp^0 strain (Fig. 6A). Thus, one might speculate that PSM mediated ROS production triggers the expression of oxidative stress genes detected in the transcriptome analysis. In this case, RSH-Syn induction should not result in the induction of these genes under anaerobic conditions, where ROS cannot be produced. However, RSH-Syn induction resulted in the same transcriptional pattern no matter whether bacteria were grown with or without oxygen (Fig. 6B). Thus, (pp)pGpp mediated gene alterations of the selected marker genes are not a consequence of ROS generation by PSMs.

These data indicate that (pp)pGpp simultaneously activates the ROS producing PSMs as well as ROS defense systems to prepare the cells to withstand oxidative stress (Fig. 6C). To verify this hypothesis we challenged wild type and mutants deficient in (pp)pGpp synthesis with H_20_2. The (pp)pGpp^0 strain was indeed more sensitive towards oxidative stress with a minimal inhibitory concentration (MIC) of 3.2 mM H_20_2 compared to an MIC of 6.4 mM H_20_2 for the wild type. Under these non-induced conditions (pp)pGpp might be derived from any of the pppGpp synthetases. Therefore, we also analyzed a *relPQ* mutant and *rsh$_{syn}$* mutant in which the synthetase domain of RSH was mutated. Both strains showed and 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. 6D). There was high variation in the lag time between biological replicates. Replicates of the *relPQ* or *rsh$_{syn}$* mutant showed a delayed lag phase and some of the replicates could not grow. The delay of lag phase was more prominent for the *relPQ* mutant compared to the *rsh$_{syn}$* mutant. Nevertheless, none of the (pp)pGpp^0 replicates could resume growth consistent with the reproducible lowered MIC of this strain indicating that (pp)pGpp indeed protects from oxidative stress.

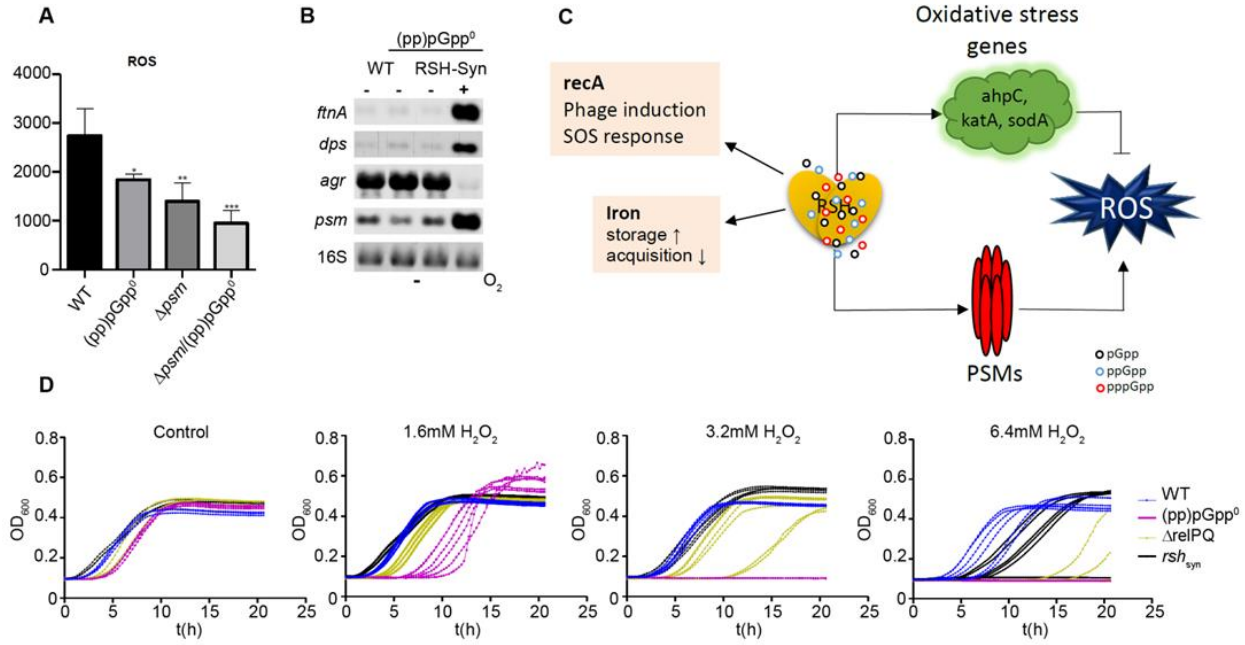

Fig.6 Functional link between stringent response and oxidative stress. A. Endogenous production of ROS in strain HG001 and derivatives ((pp)pGpp^0, *psm* and *psm*/(pp)pGpp^0 mutants). Error bars represent SD from three biological replicates. Statistical significance determined by… one-way ANOVA with Dunnett's posttest *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001. **B**. Strain HG001 and derivatives were grown anaerobically to OD_{600}=0.3 and treated for 30 min without or with 0.1µg/ml ATc ((pp)pGpp^0 mutant with inducible RSH-Syn). For Northern analysis RNA was hybridized with digoxigenin-labelled probes specific for *ftnA, dps, psmα* or *agrA*. The 16S rRNA detected in the ethidium bromide-stained gels is indicated as loading control in the bottom lane. **C.** (pp)pGpp results in up-regulation of oxidative stress genes. They are beneficial to counteract endogenous (PSMs) or exogenous (e.g. H_20_2) ROS. Upregulation of SOS and Phage genes might be a consequence of ROS accumulation e.g. by PSMs. Up-regulation of iron storage proteins may be beneficial to protect bacteria from ROS generated by the Fenton reaction. **D** WT, (pp)pGpp^0, Δ*relPQ* and *rsh*$_{syn}$ mutants were diluted from overnight culture to an OD=0.1 and challenged with different H_2O_2 concentrations and growth was monitored over time.

Discussion

We chose a genetic approach to decipher the functional consequences of (pp)pGpp synthesis without the need to apply additional stress conditions. Induction of RSH-Syn derived (pp)pGpp synthesis resulted in profound reprogramming of the transcriptional response. As expected from previous studies (Geiger et al., 2012; Reiss et al., 2012) (pp)pGpp synthesis resulted in a severe down-regulation of translational machinery and de-repression of CodY target genes. Many additional (pp)pGpp regulated genes and sRNAs were identified which are presumably important for survival of *S. aureus* during starvation conditions. Here we focused mainly on genes which were found to be activated upon (pp)pGpp synthesis in a CodY independent manner particularly p*sm*, *ftnA* and *dps*. The (pp)pGpp dependent activation of these genes occurs independent of the prototypic proteinaceous transcriptional regulators PerR, Fur, or SarA as similar (pp)pGpp effects were also detectable in regulatory mutants. The regulators are well known to be involved in the regulation of the selected genes. However, they need to be activated through e.g. oxidative stress and/or iron. (pp)pGpp function as complementary, immediate message to react to adverse conditions such as amino acid starvation or cell-walls stress. Under these conditions, up-coming oxidative stress seems to be anticipated and (pp)pGpp prepares the cells for survival e.g. ROS challenges. Indeed a $pppGpp^0$ strain is more sensitive towards H_20_2. Both RSH 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 up-regulation of *psmα and psmβ* confirming previous microarray analyses (Geiger et al., 2012). PSMs are a family of amphipathic, alpha-helical peptides that have multiple roles in staphylococcal pathogenesis and contribute a large extent to the pathogenic success of virulent staphylococci (Cheung, Joo, Chatterjee, & Otto, 2014; Peschel & Otto, 2013). They are cytotoxic, stimulate inflammatory responses and contribute to biofilm dissemination. Moreover, (pp)pGpp dependent *psm* expression within neutrophils was shown to be crucial for survival after phagocytosis (Geiger et al., 2012). However, PSMs also interact with the producer's own membrane, promote the release of membrane vesicles from the cytoplasmic membrane via an increase of membrane fluidity (Schlatterer et al., 2018; X. Wang, Thompson, Weidenmaier, & Lee, 2018),

reduce persister formation (Bojer, Lindemose, Vestergaard, & Ingmer, 2018; Xu et al., 2017) and are involved in self-toxicity via ROS formation (George et al., 2019). Interestingly, *psm* activation was not correlated with activation of the quorum sensing Agr system, the main regulator required for *psm* expression (Queck et al., 2008). 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 (W. Gao et al., 2010). Thus, (pp)pGpp mediated *psm* activation is clearly uncoupled from *agr* expression. Recently, the sRNA Teg41 (S131) (Zapf et al., 2019) and the transcriptional regulator MgrA (Jiang, Jin, & Sun, 2018) were found to interfere with *psm* expression. However, it is unlikely that they mediate the (pp)pGpp regulatory effect because expression of these regulators was not found altered in our RNAseq analysis (Supp. Tab. S1). Thus, the molecular mechanism how (pp)pGpp leads to *psm* activation has to be elucidated. *psm* promoters might be sensitive towards the concentration of the initiating nucleoside triphosphate (iNTP). The stringent response is accompanied with changes in the ATP/GTP ratios and the identity of the +1 position (A or G) dictates the activity of sensitive promoters (Krasny et al., 2008). Various sequence combinations determine whether a promoter is iNTP sensitive or not (Sojka et al., 2011). Such sequence motifs are hard to predict within the *psm* promoters. However, both p*smα* and *psmß* operons start with an A at +1 position (Queck et al., 2008).

(pp)pGpp and oxidative stress response

Genes involved in iron metabolism indicative for iron overload conditions were also highly affected by (pp)pGpp. Recently, a similar effect was reported for *Vibrio cholera* (H. Y. Kim et al., 2018). 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 reduce antibiotic-induced oxidative stress and thus tolerance and it is likely that this is also the case in *S. aureus*. Besides interfering with iron metabolism other genes involved in oxidative stress were activated by (pp)pGpp. A link between stringent response and oxidative stress response was 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* (Hanna et al., 2013), and *P. aeruginosa* (Martins et al., 2018). SOD was shown to be the key factor responsible for (pp)pGpp

mediated multidrug tolerance in *P. aeruginosa* (Martins et al., 2018). Moreover, (pp)pGpp deficient strains are often found to be more sensitive towards oxidative stress (Holley et al., 2014; Yan et al., 2009),(J. Wang et al., 2016). Also in *E. faecalis a* $pppGpp^0$ mutant is more sensitive to H_20_2.

In *S. aureus* the stringent response leads to the activation of ROS inducing toxins and simultaneous expression of the detoxifying system to protect the producer. This is likely of special advantage for the pathogen once it encounters neutrophils and elevated ROS. The PSMs are required to escape from within the cells after phagocytosis (Geiger et al., 2012; Surewaard et al., 2013). The upregulation of the oxygen stress program will help to protect from endogenous as well as exogenous ROS.

Comparison of RSH-Syn, RelQ and RelP activity

We compared the activity or RSH-Syn, RelQ and RelP. Nucleotide profiling as well as transcriptional analysis showed that induction of RelQ results in similar nucleotide changes than RSH-Syn although to a much lesser extent. RelP in contrast was equally active as RSH-Syn. Thus, RelQ activity seems to be restricted *in vivo* under our growth conditions. Comparison of RelP and RelQ from other organisms revealed that RelQ is inhibited through RNA binding and auto-activated by (pp)pGpp (Manav et al., 2018; Steinchen et al., 2018). 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. Conditions, which would relieve this restriction, remain to be determined. Analysis of purified RelP and RelQ revealed that both enzymes are equally active. The most striking difference was that RelQ can use GMP as substrate to produce pGpp, whereas no pGpp synthesis activity was detectable for purified RelP. pGpp synthesis was already described for RelQ from *E. faecalis* (Gaca, Kudrin, et al., 2015) or *Corynebacterium glutamicum* (Ruwe, Kalinowski, & Persicke, 2017). Of note, we also detected RSH dependent pGpp synthesis via either mupirocin treatment or RSH-Syn induction *in vivo*. pGpp was shown to exert inhibitory effects similar to ppGpp for e.g. enzymes involved in GTP biosynthesis (Gaca, Kudrin, et al., 2015). Former nucleotide analyses in *S. aureus* (Crosse, Greenway, & England, 2000) and other organisms (Pao & Gallant, 1979) revealed another molecule, ppGp. pGpp was found to be even more active than pppGpp regarding e.g. inhibition of some target proteins but may also exert opposite

effects (Pao & Dyess, 1981). GTP, pGpp and ppGp are difficult to differentiate due to their identical molecular weight and the biological relevance of these molecules remain to be elucidated. Nevertheless, the function of the two SAS enzymes often present together within one organism indicate that they might full-fill distinct functions. RelQ requires post-transcriptional activation and preferentially synthesizes pGpp. The different ratio of the alarmones pppGpp, ppGpp and pGpp may contribute to small differences in the stringent response out-come (Mechold et al., 2013). However, the overall changes on the nucleotide pool and transcriptional changes are largely similar between the three (pp)pGpp synthetases of *S. aureus*.

Materials and Methods

Strains and growth conditions.

Strains and plasmids are listed in Supl. Table S1. For strains carrying a resistance gene a concentration of 10µg/ml chloramphenicol, 10µg/ml erythromycin or 100µg/ml ampicillin was used only for overnight cultures. *S. aureus* strains were grown over night in chemical defined medium (CDM) (Pohl et al., 2009) and diluted to an optical density (OD_{600}) of 0.05 and grown until the early exponential phase OD_{600} = 0.3 with shaking (220rpm, 37°C). Strains carrying a plasmid with an ATc-inducible promoter were induced at OD_{600}=0.3 with 0.1µg/ml ATc for 30 min.

For anaerobic growth the strains were diluted to an OD_{600} of 0.05 in hungate tubes (Chemglass), completely filled with CDM. ATc was applied using a syringe at OD_{600}=0.03. For OD measurements, aliquots were drawn with a syringe.

Generation of (p)ppGpp^0 mutant in USA300 JE2

For the USA300 (p)ppGpp^0 mutant (USA300-229-230-263), lysates were prepared from RN4220 strains containing the mutagenesis vectors pCG229, pCG230 and pCG263, respectively (Table S3). After transduction into USA300 JE2, mutagenesis was performed as previously described (Bae & Schneewind, 2006). To avoid toxic accumulation of (p)ppGpp the genes were mutated in the order *relP, relQ* and finally *rsh*. Mutations were verified by PCR using primers enlisted in Table S4.

RNA isolation and Northern Blot analysis

RNA isolation and northern blot analysis were performed as described previously (Goerke et al., 2000). Briefly, bacteria were pelleted and resuspended in 1ml TRIzol (Thermo Fisher Scientific) and lysed using zirconia/silicia 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).

Purification of RSH-syn, RelP and RelQ

Proteins were purified as described in Gratani et al. (Gratani et al., 2018). Briefly, plasmids carrying RSH-syn, RelP or RelQ were freshly transformed into *E.coli* B21 and grown for 16 hours at RT in LB supplemented with D(+)-lactose-monohydrate (12.5g/l) and 100µg/ml ampicillin.
Cells were harvested, centrifuged (20 minutes, 3000 x g, 4 °C) and resuspended in ice-cold low-KCl buffer A supplemented with 10 µg/ml DNAse and cOmpleteTM protease inhibitor cocktail (Roche). Cells were lysed and the lysate was centrifuged (50,000 x g, 45 minutes, 4 °C). The clear supernatant was filtered (0.22-µm pore size) and loaded onto a 1-ml HisTrap HP column (GE Healthcare Life Sciences) equilibrated with high-KCl buffer A. Proteins were purified by an ÄKTA purification system (GE Healthcare Life Sciences) and eluted with an imidazole gradient to a final concentration of 500 mM. The eluted fractions were loaded on SDS-PAGE, protein collected and concentrated by an Amicon Ultracel-30K ultracentrifugal device. Size exclusion columns were pretreated with ice-cold low-KCl SEC buffer and size exclusion was performed for further protein purification (HiLoad 16/600 Superdex 200 pg, GE Healthcare Life Sciences). Proteins were concentrated and stored at -80°C.

***In vivo* nucleotide extraction**

Method was adapted from Jüngert et al, 2017 and modified. Briefly, strains were grown in CDM ,from an overnight culture and diluted to an OD_{600} 0,05 and grown in CDM until an OD_{600} of 0.3. Strains were split and treated w/wo 0.1µg/ml ATc for 30min at 37°C and 220 rpm shaking. 100ml bacterial cultures were harvested and transfered in falcon half filled with ice and centrifuged (5min, 5000 x g, 4°C). Supernatant was discarded and pellet frozen in liquid nitrogen and stored at -80°C until usage. Samples were thaw on ice and resuspended in 2M formic acid and incubated on ice for 30min. Resuspended bacteria were lysed by high speed homogenizer using zirconia/silicia beads (0,1mm diameter) and kept on ice for 30min. The aqueous phase was collected and mixed with 50mM NH_4OAc (pH 4.5) and loaded on columns (OASIS Wax cartridge 3xcc) and centrifuged (5000 x g, 5min, 4°C). Columns were pre-treated first with pure methanol and then with 50mM NH_4OAc (pH 4.5). Samples were washed first with 50mM NH_4OAc (pH 4.5) followed by a washing step with methanol. Elution was performed with a mixture of 20% methanol/70% ddH_2O/10% NH_4OH (of 30% stock

solution). Eluted nucleotides were flash frozen in liquid nitrogen and lyophilized overnight (Alpha 1-4 LSC, CHRIST). Lyophilized nucleotides were resuspended in ddH_2O and analyzed via HPLC-MS.

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

Method was used as previously described in Gratani et al., 2018. Briefly, nucleotides were analyzed using ESI-TOF (micrO-TOF II, Bruker) mass spectrometer connected to an UltiMate 3000 high-performance liquid chromatography.. 5-microliters of standards or samples were injected onto SEQuant ZIC-pHILIC column (Merck, PEEK 150 x 2.1mm, 5µm). 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 (346.06 m/z), ATP (505.99 m/z), GTP (521.98 m/z), pGpp (521.98 m/z), ppGpp (601.96 m/z) and pppGpp (681.92 m/z) were diluted from xxx to 1 mM and analyzed by HPLC-MS. 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 for 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.

Peak separation of pGpp from GTP

pGpp and GTP have the same exact mass of 522.990, but they can be distinguished by different retention times on the ZIC-pHILIC column (x min and y min respectively). To separate pGpp from GTP we used an algorithm since Prims 5 (GraphPad) was not able to separate the two peaks and calculate two different AUCs. For this reason we used an EM algorithm to separate pGpp from GTP.

Modification on R-Code and EM-Algorithm is based on Goncalo Abecasis's lecture notes on the EM algorithm (http://csg.sph.umich.edu/abecasis/class/2006/615.18.pdf,24.03.2019). Before

identifying the components, first the baseline had to be subtracted in the R-Code. Second, the density was calculated which leads to a normalized intensity with the following formula. i(t) was considered as the intensity and t time.

$$\mathrm{d}(t) = \frac{i(t)}{\sum_{\theta} i(\theta)}$$

Identification of the components.

The issue is to find all parameters of a Gaussian mixture given the data in aggregated form and given the number of components. If the goal is to find *n* components then the algorithm returns an estimate of *n* fractions λ_k, and *n* pairs (μ_k, σ_k) of parameters for the Gaussian components so that

$$f(t) = \sum_{k=1}^{n} \lambda_k \cdot \phi_{\mu_k,\sigma_k}(t)$$

where ϕ_{μ_k,σ_k} is the density of a Gaussian with mean parameter μ_k and parameter of standard deviation σ_k. In case of a chromatogram, $f(t)$ is the fitted intensity to the data, μ_k and σ_k are the measures of position and width of the k^{th} signal, whereas λ_k is the proportion of the k^{th} signal in the chromatogram. Obviously, the condition $\sum_{k=1}^{n} \lambda_k = 1$ must hold.As the signals in chromatograms do not always appear as well shaped Gaussian bell curves, a chemical component might result in a signal which is best fitted by more than one Gaussian component. To separate pGpp from GTP the peaks had to be separated in more than two components, so we split the curves in $n = 15$ components. Indeed, as there is a natural order of the components found by their parameter μ_k we had to decide upon a threshold t_c as a classification rule: All components respecting the condition $\mu_k \leq t_c$ should be classified as belonging to the first chemical component whereas the other belong to the second. The relative amount of the first chemical component in the mixture is then calculated as and for the second we get $I_2 = 1 - I_1$.

$$I_1 = \sum_{k:\mu_k \leq t_c} \lambda_k$$

H_2O_2 killing assay

Strains were grown over night in CDM. Overnight cultures were diluted in fresh CDM to an OD_{600} of 0.1 and growth followed for 24 hours with different H_2O_2 concentrations with the Infinite M200 Pro microplate reader from Tecan. MIC determination was performed according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines using CDM medium.

ROS measurement

ROS measurements were performed as described in George et al. (George et al., 2019). Briefly, bacteria equivalent to an OD_{600}=1 were harvested from overnight cultures and resuspended in 100µl DCF (2',7'-dichlorfluorescin acetate) and incubated for 50 minutes Fluorescence was measured using Tecan Infinite 200 PRO at an excitation wavelength of 488 nm and emission wavelength of 515 nm.

RNA-Seq Analysis

Strains were grown in triplicates to OD600 = 0.3, and splitted into treated (ATc, 0.1 µg/ml) and untreated control and grown for 30 min. Purified RNA was sent to Vertis Biotechnologie AG for RNASequencing 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 75bp reads. RNA-Seq analysis was performed using CLC Genomic Workbench (Qiagen). Reads were trimmed (TrueSeq-Antinsense Primer AGATCGGAAGAGCACACGTCTGAACTCCAGTCA) and mapped to reference genome HG001 (Romby). Differential gene expression was performed comparing RSH-Syn or RelQ versus the (pp)pGpp^0 mutant. Venn diagrams were performed comparing RSH-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.

Atkinson, G. C., Tenson, T., & Hauryliuk, V. (2011). The RelA/SpoT homolog (RSH) superfamily: distribution and functional evolution of ppGpp synthetases and hydrolases across the tree of life. *PLoS One, 6*(8), e23479. doi:10.1371/journal.pone.0023479

Bae, T., & Schneewind, O. (2006). Allelic replacement in Staphylococcus aureus with inducible counter-selection. *Plasmid, 55*(1), 58-63. doi:10.1016/j.plasmid.2005.05.005

Ballal, A., & Manna, A. C. (2010). Control of thioredoxin reductase gene (trxB) transcription by SarA in Staphylococcus aureus. *J Bacteriol, 192*(1), 336-345. doi:10.1128/JB.01202-09

Beljantseva, J., Kudrin, P., Andresen, L., Shingler, V., Atkinson, G. C., Tenson, T., & Hauryliuk, V. (2017). Negative allosteric regulation of Enterococcus faecalis small alarmone synthetase RelQ by single-stranded RNA. *Proc Natl Acad Sci U S A, 114*(14), 3726-3731. doi:10.1073/pnas.1617868114

Bojer, M. S., Lindemose, S., Vestergaard, M., & Ingmer, H. (2018). Quorum Sensing-Regulated Phenol-Soluble Modulins Limit Persister Cell Populations in Staphylococcus aureus. *Front Microbiol, 9*, 255. doi:10.3389/fmicb.2018.00255

Cheung, G. Y., Joo, H. S., Chatterjee, S. S., & Otto, M. (2014). Phenol-soluble modulins--critical determinants of staphylococcal virulence. *FEMS Microbiol Rev, 38*(4), 698-719. doi:10.1111/1574-6976.12057

Corrigan, R. M., Bellows, L. E., Wood, A., & Grundling, A. (2016). ppGpp negatively impacts ribosome assembly affecting growth and antimicrobial tolerance in Gram-positive bacteria. *Proc Natl Acad Sci U S A, 113*(12), E1710-1719. doi:10.1073/pnas.1522179113

Crosse, A. M., Greenway, D. L., & England, R. R. (2000). Accumulation of ppGpp and ppGp in *Staphylococcus aureus* 8325-4 following nutrient starvation. *Lett Appl Microbiol, 31*(4), 332-337. doi:lam822 [pii]

Dalebroux, Z. D., Svensson, S. L., Gaynor, E. C., & Swanson, M. S. (2010). ppGpp conjures bacterial virulence. *Microbiol Mol Biol Rev, 74*(2), 171-199. doi:10.1128/MMBR.00046-09

Dordel, J., Kim, C., Chung, M., Pardos de la Gandara, M., Holden, M. T., Parkhill, J., . . . Tomasz, A. (2014). Novel determinants of antibiotic resistance: identification of mutated loci in highly methicillin-resistant subpopulations of methicillin-resistant Staphylococcus aureus. *MBio, 5*(2), e01000. doi:10.1128/mBio.01000-13

Dozot, M., Boigegrain, R. A., Delrue, R. M., Hallez, R., Ouahrani-Bettache, S., Danese, I., . . . Kohler, S. (2006). The stringent response mediator Rsh is required for *Brucella melitensis* and *Brucella suis* virulence, and for expression of the type IV secretion system *vir*B. *Cell Microbiol, 8*(11), 1791-1802. doi:CMI749 [pii]

10.1111/j.1462-5822.2006.00749.x

Eymann, C., Homuth, G., Scharf, C., & Hecker, M. (2002). *Bacillus subtilis* functional genomics: global characterization of the stringent response by proteome and transcriptome analysis. *J Bacteriol, 184*(9), 2500-2520.

Fuchs, S., Mehlan, H., Bernhardt, J., Hennig, A., Michalik, S., Surmann, K., . . . Mader, U. (2018). AureoWiki The repository of the Staphylococcus aureus research and annotation community. *Int J Med Microbiol, 308*(6), 558-568. doi:10.1016/j.ijmm.2017.11.011

Gaca, A. O., Abranches, J., Kajfasz, J. K., & Lemos, J. A. (2012). Global transcriptional analysis of the stringent response in Enterococcus faecalis. *Microbiology, 158*(Pt 8), 1994-2004. doi:10.1099/mic.0.060236-0

Gaca, A. O., Colomer-Winter, C., & Lemos, J. A. (2015). Many means to a common end: the intricacies of (p)ppGpp metabolism and its control of bacterial homeostasis. *J Bacteriol, 197*(7), 1146-1156. doi:10.1128/jb.02577-14

Gaca, A. O., Kudrin, P., Colomer-Winter, C., Beljantseva, J., Liu, K., Anderson, B., . . . Lemos, J. A. (2015). From (p)ppGpp to (pp)pGpp: Characterization of Regulatory Effects of pGpp Synthesized by the Small Alarmone Synthetase of Enterococcus faecalis. *J Bacteriol, 197*(18), 2908-2919. doi:10.1128/JB.00324-15

Gao, W., Chua, K., Davies, J. K., Newton, H. J., Seemann, T., Harrison, P. F., . . . Howden, B. P. (2010). Two novel point mutations in clinical Staphylococcus aureus reduce linezolid susceptibility and switch on the stringent response to promote persistent infection. *PLoS Pathog, 6*(6), e1000944. doi:10.1371/journal.ppat.1000944

Gaupp, R., Ledala, N., & Somerville, G. A. (2012). Staphylococcal response to oxidative stress. *Front Cell Infect Microbiol, 2*, 33. doi:10.3389/fcimb.2012.00033

Geiger, T., Francois, P., Liebeke, M., Fraunholz, M., Goerke, C., Krismer, B., . . . Wolz, C. (2012). The stringent response of Staphylococcus aureus and its impact on survival after phagocytosis through the induction of intracellular PSMs expression. *PLoS Pathog, 8*(11), e1003016. doi:10.1371/journal.ppat.1003016

Geiger, T., Goerke, C., Fritz, M., Schafer, T., Ohlsen, K., Liebeke, M., . . . Wolz, C. (2010). Role of the (p)ppGpp synthase RSH, a RelA/SpoT homolog, in stringent response and virulence of Staphylococcus aureus. *Infect Immun, 78*(5), 1873-1883. doi:10.1128/IAI.01439-09

Geiger, T., Kastle, B., Gratani, F. L., Goerke, C., & Wolz, C. (2014). Two small (p)ppGpp synthases in Staphylococcus aureus mediate tolerance against cell envelope stress conditions. *J Bacteriol, 196*(4), 894-902. doi:10.1128/jb.01201-13

George, S. E., Hrubesch, J., Breuing, I., Vetter, N., Korn, N., Hennemann, K., . . . Wolz, C. (2019). Oxidative stress drives the selection of quorum sensing mutants in the Staphylococcus aureus population. *Proc Natl Acad Sci U S A, 116*(38), 19145-19154. doi:10.1073/pnas.1902752116

Goerke, C., Campana, S., Bayer, M. G., Doring, G., Botzenhart, K., & Wolz, C. (2000). Direct quantitative transcript analysis of the agr regulon of Staphylococcus aureus during human infection in comparison to the expression profile in vitro. *Infect Immun, 68*(3), 1304-1311.

Gratani, F. L., Horvatek, P., Geiger, T., Borisova, M., Mayer, C., Grin, I., . . . Wolz, C. (2018). Regulation of the opposing (p)ppGpp synthetase and hydrolase activities in a bifunctional RelA/SpoT homologue from Staphylococcus aureus. *PLoS Genet, 14*(7), e1007514. doi:10.1371/journal.pgen.1007514

Grosser, M. R., Weiss, A., Shaw, L. N., & Richardson, A. R. (2016). Regulatory Requirements for Staphylococcus aureus Nitric Oxide Resistance. *J Bacteriol, 198*(15), 2043-2055. doi:10.1128/jb.00229-16

Hanna, N., Ouahrani-Bettache, S., Drake, K. L., Adams, L. G., Kohler, S., & Occhialini, A. (2013). Global Rsh-dependent transcription profile of Brucella suis during stringent response unravels adaptation to nutrient starvation and cross-talk with other stress responses. *BMC Genomics, 14*, 459. doi:10.1186/1471-2164-14-459

Hauryliuk, V., Atkinson, G. C., Murakami, K. S., Tenson, T., & Gerdes, K. (2015). Recent functional insights into the role of (p)ppGpp in bacterial physiology. *Nat Rev Microbiol, 13*(5), 298-309. doi:10.1038/nrmicro3448

Heinrichs, J. H., Bayer, M. G., & Cheung, A. L. (1996). Characterization of the sar locus and its interaction with agr in Staphylococcus aureus. *J Bacteriol, 178*(2), 418-423. doi:10.1128/jb.178.2.418-423.1996

Helle, L., Kull, M., Mayer, S., Marincola, G., Zelder, M. E., Goerke, C., . . . Bertram, R. (2011). Vectors for improved Tet repressor-dependent gradual gene induction or silencing in Staphylococcus aureus. *Microbiology, 157*(Pt 12), 3314-3323. doi:10.1099/mic.0.052548-0

Herbert, S., Ziebandt, A. K., Ohlsen, K., Schafer, T., Hecker, M., Albrecht, D., . . . Gotz, F. (2010). Repair of global regulators in Staphylococcus aureus 8325 and comparative analysis with other clinical isolates. *Infect Immun, 78*(6), 2877-2889. doi:10.1128/iai.00088-10

Hobbs, J. K., & Boraston, A. B. (2019). (p)ppGpp and the Stringent Response: An Emerging Threat to Antibiotic Therapy. *ACS Infect Dis, 5*(9), 1505-1517. doi:10.1021/acsinfecdis.9b00204

Holley, C., Gangaiah, D., Li, W., Fortney, K. R., Janowicz, D. M., Ellinger, S., . . . Spinola, S. M. (2014). A (p)ppGpp-null mutant of Haemophilus ducreyi is partially attenuated in humans due to multiple conflicting phenotypes. *Infect Immun, 82*(8), 3492-3502. doi:10.1128/iai.01994-14

Irving, S. E., & Corrigan, R. M. (2018). Triggering the stringent response: signals responsible for activating (p)ppGpp synthesis in bacteria. *Microbiology, 164*(3), 268-276. doi:10.1099/mic.0.000621

Jiang, Q., Jin, Z., & Sun, B. (2018). MgrA Negatively Regulates Biofilm Formation and Detachment by Repressing the Expression of psm Operons in Staphylococcus aureus. *Appl Environ Microbiol, 84*(16). doi:10.1128/AEM.01008-18

Kastle, B., Geiger, T., Gratani, F. L., Reisinger, R., Goerke, C., Borisova, M., . . . Wolz, C. (2015). rRNA regulation during growth and under stringent conditions in Staphylococcus aureus. *Environ Microbiol, 17*(11), 4394-4405. doi:10.1111/1462-2920.12867

Katayama, Y., Azechi, T., Miyazaki, M., Takata, T., Sekine, M., Matsui, H., . . . Chambers, H. F. (2017). Prevalence of Slow-Growth Vancomycin Nonsusceptibility in Methicillin-Resistant Staphylococcus aureus. *Antimicrob Agents Chemother, 61*(11). doi:10.1128/aac.00452-17

Kazmierczak, K. M., Wayne, K. J., Rechtsteiner, A., & Winkler, M. E. (2009). Roles of rel(Spn) in stringent response, global regulation and virulence of serotype 2 Streptococcus pneumoniae D39. *Mol Microbiol, 72*(3), 590-611. doi:10.1111/j.1365-2958.2009.06669.x

Kim, H. Y., Go, J., Lee, K. M., Oh, Y. T., & Yoon, S. S. (2018). Guanosine tetra- and pentaphosphate increase antibiotic tolerance by reducing reactive oxygen

species production in Vibrio cholerae. *J Biol Chem, 293*(15), 5679-5694. doi:10.1074/jbc.RA117.000383

Krasny, L., & Gourse, R. L. (2004). An alternative strategy for bacterial ribosome synthesis: Bacillus subtilis rRNA transcription regulation. *Embo j, 23*(22), 4473-4483. doi:10.1038/sj.emboj.7600423

Krasny, L., Tiserova, H., Jonak, J., Rejman, D., & Sanderova, H. (2008). The identity of the transcription +1 position is crucial for changes in gene expression in response to amino acid starvation in Bacillus subtilis. *Mol Microbiol, 69*(1), 42-54. doi:10.1111/j.1365-2958.2008.06256.x

Kreiswirth, B. N., Lofdahl, S., Betley, M. J., O'Reilly, M., Schlievert, P. M., Bergdoll, M. S., & Novick, R. P. (1983). The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature, 305*(5936), 709-712. doi:10.1038/305709a0

Kriel, A., Bittner, A. N., Kim, S. H., Liu, K., Tehranchi, A. K., Zou, W. Y., . . . Wang, J. D. (2012a). Direct regulation of GTP homeostasis by (p)ppGpp: a critical component of viability and stress resistance. *Mol Cell, 48*(2), 231-241. doi:10.1016/j.molcel.2012.08.009

Kriel, A., Bittner, A. N., Kim, S. H., Liu, K., Tehranchi, A. K., Zou, W. Y., . . . Wang, J. D. (2012b). Direct regulation of GTP homeostasis by (p)ppGpp: a critical component of viability and stress resistance. *Molecular Cell, 48*(2), 231-241. doi:10.1016/j.molcel.2012.08.009

Liu, K., Bittner, A. N., & Wang, J. D. (2015). Diversity in (p)ppGpp metabolism and effectors. *Curr Opin Microbiol, 24*, 72-79. doi:10.1016/j.mib.2015.01.012

Majerczyk, C. D., Dunman, P. M., Luong, T. T., Lee, C. Y., Sadykov, M. R., Somerville, G. A., . . . Sonenshein, A. L. (2010). Direct targets of CodY in *Staphylococcus aureus*. *J Bacteriol, 192*(11), 2861-2877. doi:10.1128/JB.00220-10

Manav, M. C., Beljantseva, J., Bojer, M. S., Tenson, T., Ingmer, H., Hauryliuk, V., & Brodersen, D. E. (2018). Structural basis for (p)ppGpp synthesis by the Staphylococcus aureus small alarmone synthetase RelP. *J Biol Chem, 293*(9), 3254-3264. doi:10.1074/jbc.RA117.001374

Martins, D., McKay, G., Sampathkumar, G., Khakimova, M., English, A. M., & Nguyen, D. (2018). Superoxide dismutase activity confers (p)ppGpp-mediated antibiotic tolerance to stationary-phase Pseudomonas aeruginosa. *Proc Natl Acad Sci U S A, 115*(39), 9797-9802. doi:10.1073/pnas.1804525115

Matsuo, M., Hiramatsu, M., Singh, M., Sasaki, T., Hishinuma, T., Yamamoto, N., . . . Hiramatsu, K. (2019). Genetic and Transcriptomic Analyses of Ciprofloxacin-Tolerant Staphylococcus aureus Isolated by the Replica Plating Tolerance Isolation System (REPTIS). *Antimicrob Agents Chemother, 63*(2), e02019-02018. doi:10.1128/aac.02019-18

Mechold, U., Potrykus, K., Murphy, H., Murakami, K. S., & Cashel, M. (2013). Differential regulation by ppGpp versus pppGpp in *Escherichia coli*. *Nucleic Acids Res, 41*(12), 6175-6189. doi:10.1093/nar/gkt302

Nascimento, M. M., Lemos, J. A., Abranches, J., Lin, V. K., & Burne, R. A. (2008). Role of RelA of Streptococcus mutans in global control of gene expression. *J Bacteriol, 190*(1), 28-36. doi:10.1128/jb.01395-07

Novichkov, P. S., Kazakov, A. E., Ravcheev, D. A., Leyn, S. A., Kovaleva, G. Y., Sutormin, R. A., . . . Rodionov, D. A. (2013). RegPrecise 3.0--a resource for genome-scale exploration of transcriptional regulation in bacteria. *BMC Genomics, 14*, 745. doi:10.1186/1471-2164-14-745

Pao, C. C., & Dyess, B. T. (1981). Effect of unusual guanosine nucleotides on the activities of some Escherichia coli cellular enzymes. *Biochim Biophys Acta, 677*(3-4), 358-362. doi:10.1016/0304-4165(81)90247-6

Pao, C. C., & Gallant, J. (1979). A new nucleotide involved in the stringent response in *Escherichia coli*. Guanosine 5'-diphosphate-3'-monophosphate. *J Biol Chem, 254*(3), 688-692.

Peschel, A., & Otto, M. (2013). Phenol-soluble modulins and staphylococcal infection. *Nat Rev Microbiol, 11*(10), 667-673. doi:10.1038/nrmicro3110

Pohl, K., Francois, P., Stenz, L., Schlink, F., Geiger, T., Herbert, S., . . . Wolz, C. (2009). CodY in Staphylococcus aureus: a regulatory link between metabolism and virulence gene expression. *J Bacteriol, 191*(9), 2953-2963. doi:10.1128/JB.01492-08

Queck, S. Y., Jameson-Lee, M., Villaruz, A. E., Bach, T. H., Khan, B. A., Sturdevant, D. E., . . . Otto, M. (2008). RNAIII-independent target gene control by the agr quorum-sensing system: insight into the evolution of virulence regulation in Staphylococcus aureus. *Mol Cell, 32*(1), 150-158. doi:10.1016/j.molcel.2008.08.005

Reiss, S., Pane-Farre, J., Fuchs, S., Francois, P., Liebeke, M., Schrenzel, J., . . . Engelmann, S. (2012). Global analysis of the Staphylococcus aureus response to mupirocin. *Antimicrob Agents Chemother, 56*(2), 787-804. doi:10.1128/AAC.05363-11

Ruwe, M., Kalinowski, J., & Persicke, M. (2017). Identification and Functional Characterization of Small Alarmone Synthetases in Corynebacterium glutamicum. *Front Microbiol, 8*, 1601. doi:10.3389/fmicb.2017.01601

Sanchez-Vazquez, P., Dewey, C. N., Kitten, N., Ross, W., & Gourse, R. L. (2019). Genome-wide effects on Escherichia coli transcription from ppGpp binding to its two sites on RNA polymerase. *Proc Natl Acad Sci U S A, 116*(17), 8310-8319. doi:10.1073/pnas.1819682116

Schlatterer, K., Beck, C., Hanzelmann, D., Lebtig, M., Fehrenbacher, B., Schaller, M., . . . Peschel, A. (2018). The Mechanism behind Bacterial Lipoprotein Release: Phenol-Soluble Modulins Mediate Toll-Like Receptor 2 Activation via Extracellular Vesicle Release from Staphylococcus aureus. *MBio, 9*(6). doi:10.1128/mBio.01851-18

Schroder, W., Goerke, C., & Wolz, C. (2013). Opposing effects of aminocoumarins and fluoroquinolones on the SOS response and adaptability in Staphylococcus aureus. *J Antimicrob Chemother, 68*(3), 529-538. doi:10.1093/jac/dks456

Sojka, L., Kouba, T., Barvik, I., Sanderova, H., Maderova, Z., Jonak, J., & Krasny, L. (2011). Rapid changes in gene expression: DNA determinants of promoter regulation by the concentration of the transcription initiating NTP in Bacillus subtilis. *Nucleic Acids Res, 39*(11), 4598-4611. doi:10.1093/nar/gkr032

Steinchen, W., & Bange, G. (2016). The magic dance of the alarmones (p)ppGpp. *Mol Microbiol, 101*(4), 531-544. doi:10.1111/mmi.13412

Steinchen, W., Schuhmacher, J. S., Altegoer, F., Fage, C. D., Srinivasan, V., Linne, U., . . . Bange, G. (2015). Catalytic mechanism and allosteric regulation of an oligomeric (p)ppGpp synthetase by an alarmone. *Proc Natl Acad Sci U S A, 112*(43), 13348-13353. doi:10.1073/pnas.1505271112

Steinchen, W., Vogt, M. S., Altegoer, F., Giammarinaro, P. I., Horvatek, P., Wolz, C., & Bange, G. (2018). Structural and mechanistic divergence of the small (p)ppGpp synthetases RelP and RelQ. *Sci Rep, 8*(1), 2195. doi:10.1038/s41598-018-20634-4

Sun, F., Ding, Y., Ji, Q., Liang, Z., Deng, X., Wong, C. C., . . . He, C. (2012). Protein cysteine phosphorylation of SarA/MgrA family transcriptional regulators mediates bacterial virulence and antibiotic resistance. *Proc Natl Acad Sci U S A, 109*(38), 15461-15466. doi:10.1073/pnas.1205952109

Surewaard, B. G., de Haas, C. J., Vervoort, F., Rigby, K. M., DeLeo, F. R., Otto, M., . . . Nijland, R. (2013). Staphylococcal alpha-phenol soluble modulins contribute to neutrophil lysis after phagocytosis. *Cell Microbiol, 15*(8), 1427-1437. doi:10.1111/cmi.12130

Wang, J., Tian, Y., Zhou, Z., Zhang, L., Zhang, W., Lin, M., & Chen, M. (2016). Identification and Functional Analysis of RelA/SpoT Homolog (RSH) Genes in Deinococcus radiodurans. *J Microbiol Biotechnol, 26*(12), 2106-2115. doi:10.4014/jmb.1601.01017

Wang, X., Thompson, C. D., Weidenmaier, C., & Lee, J. C. (2018). Release of Staphylococcus aureus extracellular vesicles and their application as a vaccine platform. *Nat Commun, 9*(1), 1379. doi:10.1038/s41467-018-03847-z

Wolz, C., Geiger, T., & Goerke, C. (2010). The synthesis and function of the alarmone (p)ppGpp in firmicutes. *Int J Med Microbiol, 300*(2-3), 142-147. doi:10.1016/j.ijmm.2009.08.017

Wu, J., & Xie, J. (2009). Magic spot: (p) ppGpp. *J Cell Physiol, 220*(2), 297-302. doi:10.1002/jcp.21797

Xu, T., Wang, X. Y., Cui, P., Zhang, Y. M., Zhang, W. H., & Zhang, Y. (2017). The Agr Quorum Sensing System Represses Persister Formation through Regulation of Phenol Soluble Modulins in Staphylococcus aureus. *Front Microbiol, 8*, 2189. doi:10.3389/fmicb.2017.02189

Yan, X., Zhao, C., Budin-Verneuil, A., Hartke, A., Rince, A., Gilmore, M. S., . . . Pichereau, V. (2009). The (p)ppGpp synthetase RelA contributes to stress adaptation and virulence in Enterococcus faecalis V583. *Microbiology, 155*(Pt 10), 3226-3237. doi:10.1099/mic.0.026146-0

Zapf, R. L., Wiemels, R. E., Keogh, R. A., Holzschu, D. L., Howell, K. M., Trzeciak, E., . . . Carroll, R. K. (2019). The Small RNA Teg41 Regulates Expression of the Alpha Phenol-Soluble Modulins and Is Required for Virulence in Staphylococcus aureus. *MBio, 10*(1). doi:10.1128/mBio.02484-18

Zhu, M., Pan, Y., & Dai, X. (2019). (p)ppGpp: the magic governor of bacterial growth economy. *Curr Genet*. doi:10.1007/s00294-019-00973-z

Zielinska, A. K., Beenken, K. E., Joo, H. S., Mrak, L. N., Griffin, L. M., Luong, T. T., . . . Smeltzer, M. S. (2011). Defining the strain-dependent impact of the Staphylococcal accessory regulator (sarA) on the alpha-toxin phenotype of Staphylococcus aureus. *J Bacteriol, 193*(12), 2948-2958. doi:10.1128/jb.01517-10

Acknowledgements

We thank Isabell Samp for excellent technical assistant, Ulrich Schoppmeier, Dominik Rejman. The work was supported by Grants from the Deutsche Forschungsgemeinschaft: TR34/B1 to C.W. and U.M., TRR261/A06 and SFB766/Z01 to C.M., SPP1879 to C. W. and Infrastructural funding from Cluster of Excellence EXC 2124 “Controlling Microbes to Fight Infections”.

Supplementary information

Table S1, S2 and raw data can be found on the disc CD “Reads & Excel files” provided on the last page of this thesis.

Table S3: Strains and plasmids

Strains	Description	Source/ Reference
E.Coli		
BL21	fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3= λ sBamHIo ΔEcoRI-B int:: (lacI::PlacUV5::T7 gene1) i21 Δnin5, competent for protein expression	NEB
S.aureus		
RN4220	Restriction deficient derivate of 8325-4, $rK^{-}mK^{+}$	(Kreiswirth et al., 1983)
Ne1193	Tnbursa::*sarA erm*	NARSA
NE99	Tnbursa::*fur erm*	NARSA
NE665	Tnbursa::*perR erm*	NARSA
HG001	RN1 derivate, rsbU repaired, tcaR	(Pohl et al., 2009) (Herbert et al., 2010)
HG001-86	Mutation in the synthetase domain of *rel*	(Geiger et al., 2010)
HG001-229-230	Mutation in the synthetase domain of *relP* and *relQ* ($\Delta relP_{syn}$ $\Delta relQ_{syn}$)	(Geiger et al., 2014)
HG001 229-230-263	Mutation in the synthetase domain of *relP* ,*relQ* and complete deletion of *rel* ($\Delta relP_{syn}$ $\Delta relQ_{syn}$ Δrel)	(Geiger et al., 2014)
HG001 *fur*	Tnbursa::*fur erm*	This work
HG001 229-230-263 *fur*	Mutation in the synthetase domain of *relP* ,*relQ* and complete deletion of *rel* ($\Delta relP_{syn}$ $\Delta relQ_{syn}$ Δrel) Tnbursa::*fur erm*	This work
HG001 *perR*	Tnbursa::*perR erm*	This work

HG001 229-230-263 *perR*	Mutation in the synthetase domain of *relP* ,*relQ* and complete deletion of *rel* ($\Delta relP_{syn}$ $\Delta relQ_{syn}$ Δrel) Tnbursa::*perR erm*	This work
HG001 *psmα psmβ*	*psmα1-4::tetM, psmβ1-2::ermC*	(Geiger et al., 2012)
HG001 229-230-263 *psmα psmβ*	Mutation in the synthetase domain of *relP* ,*relQ* and complete deletion of *rel* ($\Delta relP_{syn}$ $\Delta relQ_{syn}$ Δrel) *psmα1-4::tetM, psmβ1-2::ermC*	This work
USA300 JE2	USA300 derivative, cured of all plasmids	NARSA
USA300 JE2 229-230-263	Mutation in the synthetase domain of *relP* ,*relQ* and complete deletion of *rel* ($\Delta relP_{syn}$ $\Delta relQ_{syn}$ Δrel)	This work
Plasmids	**Description**	**Source/ Reference**
pET15b	Protein expression vector, ampicillin resistance	Novagen
pKOR1	ATc-inducible mutagenisis vector, chloramphenicol resistance	(Bae & Schneewind, 2006)
pCG248	anhydrotetracyclin (ATc) inducible vector, chloramphenicol resistance	(Helle et al., 2011) (Schroder, Goerke, & Wolz, 2013)
pCG258	relP cloned into pCG248	(Geiger et al., 2014)
pCG259	relQ cloned into pCG248	(Geiger et al., 2014)
pCG327	N-terminal domain of Rel with hydrolase mutated	(Gratani et al., 2018)
pCG229	pKOR1 with integrated, mutated *relP*	(Geiger et al., 2014)

pCG230	pKOR1 with integrated, mutated *relQ*	(Geiger et al., 2014)
pCG263	pKOR1 with integrated, mutated *rel*	(Geiger et al., 2014)
pCG551	N-terminal domain of Rel with hydrolase mutated cloned into pET15b	(Gratani et al., 2018)
pCG121	relP cloned into pET15b	(Geiger et al., 2014)
pCG122	relQ cloned into pET15b	(Geiger et al., 2014)

Table S4: Oligonucleotides

Purpose and Description	**Template**	**Name**	**Sequence**
verification of *relP* synthase mutant	USA300-229-230-263	relPDIG-for relPDIG-rev	GTCGCACATTCTTTCAGT CGTTATTAGGTTTCGTAGAGTT
verification of *relQ* synthase mutant	USA300-229-230-263	relQDIGfor2 relQDIGrev2	TTCGTAACACTAAAGAAAGTGG GCGTGTAATATTTTTGAGCT
verification of *rsh* mutant	USA300-229-230-263	rel431for relLC4rev	GCGTGGCTTTATCATTGG ACTTCAACCATCATTCGG
Verification of *perR* mutant	HG001 *perR* HG001 229-230-263 *perR*	perR-for TnUpstream	TGAACTAGAAGAATCAATTGCATCA CTCGATTCTATTAACAAGGG
Verification of *fur* mutant	HG001 *fur* HG001 229-230-263 *fur*	furtnfor TnBuster	GCACGTTTCACACACACCAT GCTTTTTCTAAATGTTTTTTAAGTAAATCAAGTAC
Verification of *sarA* mutant	HG001 *sarA*	sarAtnfor TnUpstream	GTTGTTTGCTTCAGTGATTCGT CTCGATTCTATTAACAAGGG

	HG001 229-230-263 *sarA*		
Verification of *psmα/β* mutant	HG001 229-230-263 *psm*		(Geiger et al., 2012)
Creation of dig-labeled probe *ftnA*	WT HG001	ftnADig-for ftnADig-rev	GAGTACTTTGCAGCACACGC CATTGCTGTCATCGCCGATAC
Creation of dig-labeled probe *dps*	WT HG001	mrgADig-for mrgADig-rev	GCTACACAATTTCCACTGGT CATACCTATAAACATATCTTC
Creation of dig-labeled probe *psm*/*agr*			(Geiger et al., 2012)

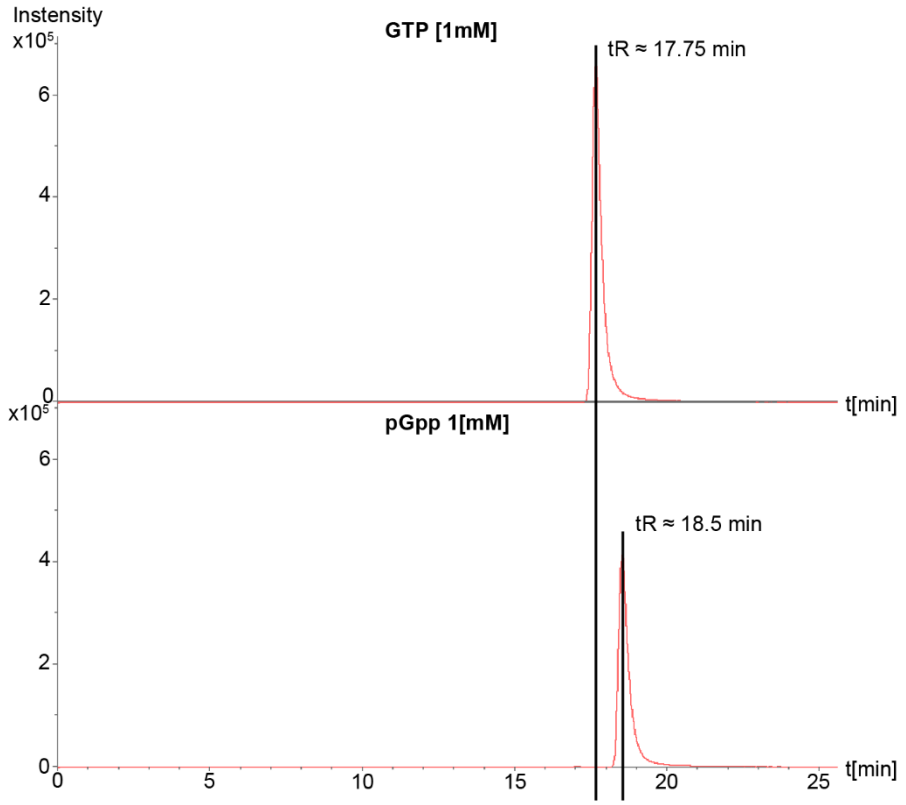

Fig. S1: Differentiation of GTP and pGpp via mass spectrometry. Separation of GTP and pGpp in a nucleotide mix is possible by the retention time. 1mM of purified GTP and pGpp were analyzed via mass spectrometry (ESI-TOF). Both nucleotides have a m/z of 521.98 but different retention times. GTP has a retention time tR of ≈ 17.75 min and pGpp a tR ≈ 18.5min.

7.2 Publication 2

Gratani FL, **Horvatek P**, Geiger T, Borisova M, Mayer C, Grin I, Wagner S, Steinchen W, Bange G, Velic A, Maček B, Wolz C. " Regulation of the opposing (p)ppGpp synthetase and hydrolase activities in a bifunctional RelA/SpoT homologue from Staphylococcus aureus." PLoS Genetics 2018 Jul 9;14(7):e1007514. doi: 10.1371/journal.pgen.1007514. eCollection 2018 Jul.

PLOS GENETICS

RESEARCH ARTICLE

Regulation of the opposing (p)ppGpp synthetase and hydrolase activities in a bifunctional RelA/SpoT homologue from *Staphylococcus aureus*

Fabio Lino Gratani[1], Petra Horvatek[1], Tobias Geiger[1¤], Marina Borisova[1], Christoph Mayer[1], Iwan Grin[1,2], Samuel Wagner[1,2], Wieland Steinchen[3], Gert Bange[3], Ana Velic[4], Boris Maček[4], Christiane Wolz[1]*

1 Interfaculty Institute of Microbiology and Infection Medicine, University of Tuebingen, Tuebingen, Germany, **2** German Centre for Infection Research, Partner Site Tuebingen, Tuebingen, Germany, **3** Center for Synthetic Microbiology (SYNMIKRO) & Dept. of Chemistry, Philipps-University, Marburg, Germany, **4** Quantitative Proteomics and Proteome Center Tuebingen, Tuebingen, Interfaculty Institute for Cell Biology, University of Tuebingen, Tuebingen, Germany

¤ Current address: Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven, CT, United States of America
* christiane.wolz@med.uni-tuebingen.de

OPEN ACCESS

Citation: Gratani FL, Horvatek P, Geiger T, Borisova M, Mayer C, Grin I, et al. (2018) Regulation of the opposing (p)ppGpp synthetase and hydrolase activities in a bifunctional RelA/SpoT homologue from *Staphylococcus aureus*. PLoS Genet 14(7): e1007514. https://doi.org/10.1371/journal.pgen.1007514

Editor: Danielle A. Garsin, The University of Texas Health Science Center at Houston, UNITED STATES

Received: March 7, 2018

Accepted: June 25, 2018

Published: July 9, 2018

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG), www.dfg.de: TR34/B1 to CW; DFG SFB766/A15, and GRK1708/B2 to CM; SFB766/Z02 to BM; SPP1879 to GB, SFB766 to SW. The funders had no role in the study design, data collection and

Abstract

The stringent response is characterized by (p)ppGpp synthesis resulting in repression of translation and reprogramming of the transcriptome. In *Staphylococcus aureus*, (p)ppGpp is synthesized by the long RSH (RelA/SpoT homolog) enzyme, Rel_{Sau} or by one of the two short synthetases (RelP, RelQ). RSH enzymes are characterized by an N-terminal enzymatic domain bearing distinct motifs for (p)ppGpp synthetase or hydrolase activity and a C-terminal regulatory domain (CTD) containing conserved motifs (TGS, DC and ACT). The intramolecular switch between synthetase and hydrolase activity of Rel_{Sau} is crucial for the adaption of *S. aureus* to stress (stringent) or non-stress (relaxed) conditions. We elucidated the role of the CTD in the enzymatic activities of Rel_{Sau}. Growth pattern, transcriptional analyses and *in vitro* assays yielded the following results: i) *in vivo*, under relaxed conditions, as well as *in vitro*, the CTD inhibits synthetase activity but is not required for hydrolase activity; ii) under stringent conditions, the CTD is essential for (p)ppGpp synthesis; iii) Rel_{Sau} lacking the CTD exhibits net hydrolase activity when expressed in *S. aureus* but net (p)ppGpp synthetase activity when expressed in *E. coli*; iv) the TGS and DC motifs within the CTD are required for correct stringent response, whereas the ACT motif is dispensable, v) Co-immunoprecipitation indicated that the CTD interacts with the ribosome, which is largely dependent on the TGS motif. In conclusion, Rel_{Sau} primarily exists in a synthetase-OFF/hydrolase-ON state, the TGS motif within the CTD is required to activate (p)ppGpp synthesis under stringent conditions.

analysis, decision to publish, or preparation of manuscript.

Competing interests: The authors have declared that no competing interests exist.

Author summary

The stringent response is a general stress response, which allows bacteria to survive nutrient limited conditions and to better tolerate antibiotic treatment. In the human pathogen, *Staphylococcus aureus*, the stringent response plays an important role for virulence, phagosomal escape and antibiotic tolerance. The response is initiated by the synthesis of the nucleotide derivative (p)ppGpp which in turn leads to growth arrest and reprogramming of gene expression. However, a rapid and controlled inactivation of these growth inhibitory molecules is equally important for the organism. (p)ppGpp synthesis as well as hydrolysis is accomplished by a bi-functional RelA/SpoT homolog, Rel_{Sau} bearing distinct synthetase, hydrolase and sensory domains. We elucidated how the C-terminal sensory domain of Rel_{Sau} controls the intermolecular switch between hydrolase and synthetase activities in *S. aureus*. The switch is crucial for the appropriate response of *S. aureus* to adapt to changing environment encountered during infection.

Introduction

Bacteria react to nutrient limitation via a stress response that is characterized by the synthesis of pyrophosphorylated GTP (pppGpp) or GDP (ppGpp) (previously reviewed in [1,2,3,4,5,6,7,8,9,10,11]). Synthesis of (p)ppGpp, induced under these stress conditions (stringent conditions), results in many physiological changes, including inhibition of rRNA synthesis, replication and translation but also activation or repression of various genes. In many pathogenic bacteria, (p)ppGpp influences virulence, persistence and host interaction (see reviews [9,10]).

(p)ppGpp is synthesized by cytoplasmic enzymes that contain a conserved synthetase domain. RelA of *Escherichia coli* was the first such enzyme described and has been shown to synthesize (p)ppGpp under conditions of amino acid limitation [12]. *E. coli* and many other gram-negative bacteria possess an additional enzyme, SpoT, that possesses (p)ppGpp synthetase and hydrolase activities. The (p)ppGpp synthetase activity of SpoT is stimulated by various conditions, e.g. fatty acid deprivation [13,14]. In Firmicutes, homologous enzymes (Rel) constitute a distinct class of (p)ppGpp synthetases [11,15,16]. RelA, SpoT and Rel enzymes all belong to RSH (for RelA/SpoT homolog) superfamily [15,17]. Similar to SpoT, the Rel enzymes from Firmicutes are bifunctional proteins with (p)ppGpp synthetase and hydrolase activities; however, similar to RelA, the synthetase activity of these enzymes is stimulated upon amino acid starvation [18,19]. RSH enzymes share a multi-domain architecture with a C-terminal regulatory domain (CTD) and an N-terminal enzymatic domain (NTD) containing synthetase and hydrolase motifs. The only available crystal structure of an RSH enzyme is that of the NTD of Rel from *Streptococcus equisimilis* [20]. The structure indicates two conformations of the enzyme, corresponding to the reciprocal active states of the enzyme: (p)ppGpp-synthetase-ON/hydrolase-OFF (stringent) and synthetase-OFF/hydrolase-ON (relaxed). It has been proposed that the CTD is involved in reciprocal regulation of the enzymatic states. The current model suggests that under non-stringent (relaxed) conditions, the interaction of the CTD with the NTD maintains the enzyme in the synthetase-OFF/hydrolase-ON conformation [21,22,23] The CTD of RelA stimulates (p)ppGpp synthesis in a ribosome-dependent manner when uncharged tRNA, as a consequence of amino acid limitation, is located in the ribosomal A-site [24,25]. Interestingly, Rel from *S. equisimilis* is responsive to amino acid starvation only within its native genetic background and not when expressed in *E. coli* [13,21]. Bioinformatic analyses have revealed the presence of three conserved motifs within the CTDs of RSHs: TGS, ACT and DC. The TGS motif (named after the presence in ThrRS, GTPases, and SpoT) was shown to be

responsible for the interaction of SpoT with the acyl-carrier protein. The ACT motif (named after three of the allosterically regulated enzymes in which this domain is found: aspartate kinase, chorismate mutase and TyrA) was proposed to be a conserved regulatory ligand-binding fold [26,27]. Recently, major insights into the ribosome-RelA structure were provided by cryo-EM analyses [28,29,30]. The structures revealed that RelA adopts an open conformation in which the CTD is intertwined around an A-site tRNA within the intersubunit cavity of the ribosome, and the NTD extends into the solvent. The structures support a model in which association of monomeric RelA with the ribosome relieves the autoinhibitory effect of the CTD on the NTD. It was hypothesized that autoinhibition in the unbound state is mediated by oligomerization of RelA. Oligomerization was previously demonstrated to occur via a conserved aspartate-cysteine motif (DC) in the CTD [31,32,33]. Interaction of monomeric RelA with the ribosome and putative RelA oligomerization in the unbound state indicate that the switching of enzymatic activities occurs via a complex mechanism that has not yet been elucidated.

In the human pathogen *Staphylococcus aureus*, the stringent response plays an important role in virulence [18], phagosomal escape [34] and antibiotic tolerance [35]. In *S. aureus*, in addition to Rel_{Sau}, two enzymes with (p)ppGpp synthetase activity (RelP and RelQ) are present. These enzymes form homotetramers that lack the CTD and the hydrolase domain [36,37,38] and are transcriptionally induced under conditions of cell-wall stress [35]. The basal (p)ppGpp level produced by these enzymes is controlled by the hydrolase activity of Rel_{Sau} [35]. The phenotypic consequences of (p)ppGpp accumulation vary among species and can be mediated by different mechanisms. In *S. aureus*, as in other Firmicutes, (p)ppGpp regulates transcription by an indirect mechanism that strongly relies on the lowering of intracellular GTP levels [39,40,41]. Low GTP levels lead to de-repression of the CodY regulon. CodY, when loaded with GTP and branched-chain amino acids, acts as a repressor of a variety of genes, e.g., genes involved in amino acid synthesis and virulence [41] A decrease in GTP levels could also lead to the repression of sensitive GTP-initiating promoters (e.g., those of stable RNA genes) [42,43]. All these studies illustrate the complex role of (p)ppGpp during the bacterial life cycle. The cellular concentration of (p)ppGpp has to be tightly regulated not only to support survival under stressed conditions but also to avoid toxicity under relaxed conditions. The molecular switch between the synthetase and hydrolase activities of Rel_{Sau} is crucial for the maintenance of this balance.

Here, we aim to elucidate the role of the CTD in controlling the activity of Rel of the major human pathogen *S. aureus* (Rel_{Sau}) *in vivo*. We show that the (p)ppGpp synthetase activity is restricted in *S. aureus* and that the synthetase is activated only upon interaction of the CTD with ribosomal partners under stringent conditions. The TGS and DC motifs within the CTD are essential for the enzymatic switch to the synthetase-ON state and play a major role in the interaction between Rel_{Sau} and the translational apparatus.

Results

CTD-deleted Rel_{Sau} is in a synthetase-OFF/hydrolase-ON state in its native *S. aureus* background

We aimed to analyze the role of the CTD of Rel_{Sau} in the stringent response in *S. aureus*. In Rel_{Sau} the canonical domains and motifs could be identified through alignment with RelA and SpoT from *E. coli* (Fig 1A). We first established a readout system for (p)ppGpp activity. To this end, we analyzed strain HG001 (wild type) as well as an isogenic mutant of this strain that carries mutations in all three (p)ppGpp enzymes (full deletion of *rel*, synthetase mutation in *relP* and *relQ*) and thus is unable to synthesize pppGpp (designated $(p)ppGpp^0$) [35]. The mutant

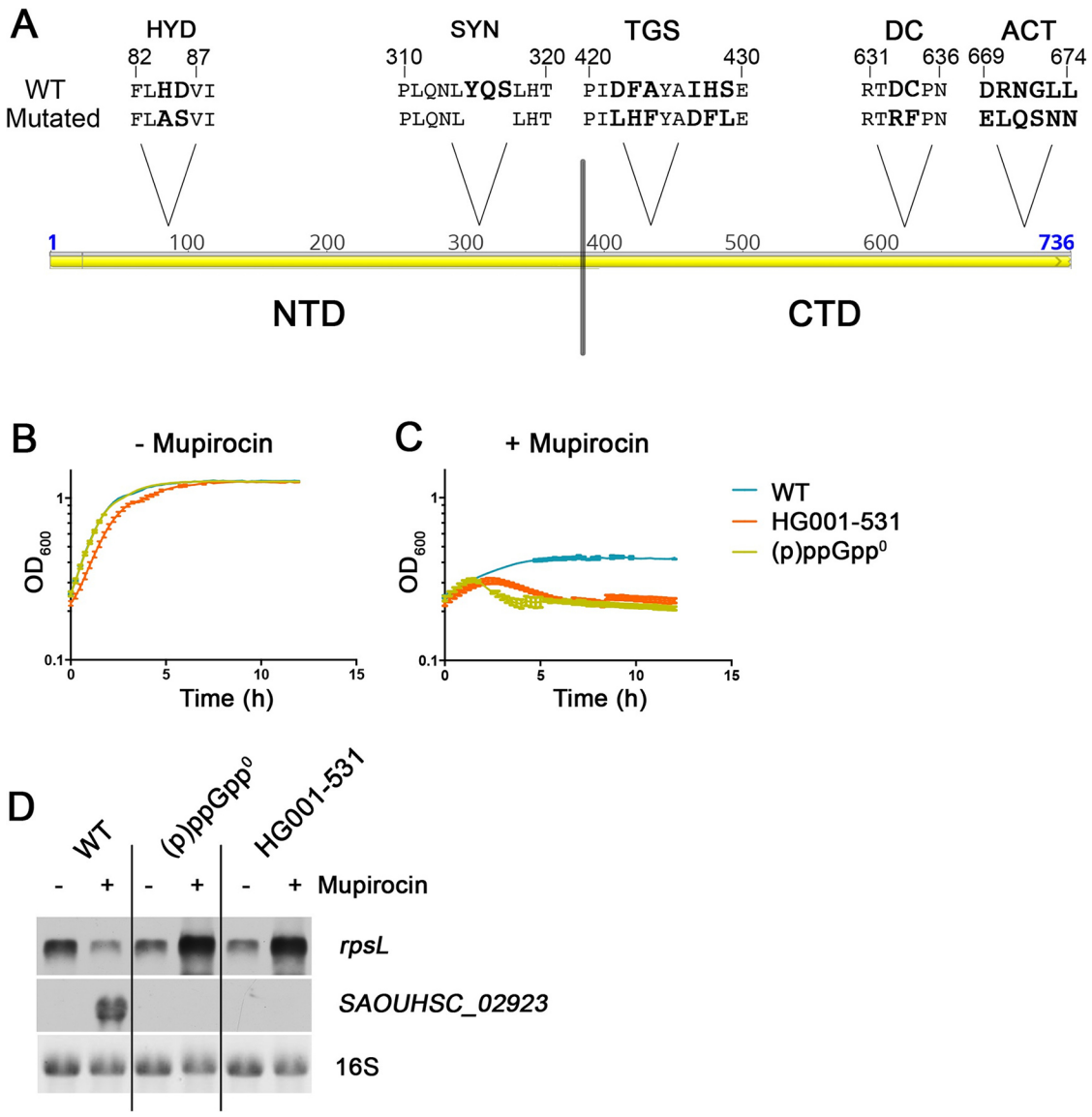

Fig 1. Experimental set-up for synthetase activity in *S. aureus*. (A) The molecular architecture of Rel_{Sau} including conserved and mutated motifs. *S. aureus* strains HG001 wild type (WT), (p)ppGpp^0 and HG001-531 (*rel* CTD deleted) were grown in rich medium to OD_{600} = 0.3 and split in cultures with and without 0.3 μg/ml mupirocin. Growth was monitored after reaching OD_{600} = 0.3 without (B) or with mupirocin (C).For Northern analysis (D) RNA was isolated from bacteria 30 minutes after reaching OD_{600} = 0.3 and hybridized with digoxigenin-labelled probes specific for gene encoding ribosomal protein RpsL and the CodY target gene SAOUHSC_02923. The 16S rRNA detected in the ethidium bromide-stained gels is indicated as loading control in the bottom lane.

https://doi.org/10.1371/journal.pgen.1007514.g001

exhibits no phenotypic difference compared to the wild type when grown in rich medium (Fig 1B). The stringent response can be evoked by mupirocin, an inhibitor of isoleucyl-tRNA synthetase [18,34]. (p)ppGpp synthesis results in higher tolerance towards mupirocin. The (p)ppGpp^0 strain exhibited a typical decline in OD_{600} when treated with mupirocin (Fig 1C). Furthermore, synthesis of (p)ppGpp results in repression of genes coding for ribosomal proteins (e.g., *rpsL*) and de-repression of the CodY target genes (e.g., SAOUHSC_02923, a putative amino acid transporter) (Fig 1D). Therefore, we used the enhanced mupirocin tolerance and typical transcription pattern (*rpsL* down, SAOUHSC_02923 up) as a readout for (p)ppGpp synthesis in *S. aureus*. As a first approach, we deleted the CTD of the wild-type Rel_{Sau}

(SAOUHSC_01742) to generate strain HG001-531. In contrast to a full-length *rel* deletion (Geiger et al., 2014a), truncation of the CTD had only a slight effect on growth (Fig 1C). It has been previously shown that Rel_{Sau} is essential due to its hydrolase function [35]. Thus, the CTD does not seem to impede the hydrolase activity *in vivo*. Northern blot analysis revealed that the CTD mutant was unable to elicit a mupirocin-induced stringent response (Fig 1B). The transcriptional pattern of the marker genes *rpsL* and SAOUHSC_02923 as well as the mupirocin tolerance (Fig 1D) of the CTD mutant were indistinguishable from those of the (p)$ppGpp^0$ strain. This finding is consistent with the general assumption that the CTD is required for sensing amino acid deprivation. However, hydrolase activity seems to be hardly effected by the CTD.

Next, we complemented the (p)$ppGpp^0$ strain with anhydrotetracycline (ATc)-inducible full-length and truncated *rel* constructs (Fig 2A) and analyzed the effects under relaxed growth conditions (exponential growth phase in nutrient-rich medium). As a positive control, we induced *relQ* expression. RelQ is a small synthetase without a regulatory CTD and thus can activate the stringent response in a (p)$ppGpp^0$ mutant by transcriptional induction alone. This activation was demonstrated by downregulation of *rpsL* and upregulation of SAOUHSC_02923 (Fig 2B) and by the immediate growth arrest after *relQ* induction (Fig 2C).

In contrast to *relQ*, transcriptional induction of full-length *rel* showed no effect on the transcription of marker genes or on growth (Fig 2B and 2D). This finding confirms that additional post-transcriptional activation is required to activate the synthetase activity. Induction of a construct lacking the CTD also failed to induce the stringent response phenotype (Fig 2B and 2D). At first glance, these results may indicate that under relaxed conditions, the enzymatic domain of Rel_{Sau}, with or without the CTD, is tightly held in a synthetase-OFF conformation. Alternatively, the hydrolase might be hyperactive, so any (p)ppGpp synthesized would be immediately degraded. To test this hypothesis, we mutated the hydrolase domain in full-length and CTD-deleted *rel* constructs. Indeed, both full-length and truncated *rel* lacking the hydrolase domain elicited a stringent response pattern similar to that of the wild type, as indicated by transcriptional and growth analyses (Fig 2B and 2D). Thus, we presume that there might be some synthetase activity under relaxed growth conditions. However, due to hydrolase activity, any (p)ppGpp present is efficiently degraded under these conditions.

CTD of Rel_{Sau} does not impact hydrolase activity *in vivo*

To analyze the hydrolase activity of Rel_{Sau} *in vivo*, we used a conditional *rel* mutant strain (HG001-55) [18] in which genomic *rel* was placed under an IPTG-inducible promoter complemented with different rel constructs (Fig 3A). Without IPTG, the *rel* mutant is unable to grow (Fig 3B) because it cannot degrade the (p)ppGpp synthesized by RelP and RelQ [35]. We introduced ATc-inducible full-length or truncated *rel* into the conditional *rel* mutant and monitored growth after ATc induction. As expected, constructs with mutated hydrolase could not rescue the growth defect of HG001-55 (Fig 3C). However, full-length and CTD-truncated *rel*, with intact hydrolase, fully complemented the growth defect of the conditional *rel* mutant. These results show that the hydrolase was constitutively active, independent of the presence of the CTD. In summary, the data indicate that under relaxed conditions, the wild-type Rel_{Sau} enzyme, with or without sensory domain, is tightly held in the hydrolase-ON state.

Role of the CTD in the enzymatic activity of Rel_{Sau} *in vitro*

To confirm the data from the *in vivo* experiments under relaxed conditions, full-length or CTD-truncated Rel_{Sau} proteins (with or without hydrolase domains) were purified and tested *in vitro* for enzymatic activities (Fig 4A and 4B). In the synthetase reaction, pyrophosphate is

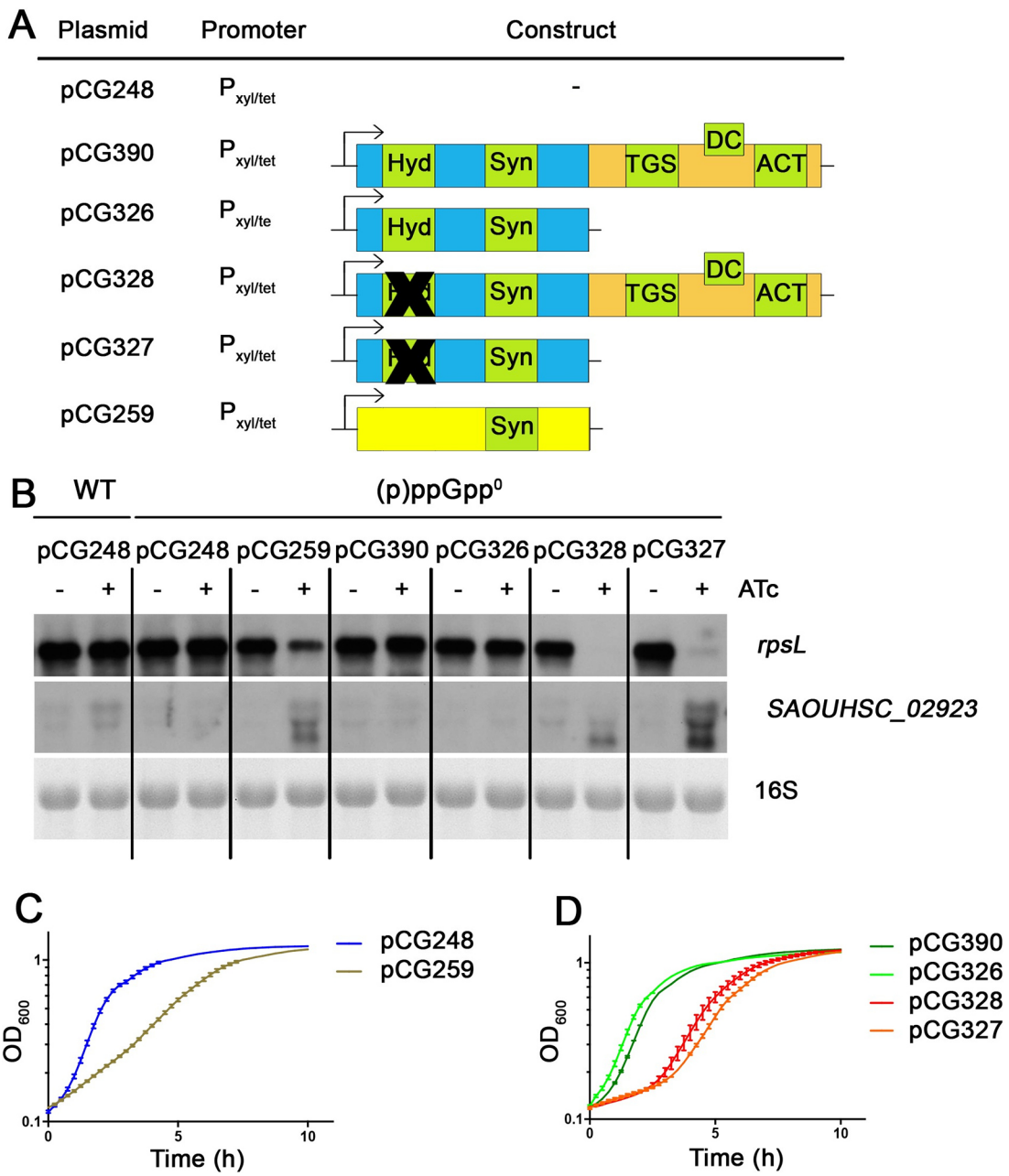

Fig 2. Influence of the CTD on the synthetase activity *in vivo*. ATc inducible *rel* with and without CTD or hydrolase domain and *relQ* (A) were expressed in the (p)ppGpp^0 mutant and compared to WT HG001. The black crosses (A) represent mutations as indicated in Fig 1A. Strains were grown in rich medium to OD_{600} = 0.3 and then split in cultures with and without 0.1 μg/ml ATc. For Northern analysis (B) RNA was isolated from bacteria 30 minutes after reaching OD_{600} = 0.3 and hybridized with digoxigenin-labelled probes. The 16S rRNA detected in the ethidium bromide-stained gels is indicated as loading control in the bottom lane. *rpsL* and SAOUHSC_02923 are control markers for the induction by (p)ppGpp Growth was monitored after addition of 0.1 μg/ml ATc. The (p)ppGpp^0 was complemented with different plasmids: empty vector (pCG248) and *relQ* (pCG259), as controls (C), and different *rel* constructs (D).

https://doi.org/10.1371/journal.pgen.1007514.g002

transferred from ATP to GTP, yielding AMP and pppGpp. The presence of both products was measured by HPLC-MS. AMP production was detectable with all constructs (Fig 4C); however, the AMP levels were significantly higher for the constructs that lacked the CTD (Fig 4C),

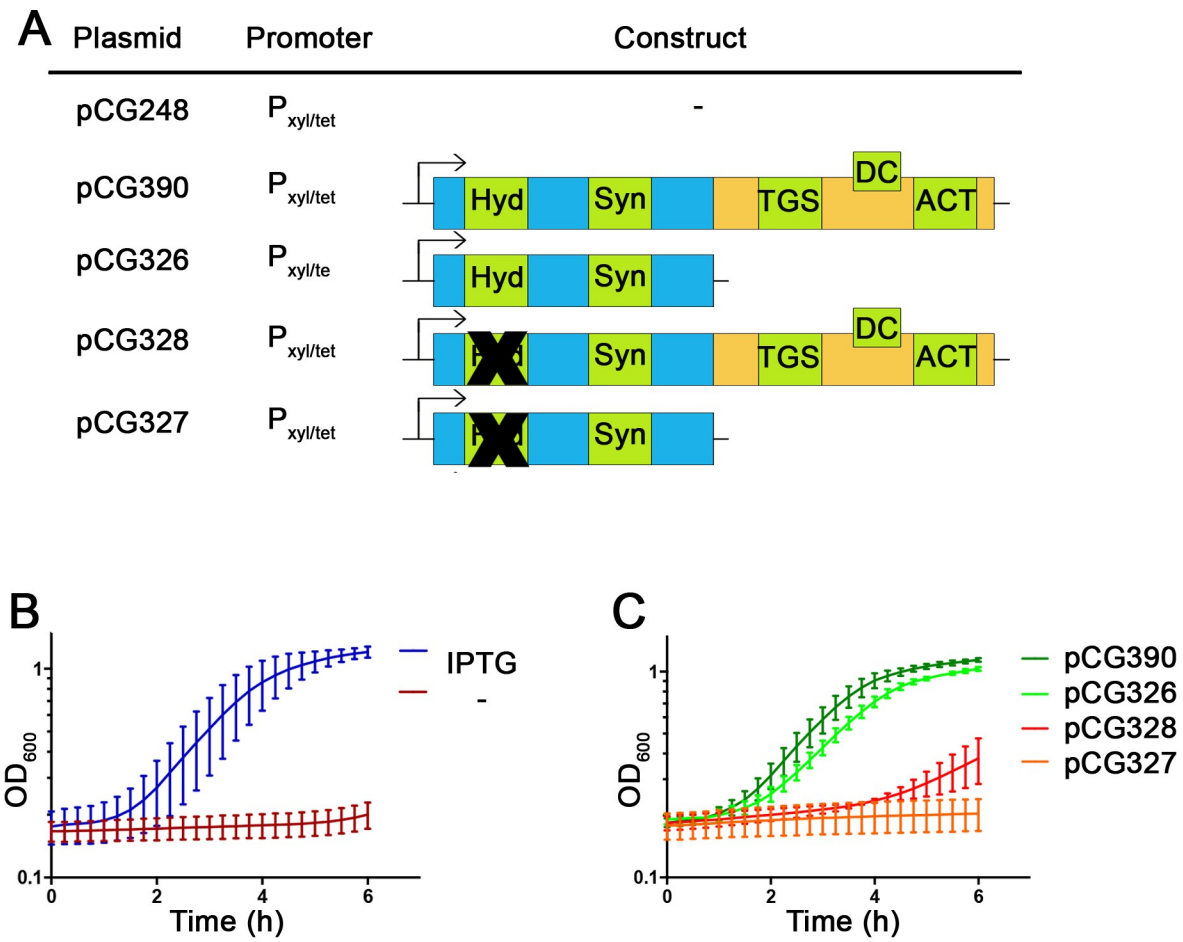

Fig 3. CTD of Rel_{Sau} has no impact on hydrolase activity *in vivo*. The conditional *rel* mutant HG001-55 (inducible by IPTG) was complemented with ATc inducible *rel* constructs (A). Strains were grown during preculture in presence of IPTG (0.5 mM) and were diluted to an initial OD_{600} = 0.1 and growth monitored over time. As control, strain complemented with empty vector was grown with and without IPTG to illustrate the essentially of the Rel hydrolase (B). *Rel* constructs were grown without IPTG but addition of ATc (0.1 μg/ml) (C).

https://doi.org/10.1371/journal.pgen.1007514.g003

indicating that the CTD negatively interferes with synthetase activity. Interestingly, pppGpp production was not detected for constructs with intact hydrolase (Fig 4D). However, proteins with mutated hydrolases synthesized detectable amounts of (p)ppGpp. The enzyme lacking the CTD showed slightly higher pppGpp synthetase activity than the full-length Rel_{Sau} supporting the inhibitory effect of the CTD on the synthetase domain. Thus, Rel_{Sau} exhibits strong hydrolase activity, which prevents pppGpp accumulation. This finding was confirmed by the rapid degradation of pppGpp (Fig 4E) and ppGpp (Fig 4F) by full-length and CTD deleted Rel_{Sau}. Notably, Rel_{Sau} preferentially degraded ppGpp over pppGpp. The CTD apparently has a minor impact on hydrolase activity.

Role of the CTD of Rel_{Sau} in *E. coli*

Our analysis of Rel_{Sau} in its native background seemed to be inconsistent with the results of previous studies, in which different CTD-deleted enzymes from other organisms were expressed in *E. coli* [20,21,44,45]. These studies indicated that RSH enzymes that lack CTDs are in a synthetase-ON/hydrolase-OFF state. Thus, based on these studies, we also expressed

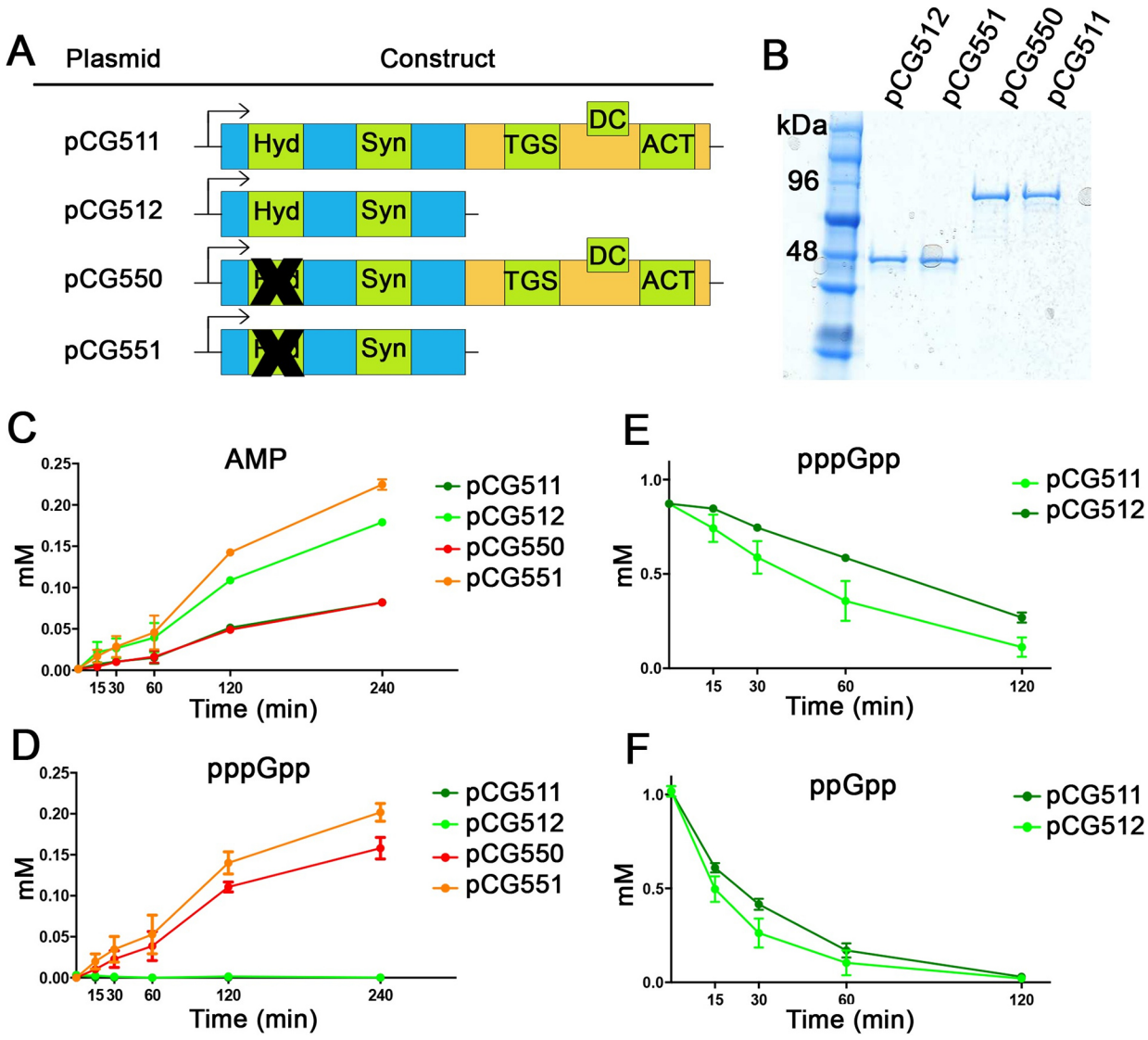

Fig 4. Role of the CTD on the enzymatic activity *in vitro*. 2 μM of purified proteins with and without CTD or hydrolase domain (A) were loaded on SDS-PAGE, Rel_{Sau} (85 KDa) and CTD-deleted (48KDa) (B) and used to assay synthetase (C, D) and hydrolase activity (E, F). The black crosses (A) represent mutations as indicated in Fig 1A. Synthetase activity was determined in the presence of the two substrates ATP and GTP and 2 μM of enzymes. The reaction products AMP (C) and pppGpp (D) were monitored over time at 37˚C by HPLC-MS. Hydrolase activity was assayed in the presence of pppGpp (E) or ppGpp (F) and 0.1 μM of enzymes. Decrease of 1 mM of pppGpp or ppGpp was monitored for 120 minutes at 37˚C.

https://doi.org/10.1371/journal.pgen.1007514.g004

full-length and CTD-deleted *rel* in *E. coli* using an arabinose-inducible promoter. We tested the capacity of different *rel* constructs (Fig 5A) to complement the defective phenotype of MG1655, a *relA/spoT* mutant, under stringent conditions (Fig 5B and 5C). Full-length and CTD-deleted Rel_{Sau} were able to complement the *relA/spoT* mutation. The complementation could be attributed to (p)ppGpp synthetase activity: mutation within the synthetase domain abolished complementation, whereas mutation within the hydrolase domain did not affect the complementation assay. Thus, in *E. coli*, CTD-deleted Rel_{Sau}, similar to other RSH enzymes, is predominantly in a synthetase-ON/hydrolase-OFF state, whereas in *S. aureus*, this enzyme is primarily in a hydrolase-ON state.

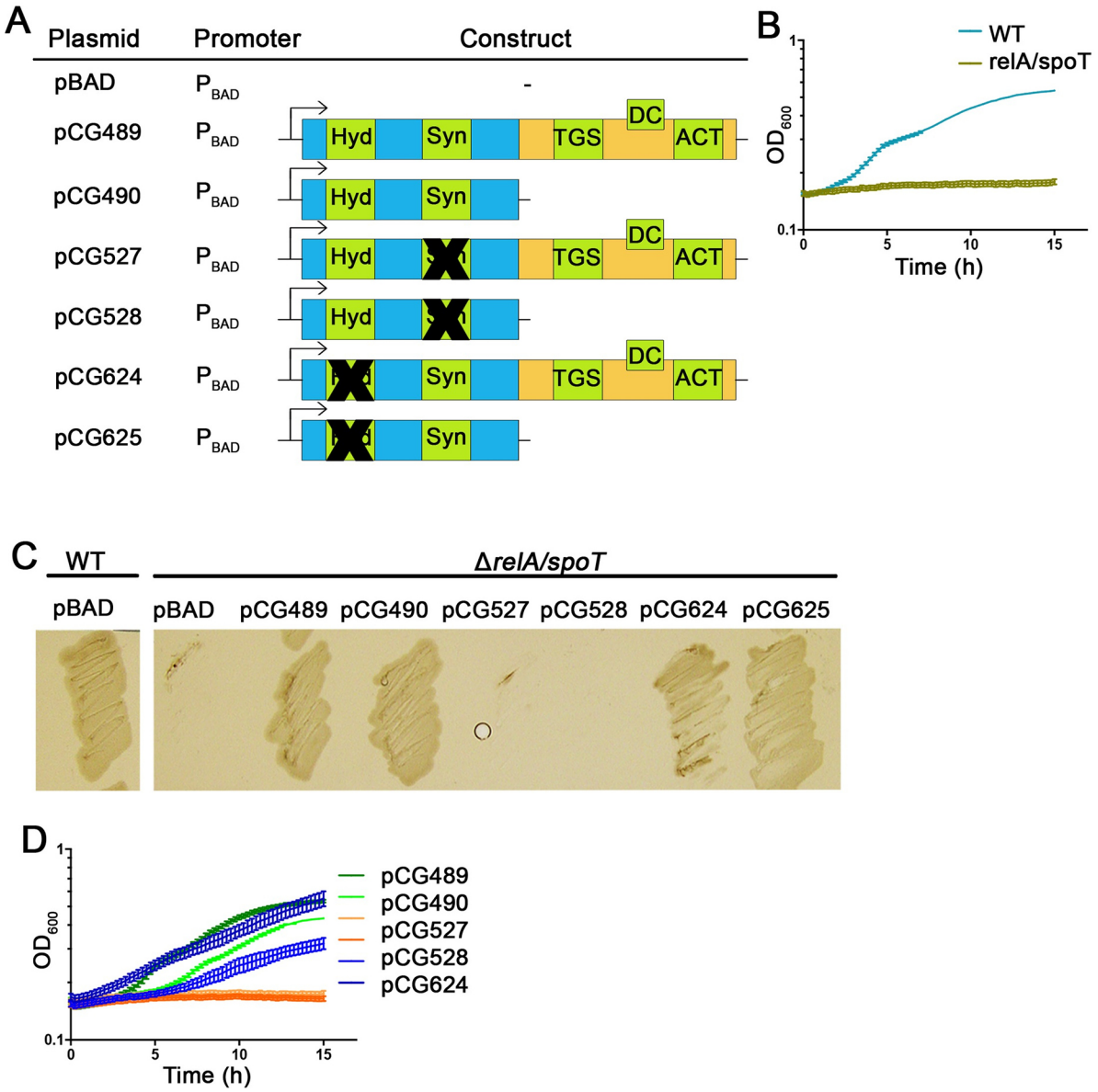

Fig 5. Role of CTD of Rel$_{Sau}$ in *E. coli*. *rel* from *S. aureus* with and without CTD, hydrolase or synthetase domain (A) were expressed in *relA*/*spoT* mutant of *E. coli* MG1655. The black crosses (A) represent mutations as indicated in Fig 1A. As controls WT and *relA*/*spoT* mutant were transformed with empty vector (B and C). Strains were grown under stringent conditions streaked on M9 agar plates (C) or M9 medium for growth analysis (D). Under these conditions the *relA*/*spoT* mutant was unable to grow.

https://doi.org/10.1371/journal.pgen.1007514.g005

Role of CTD motifs for sensing *in vivo*

Within the CTDs of RSHs, several conserved motifs can be identified. The conserved TGS, DC and ACT motifs of Rel$_{Sau}$ were predicted based on sequence alignments, and the critical residues of these motifs were mutated (Fig 1A). Wild-type and CTD-mutated *rel* were cloned to be under the control of the native *rel* promoter and introduced into the (p)ppGpp^0 strain (Fig 6A). The stringent response upon mupirocin treatment was analyzed by Northern blotting and growth analysis (Fig 6B and 6C). A (p)ppGpp^0 strain containing the empty vector showed the typical decrease in OD_{600} after mupirocin treatment. Induction of full-length *rel* in the (p)

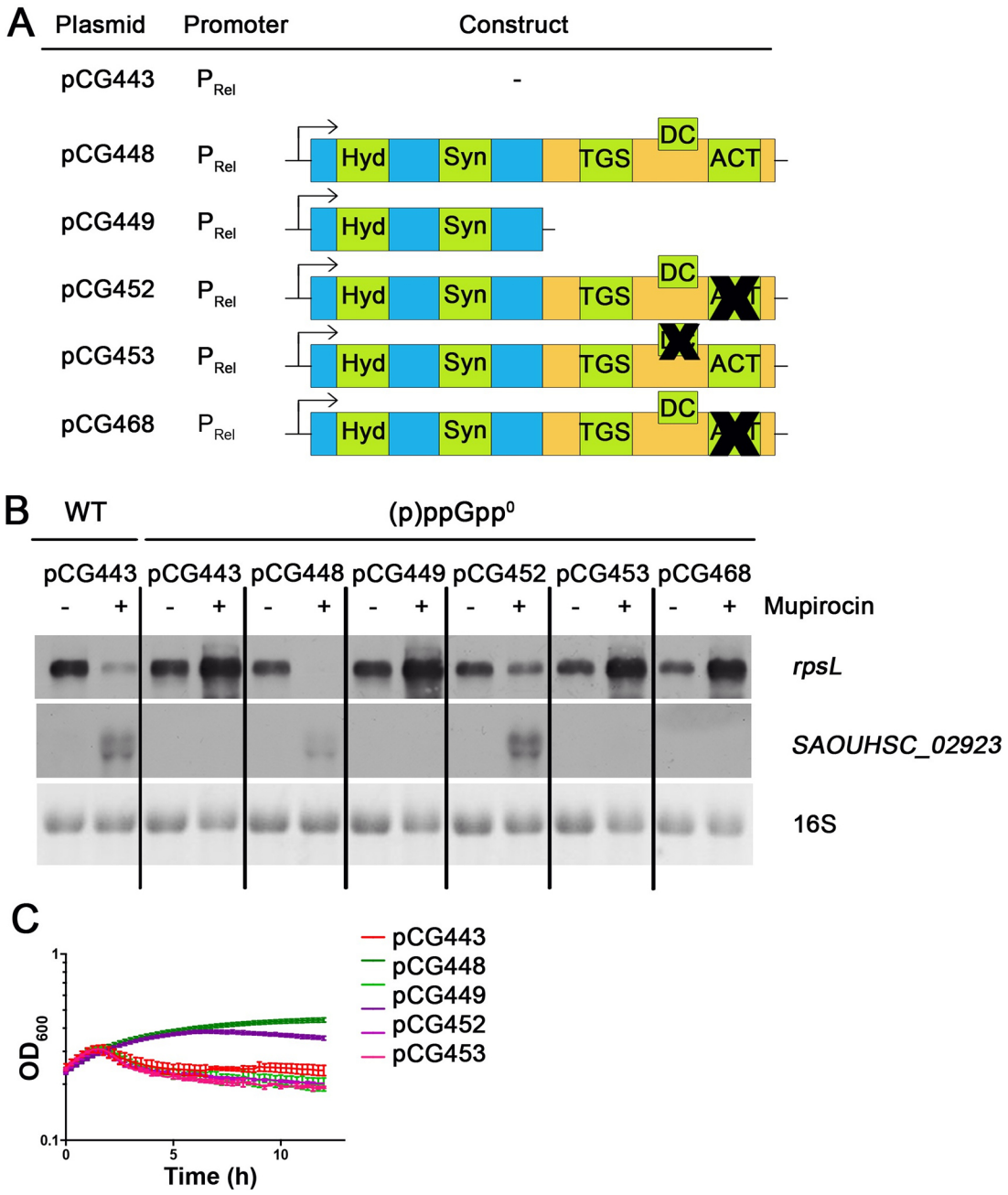

Fig 6. Role of CTD motifs for sensing of Rel$_{Sau}$ *in vivo*. (p)ppGpp^0 mutant was complemented with different *rel* constructs expressed from the native promoter (A). The black crosses (A) represent mutations as indicated in Fig 1A. Strains were grown in rich medium to OD_{600} = 0.3 and then split in cultures with and without 0.3 μg/ml mupirocin. For Northern analysis (B) RNA was isolated from cells 30 minutes after reaching OD_{600} = 0.3 and hybridized with digoxigenin-labeled probes. The 16S rRNA detected in the ethidium bromide-stained gels is indicated as loading control in the bottom lane. Growth was monitored after reaching OD_{600} = 0.3 after which 0.3. μg/ml mupirocin was added (C).

https://doi.org/10.1371/journal.pgen.1007514.g006

ppGpp^0 mutant fully complemented the mutant phenotype, whereas the CTD-deleted *rel* was unable to do so. Mutation of the ACT motif resulted in slightly impaired complementation. However, mutation of the TGS or DC motif resulted in complete inactivation of the stringent response. Expression of these mutated *rel* genes resulted in a phenotype that was not

distinguishable from the phenotype of the (p)ppGpp^0 strain in terms of growth and gene expression pattern. Thus, the TGS and DC motifs are required for stringent response, while the ACT motif plays only a minor role.

Co-immunoprecipitation (Co-IP) of Rel$_{Sau}$

We aimed to analyze the role of the conserved motifs within the CTD for interaction with cytosolic proteins. Therefore, we performed Co-IP experiments using whole-cell lysates of (p)ppGpp^0 mutants expressing wild-type or mutated (ACT, DC and TGS see Fig 1A) versions of Rel$_{Sau}$. For each pull-down experiment, the wild-type or mutant Rel$_{Sau}$ was the most abundant protein detected, with no significant difference observed between wild type and mutant proteins (Data S1 Dataset) and the expression of all proteins was similar as shown by Western blot analysis (Blot in S1 Fig). Mainly ribosomal proteins were co-immuno-precipitated with native Rel$_{Sau}$. When Rel$_{Sau}$ with mutated TGS motif was used as bait significant less proteins were enriched (Fig 7 first column). Most of these putative TGS interacting proteins were also found to be effected when Rel$_{Sau}$ harboring mutations in ACT or DC motifs were used, although to a lesser extent. Immuno-precipitated proteins that were strongly influenced by the TGS mutation are ribosomal proteins, proteins associated with RNA degradation and proteins involved in DNA-related pathways. In summary, the results indicate that all three motifs within the CTD work together to dock Rel$_{Sau}$ onto the translational apparatus. The strongest interaction is mediated by TGS, whereas the ACT motif seems to have a low impact. The TGS motif seems to mediate also interaction with non-ribosomal proteins.

Discussion

RSH enzymes are major players in the synthesis and hydrolysis of the second messenger (p)ppGpp. There is still limited information about the molecular switch that regulates the two activities, both present in long RSH enzymes. Here, we analyzed how the CTD of Rel$_{Sau}$ influences the enzymatic activities *in vivo*. We showed that Rel$_{Sau}$ exists primarily in a synthetase-OFF/hydrolase-ON conformation. Only under stringent growth conditions was the switching to the synthetase-ON conformation detectable, and this switching occurred only when the CTD possessed intact TGS and DC motifs.

In *S. aureus* and probably in other Firmicutes, Rel combines the functions of the two prototypic RSH enzymes, RelA and SpoT, from Proteobacteria. The synthetase activity is needed to elicit a stringent response phenotype, presumably via interaction with ribosomes and uncharged tRNA, as previously shown for RelA [24,25]. However, similar to SpoT, Rel$_{Sau}$ also possesses strong hydrolase activity, which is necessary to counteract the (p)ppGpp production by the small synthetases RelP and RelQ present in Firmicutes. The equilibrium between these two activities needs to be tightly regulated in order to attain an appropriate level of (p)ppGpp based on the growth conditions. So far, potential differences between RelA, SpoT and Rel associated with the molecular switch could not be inferred from the sequence or *in vitro* analyses. *In vivo* activities of different RSH enzymes were mainly analyzed by heterologous expression in *E. coli*. These analyses indicated that without CTDs, RSH enzymes possess strong synthetase activity [21,44,45]. Similarly, Rel$_{Sau}$, with or without the CTD, can complement an *E. coli relA/spoT* mutant, also indicating that (p)ppGpp synthesis can occur with or without the CTD. However, analysis of the same construct in the native background clearly showed that Rel$_{Sau}$ lacking the CTD is tightly held in the hydrolase-ON state, and synthetase activity is detectable only in constructs that lack the hydrolase. Thus, Rel$_{Sau}$, with or without the CTD, exhibits net (p)ppGpp synthetase activity when expressed in *E. coli* but net hydrolase activity when expressed in *S. aureus*. It would be interesting to see whether enzymes from other organisms

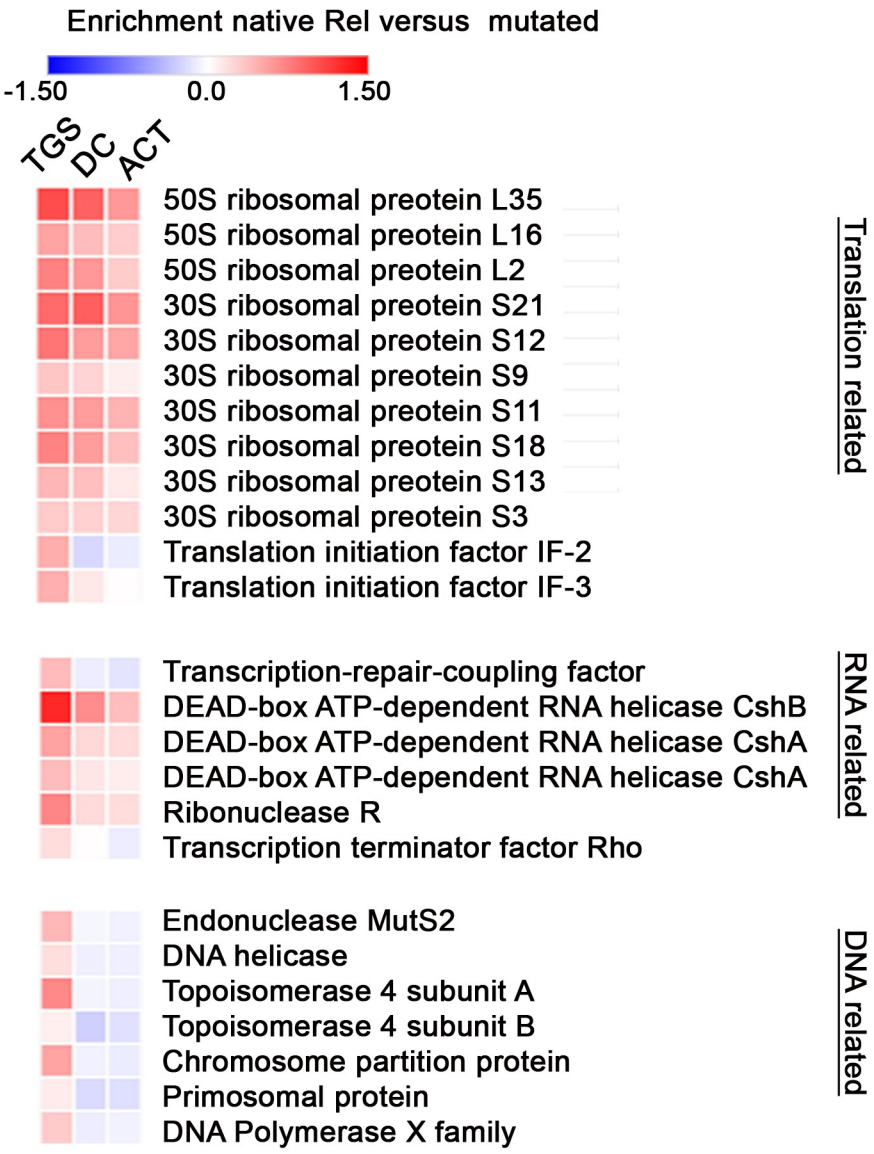

Fig 7. Influence of the CTD motifs on Rel_{Sau} interactions. *S. aureus* (p)ppGpp^0 was complemented with native and mutated Rel_{Sau}. Cell lysates were mixed with magnetic beads, coated with Rel_{Sau} antibodies and enriched proteins identified by MS. Proteins significantly (t-test difference > 0.05) less abundant using TGS mutated versus native Rel_{Sau} as bait are shown in the first column. The effects of the DC or ACT mutation on interaction with these proteins are shown in the second and third column, respectively.

https://doi.org/10.1371/journal.pgen.1007514.g007

show a similar discrepancy between *E. coli* and native backgrounds. Our results indicated that the enzymatic activity of Rel_{Sau} is influenced by species-specific interactions of the enzymatic NTD with unknown factors. To date, there is no evidence that the NTD alone interacts with the ribosome. Thus, other interaction partners or intracellular properties of the NTD should be elucidated in the future. An alternative possibility is that less (p)ppGpp is needed to complement the phenotype of a pppGpp mutant in *E. coli* allowing growth even if Rel_{Sau} has a weak synthetase. However, this is not supported by our in vitro results, showing that synthetase activity is only detectable when the hydrolase is mutated in constructs with or without CTD. Analysis of full-length or truncated Rel_{Sau} *in vitro* largely confirmed the results obtained with

the in vivo data obtained in *S. aureus*. The presence of an intact hydrolase abrogates the synthetase activity. Synthesis of pppGpp was detectable only in hydrolase-deficient constructs. Moreover, we show that the CTD has an inhibitory effect on synthetase activity since truncated versions of Rel_{Sau} showed higher accumulation of the reaction products AMP and pppGpp compared to the full-length enzyme. However, the CTD had only a minor impact on the strong hydrolase activity of the purified enzymes. Interestingly, Rel_{Sau} preferentially hydrolyzes ppGpp over pppGpp. *In vivo*, it was shown that RelP and RelQ mainly produce ppGpp [35], which is toxic at high concentrations and requires efficient hydrolysis. This observation could explain the preference of Rel_{Sau} for ppGpp hydrolysis.

The Co-IP results indicate that Rel_{Sau} interacts with the translation machinery and that the TGS strongly influence this interaction. This is largely consistent with previous data obtained for RelA [24,25,28,29,30]. Among the 10 detected interacting ribosomal proteins, L16, S13, and S12 are homologous to *E. coli* proteins that have been previously identified to interact with RelA [28,29,30]. Of note, the TGS motif also seems to hamper the putative interaction of Rel_{Sau} with other proteins of the RNA and DNA pathways. Whether such interactions are specific and involved in the molecular function of Rel_{Sau} remains to be investigated.

The *in vivo* analyses combined with the Co-IP results provided some clues regarding the roles of the different motifs of the CTD of Rel_{Sau}. Of the three motifs, the TGS motif showed the strongest effect, and the ACT motif showed the weakest effect, on (p)ppGpp activation and ribosomal interaction. Thus, the role of the ACT motif remains to be elucidated but seems to be minor. The TGS motif is clearly required for synthetase activation, most likely interacts with the ribosome to sense whether or not the tRNA in the A-site is aminoacetylated as shown for RelA [28,29,30,46]. Similar to the TGS motif, the DC motif was also found to be required for synthetase activity and to influence interactions with ribosomal proteins. DC has also been reported to interact with 23S rRNA ASF [28] which is critical to RelA activation in E. coli [46], presumably through stabilizing ribosome interaction.

This finding contradicts the simple model in which the DC motif causes oligomerization and thereby autoinhibition [31,32,33]. This would imply that DC mutation alleviates autoinhibition leading to increased synthase activity. In contrast, our data showed that the DC-mutated Rel_{Sau} is held in a synthetase-OFF state. Thus, our data support a model in which the DC motif participates in specific activation upon ribosomal contact, and that this interaction is involved in the intramolecular switch.

Materials and methods

Strains and growth conditions

Strains and plasmids are listed in the table in S1 Table. For strains carrying resistance genes, antibiotics (10 μg/ml erythromycin, 5 μg/ml tetracycline, 10 μg/ml chloramphenicol, and 100 μg/ml ampicillin) were used only in precultures. For the conditional mutant HG001-55, IPTG (final concentration of 0.5 mM) was added only in the preculture. *S. aureus* strains were grown in CYPG (10 g/l casamino acids, 10 g/l yeast extract, 5 g/l NaCl, 0.5% glucose and 0.06 M phosphoglycerate) medium [47]. Bacteria from an overnight culture were diluted to an initial optical density (OD_{600}) of 0.05 in fresh medium and grown with shaking (220 rpm) at 37˚C to the desired growth phase. Expression of cloned proteins was induced in exponential phase (OD_{600} = 0.3) with 0.1 μg/ml anhydrotetracycline (ATc) and in stringent conditions by addition of 0.3 μg/ml mupirocin. *E. coli* strains were grown in an overnight preculture in LB medium. Stringent conditions were applied by growing cells in modified M9 medium (33.7 mM $NaHPO_4$, 22 mM KH_2PO_4, 8.55 mM NaCl, 9.35 mM NH_4Cl, 1 mM $MgSO_4$, 0.3 mM $CaCl_2$, 1 μg/ml thiamine hydrochloride, 0.4% glycerol, 1 mM serine, 1 mM methionine and 1

mM glycine) [48]. For growth on solid media, single colonies grown on LB agar were streaked on M9 agar plates. For growth curve analyses, bacteria were inoculated to the desired OD_{600} (*S. aureus* initial OD_{600} = 0.05; *E. coli* initial OD_{600} = 0.1) in a 96-well plate, and growth was monitored in an Infinite M200 Pro microplate reader (Tecan).

Construction of vectors for expression of full-length and truncated *rel*

All oligonucleotides are listed in S2 Table. ATc-inducible plasmids, derived from pCG248, were generated with a restriction enzyme cloning strategy. Amplicons and vector were digested with EcoRI restriction enzyme. Substitution of the hydrolase domain and the ACT, TGS and DC mutations were achieved by overlapping PCR. For expression of the *rel* constructs under the native promoter, the shuttle vector pCG443 was designed based on pJL77 [49]. pJL77 was digested with AscI and SphI to remove the previous insert, including the promoter. The *rel* promoter was amplified from genomic DNA, digested using the same restriction enzymes, and ligated to generate pCG443. Full-length, truncated and mutated versions of *rel* were subcloned from the pCG248 plasmids into AscI-digested pCG443 by Gibson assembly [50].

All inserts were verified by sequencing (4base lab AG advanced molecular analysis), electroporated into the restriction-deficient *S. aureus* strain RN4220, and then transduced into the final *S. aureus* strains. All *S. aureus* strains were tested by PCR for the presence of the correct plasmid.

For expression in *E. coli*, different derivatives of *rel* were cloned into the EcoRI site of pBAD30 via Gibson assembly using the oligonucleotides listed in Table S2. Resulting vectors were verified by sequencing and moved to MG1655 *E. coli* strains (wild type and *relA/spoT* mutant) [51,52].

For protein purification, different *rel* derivatives were subcloned from the pCG248-based plasmids into BamHI-digested pET15b using Gibson assembly.

Generation of *S. aureus* mutant strains

The markerless *rel* CTD-deletion mutant was obtained using the ATc-inducible suicide vector pBASE6 [34]. Deletion was introduced by overlapping PCR with the primers listed in S2 Table, and the amplicon was cloned into BglII- and SalI-digested pBASE6 by Gibson assembly. The resulting plasmid was verified by sequencing and electroporated into RN4220, from which the plasmid was transduced into HG001. Mutagenesis was performed as described previously [34]. Mutation was verified by PCR.

RNA isolation and Northern blot analysis

RNA isolation and Northern blot analysis were performed as described previously [53]. Briefly, 5 ml of bacteria were collected at the desired time point (30 minutes after induction) and centrifuged. The pellet was resuspended in 1 ml of TRIzol reagent (Thermo Fisher Scientific) with 0.5 ml of zirconia/silica beads (0.1-mm diameter) and lysed using a high-speed homogenizer (Thermo Fisher Scientific). RNA was isolated following the instructions provided by the TRIzol manufacturer. For the detection of specific transcripts on the Northern blot, digoxigenin-labeled probes were generated using the DIG-labeling PCR Kit as described by the manufacturer (Roche Life Science).

Purification of different Rel_{Sau} constructs

E. coli BL21 (DE3) (New England Biolabs) cells that were freshly transformed with plasmids carrying full-length *rel* constructs were grown for 16 hours at room temperature under

constant shaking (150 rpm) in LB medium supplemented with D(+)-lactose-monohydrate (12.5 g/l) and ampicillin (100 μg/ml). Cells were harvested (20 minutes, 3000 x g, 4˚C) and resuspended in ice-cold high-KCl buffer A (20 mM HEPES (pH 7.4), 20 mM NaCl, 20 mM $MgCl_2$, 1 M KCl, 30% (v/v) glycerol, and 40 mM imidazole) supplemented with 10 μg/ml DNAse and cOmplete protease inhibitor cocktail (Roche). Cells were lysed by a French press at 1000 psi. The lysate was centrifuged (50,000 x g, 45 minutes, 4˚C), and the clear supernatant was filtered (0.22-μm pore size) before being loaded onto a 1-ml HisTrap HP column (GE Healthcare Life Sciences) equilibrated with high-KCl buffer A. Purification was performed with an ÄKTA purification system (GE Healthcare Life Sciences), and elution was carried out with an imidazole gradient to a final concentration of 500 mM. Fractions were analyzed by SDS-PAGE, and the fractions containing the protein of interest were collected and concentrated to 5 ml with an Amicon Ultracel-50K ultracentrifugal device, with a cut-off of 50 kDa (Merck Millipore). Protein was further purified by size-exclusion chromatography (HiLoad 16/600 Superdex 200 pg, GE Healthcare Life Sciences). The size-exclusion column was previously equilibrated with ice-cold high-KCl SEC buffer (20 mM HEPES (pH 7.0), 20 mM NaCl, 20 mM $MgCl_2$, 1 M KCl, and 30% (v/v) glycerol). Protein-containing fractions were pooled, concentrated by ultra-filtration with a 50-kDa cut-off, aliquoted and stored at -80˚C. For purification of CTD-truncated constructs, the same procedure was followed using different buffers: low-KCl buffer A (20 mM HEPES (pH 7.4), 200 mM NaCl, 20 mM $MgCl_2$, 20 mM KCl, 30% (v/v) glycerol, and 40 mM imidazole) for affinity purification and low-KCl SEC buffer (20 mM HEPES (pH 7.0), 200 mM NaCl, 20 mM $MgCl_2$, 20 mM KCl, and 30% (v/v) glycerol) for size exclusion. For concentration of truncated Rel_{Sau}, Amicon Ultracel-30K (Merck Millipore) was used.

In vitro assay for enzymatic activity and HPLC-MS analysis

Synthetase assays were performed in reaction buffer (20 mM HEPES (pH 7.0), 200 mM NaCl, 20 mM $MgCl_2$, and 20 mM KCl) with 1 mM ATP, 1 mM GTP and 2 μM purified enzyme. Hydrolase assays were performed in the same reaction buffer with 1 mM ppGpp or 1 mM pppGpp (both from Jena Biosciences) and 0.1 μM purified enzyme. Assays were performed at 37˚C; aliquots were taken at the indicated times; and the enzyme reactions were stopped by addition of an equal volume of chloroform. The mixtures were briefly vortexed and centrifuged (3 minutes, 11,000 × g). The aqueous phase containing the nucleotides was collected and stored at -20˚C prior to analysis. Nucleotide analysis was performed using an ESI-TOF mass spectrometer (micrO-TOF II, Bruker) operated in negative-ion mode and connected to an UltiMate 3000 high-performance liquid chromatography (HPLC) system (Dionex). 5 μl of each sample at 10˚C was injected onto the SeQuant ZIC-pHILIC column (Merck, PEEK 150 × 2.1 mm, 5 μm), and the system was run at 30˚C as previously described [43]. The following 40-minute gradient program was used at a flow rate of 0.2 ml/min: 5 minutes of 82% buffer A (CH_3CN) and 18% buffer B (100 mM $(NH_4)_2CO_3$, pH 9.2); 25 minutes of a linear gradient to 42% buffer A; and finally, 10 minutes of 82% buffer A. The DataAnalysis program (Bruker) was used to present the nucleotide masses as extracted-ion chromatograms, and the peak areas were calculated and quantified with Prism 5 (GraphPad). Dilution series of commercially available nucleotides ppGpp (m/z, 601.95), pppGpp (m/z, 681.92) and AMP (m/z, 346.06) were used for calibration to quantify the amounts of nucleotides in the reactions.

Co-IP of Rel_{Sau} with native *S. aureus* proteins

To generate Rel_{Sau}-specific antibodies, 0.5 mg of purified full-length protein was sent to Davids Biotechnologie GmbH to generate antiserum and affinity-purified IgG. The specificity of the

IgG was verified by Western blot analysis (Blot in S1 Fig). For Co-IP, bacteria were grown in 100 ml of CYPG medium to an OD_{600} of 1 and centrifuged (5,000 μ g, 5 minutes). The pellet was washed 2 times with PBS and resuspended in 500 μl of cold Co-IP buffer (20 mM HEPES (pH 7.0), 200 mM NaCl, 20 mM MgCl, 20 mM KCl, 0.5 mM DTT, 0.2% (v/v) Tween 20 and cOmplete protease inhibitor cocktail). The resuspended pellet was lysed with 0.5 ml of zirconia-silica beads (0.1 mm diameter) using a high-speed homogenizer (two times, 6,500 rpm, 20 s). Lysed cells were centrifuged for 1 hour at 14,000 x g at 4˚C, and the supernatant was aliquoted (100 μl) and frozen at -80˚C. Co-IP was performed with Dynabeads (Thermo Fisher Scientific) following the manufacturer's instructions with some minor modifications. Briefly, 50 μl of Dynabeads slurry was used for each sample. The storage solution was removed, and the beads were incubated with 30 μg of Anti-Rel_{Sau} IgG resuspended in PBS (pH 7.4) with 0.02% Tween 20 for 30 minutes at room temperature under constant rotation. Coated beads were pelleted using a magnetic rack; the supernatant was removed; and 100 μl of the cell lysates were added and incubated for 30 minutes at room temperature under constant rotation. After incubation, the beads were gently washed 3 times with Co-IP buffer using a magnetic rack. Washing solution was removed, and the beads were resuspended in SDS sample buffer, boiled at 95˚C for 5 minutes, and run approximately 1 cm into an SDS-PAGE gel. The gel slice was subsequently analyzed by mass spectrometry.

Quantitative label-free proteomics

Three biological replicates of (p)ppGpp^0 complemented with WT and mutant Rel_{Sau} were analyzed. Gel slices were digested as described previously [54]. Peptide mixtures were then separated on an EasyLC nano-HPLC (Proxeon Biosystems) coupled to an LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) as described elsewhere [55] with the following modifications: peptides were eluted with an 87-min segmented gradient of 5–33–90% HPLC solvent B (80% acetonitrile in 0.5% acetic acid). Each sample was run in triplicate. The acquired MS spectra were processed with the MaxQuant software package, version 1.5.2.8 [56] with the integrated Andromeda search engine [57] as described previously [55]. Database searches were performed against a target-decoy *S. aureus* all-strains database obtained from UniProt, containing 126,225 protein entries and 248 commonly observed contaminants. The label-free algorithm was enabled, as was the "match between runs" option [58]. Label-free quantification (LFQ) protein intensities from the MaxQuant data output were used for relative protein quantification. Downstream bioinformatic analysis (ANOVA and two-sample t-tests) was performed using the Perseus software package, version 1.5.0.15. $P < 0.05$ was considered to be statistically significant. For the heatmap, among the 4 different proteins, those that showed significant differences according to ANOVA were selected (Data in S1 Dataset). For these selected candidates, the t-test differences, indicating changes in the amount, were calculated between the protein immunoprecipitated with WT or mutant Rel_{Sau} and plotted on the heatmap.

Statistical analysis

The results for the growth and *in vitro* analyses represent the mean ± SD of at least three biological replicates. Significance was calculated using Prism 5 by one-way ANOVA with Bonferroni correction.

Supporting information

S1 Fig. Immunoblot showing the expression level of the different Rel_{Sau} constructs. *S. aureus* (p)ppGpp^0 complemented with Rel_{Sau}, wild type and different domain mutants, under

the native promoter. Strains were grown in rich medium to OD_{600} = 1 and then harvested. For Worthern analysis, lysate was obtained and the different Rel_{Sau} constructs were detected with anti-Rel specific antibody.
(TIF)

S1 Table. Strains and plasmids.
(DOCX)

S2 Table. Oligonucleotides.
(DOCX)

S1 Dataset. Comparison of Co-immune-precipitation results using Rel_{Sau} and TGS, ACT and DC mutated Rel_{Sau} as bait.
(XLSX)

S1 Protocol. Protocol for Rel-specific Western blot.
(DOCX)

Acknowledgments

We thank Natalya Korn, Isabell Samp and Vittoria Bisanzio for excellent technical assistance. We thank Elsa Germain–Maisonneuve for providing *E. coli* strains and vectors.

Author Contributions

Conceptualization: Fabio Lino Gratani, Christiane Wolz.

Data curation: Fabio Lino Gratani, Marina Borisova, Ana Velic.

Formal analysis: Fabio Lino Gratani, Christoph Mayer.

Funding acquisition: Christoph Mayer, Samuel Wagner, Gert Bange, Boris Maček, Christiane Wolz.

Investigation: Fabio Lino Gratani, Petra Horvatek.

Methodology: Fabio Lino Gratani, Petra Horvatek, Tobias Geiger, Marina Borisova, Iwan Grin, Wieland Steinchen, Ana Velic.

Project administration: Christiane Wolz.

Supervision: Christoph Mayer, Iwan Grin, Samuel Wagner, Gert Bange, Boris Maček, Christiane Wolz.

Validation: Fabio Lino Gratani, Christoph Mayer.

Visualization: Fabio Lino Gratani.

Writing – original draft: Fabio Lino Gratani, Christiane Wolz.

Writing – review & editing: Christiane Wolz.

References

1. Cashel M (1975) Regulation of bacterial ppGpp and pppGpp. Annual review of microbiology 29: 301–318. https://doi.org/10.1146/annurev.mi.29.100175.001505 PMID: 170852
2. Chatterji D, Ojha AK (2001) Revisiting the stringent response, ppGpp and starvation signaling. Current opinion in microbiology 4: 160–165. PMID: 11282471
3. Wagner R (2002) Regulation of ribosomal RNA synthesis in *E. coli*: effects of the global regulator guanosine tetraphosphate (ppGpp). J Mol Microbiol Biotechnol 4: 331–340. PMID: 11931566

4. Gralla JD (2005) *Escherichia coli* ribosomal RNA transcription: regulatory roles for ppGpp, NTPs, architectural proteins and a polymerase-binding protein. Molecular microbiology 55: 973–977. https://doi.org/10.1111/j.1365-2958.2004.04455.x PMID: 15686546
5. Magnusson LU, Farewell A, Nystrom T (2005) ppGpp: a global regulator in *Escherichia coli*. Trends in microbiology 13: 236–242. https://doi.org/10.1016/j.tim.2005.03.008 PMID: 15866041
6. Braeken K, Moris M, Daniels R, Vanderleyden J, Michiels J (2006) New horizons for (p)ppGpp in bacterial and plant physiology. Trends in microbiology 14: 45–54. https://doi.org/10.1016/j.tim.2005.11.006 PMID: 16343907
7. Jain V, Kumar M, Chatterji D (2006) ppGpp: stringent response and survival. J Microbiol 44: 1–10. PMID: 16554711
8. Potrykus K, Cashel M (2008) (p)ppGpp: still magical? Annual review of microbiology 62: 35–51. https://doi.org/10.1146/annurev.micro.62.081307.162903 PMID: 18454629
9. Srivatsan A, Wang JD (2008) Control of bacterial transcription, translation and replication by (p)ppGpp. Current opinion in microbiology 11: 100–105. https://doi.org/10.1016/j.mib.2008.02.001 PMID: 18359660
10. Wu J, Xie J (2009) Magic spot: (p) ppGpp. J Cell Physiol 220: 297–302. https://doi.org/10.1002/jcp.21797 PMID: 19391118
11. Wolz C, Geiger T, Goerke C (2010) The synthesis and function of the alarmone (p)ppGpp in firmicutes. International journal of medical microbiology: IJMM 300: 142–147. https://doi.org/10.1016/j.ijmm.2009.08.017 PMID: 19783475
12. Cashel M (1969) The control of ribonucleic acid synthesis in *Escherichia coli*. IV. Relevance of unusual phosphorylated compounds from amino acid-starved stringent strains. The Journal of biological chemistry 244: 3133–3141. PMID: 4893338
13. Battesti A, Bouveret E (2009) Bacteria possessing two RelA/SpoT-like proteins have evolved a specific stringent response involving the acyl carrier protein-SpoT interaction. Journal of bacteriology 191: 616–624. https://doi.org/10.1128/JB.01195-08 PMID: 18996989
14. Battesti A, Bouveret E (2006) Acyl carrier protein/SpoT interaction, the switch linking SpoT-dependent stress response to fatty acid metabolism. Molecular microbiology 62: 1048–1063. https://doi.org/10.1111/j.1365-2958.2006.05442.x PMID: 17078815
15. Atkinson GC, Tenson T, Hauryliuk V (2011) The RelA/SpoT homolog (RSH) superfamily: distribution and functional evolution of ppGpp synthetases and hydrolases across the tree of life. PloS one 6: e23479. https://doi.org/10.1371/journal.pone.0023479 PMID: 21858139
16. Mittenhuber G (2001) Comparative genomics and evolution of genes encoding bacterial (p)ppGpp synthetases/hydrolases (the Rel, RelA and SpoT proteins). J Mol Microbiol Biotechnol 3: 585–600. PMID: 11545276
17. Steinchen W, Bange G (2016) The magic dance of the alarmones (p)ppGpp. Molecular microbiology 101: 531–544. https://doi.org/10.1111/mmi.13412 PMID: 27149325
18. Geiger T, Goerke C, Fritz M, Schafer T, Ohlsen K, et al. (2010) Role of the (p)ppGpp synthase RSH, a RelA/SpoT homolog, in stringent response and virulence of *Staphylococcus aureus*. Infection and immunity 78: 1873–1883. https://doi.org/10.1128/IAI.01439-09 PMID: 20212088
19. Wendrich TM, Marahiel MA (1997) Cloning and characterization of a *rel*A/*spo*T homologue from *Bacillus subtilis*. Molecular microbiology 26: 65–79. PMID: 9383190
20. Hogg T, Mechold U, Malke H, Cashel M, Hilgenfeld R (2004) Conformational antagonism between opposing active sites in a bifunctional RelA/SpoT homolog modulates (p)ppGpp metabolism during the stringent response. Cell 117: 57–68. PMID: 15066282
21. Mechold U, Murphy H, Brown L, Cashel M (2002) Intramolecular regulation of the opposing (p)ppGpp catalytic activities of Rel(Seq), the Rel/Spo enzyme from *Streptococcus equisimilis*. Journal of bacteriology 184: 2878–2888. https://doi.org/10.1128/JB.184.11.2878-2888.2002 PMID: 12003927
22. Schreiber G, Metzger S, Aizenman E, Roza S, Cashel M, et al. (1991) Overexpression of the relA gene in Escherichia coli. The Journal of biological chemistry 266: 3760–3767. PMID: 1899866
23. Jain V, Saleem-Batcha R, China A, Chatterji D (2006) Molecular dissection of the mycobacterial stringent response protein Rel. Protein Sci 15: 1449–1464. https://doi.org/10.1110/ps.062117006 PMID: 16731979
24. Haseltine WA, Block R (1973) Synthesis of guanosine tetra- and pentaphosphate requires the presence of a codon-specific, uncharged transfer ribonucleic acid in the acceptor site of ribosomes. Proceedings of the National Academy of Sciences of the United States of America 70: 1564–1568. PMID: 4576025
25. Wendrich TM, Blaha G, Wilson DN, Marahiel MA, Nierhaus KH (2002) Dissection of the mechanism for the stringent factor RelA. Mol Cell 10: 779–788. PMID: 12419222

26. Chipman DM, Shaanan B (2001) The ACT domain family. Current Opinion in Structural Biology 11: 694–700. PMID: 11751050

27. Grant GA (2006) The ACT domain: a small molecule binding domain and its role as a common regulatory element. J Biol Chem 281: 33825–33829. https://doi.org/10.1074/jbc.R600024200 PMID: 16987805

28. Loveland AB, Bah E, Madireddy R, Zhang Y, Brilot AF, et al. (2016) Ribosome*RelA structures reveal the mechanism of stringent response activation. eLife 5.

29. Brown A, Fernandez IS, Gordiyenko Y, Ramakrishnan V (2016) Ribosome-dependent activation of stringent control. Nature 534: 277–280. https://doi.org/10.1038/nature17675 PMID: 27279228

30. Arenz S, Abdelshahid M, Sohmen D, Payoe R, Starosta AL, et al. (2016) The stringent factor RelA adopts an open conformation on the ribosome to stimulate ppGpp synthesis. Nucleic acids research 44: 6471–6481. https://doi.org/10.1093/nar/gkw470 PMID: 27226493

31. Gropp M, Strausz Y, Gross M, Glaser G (2001) Regulation of *Escherichia coli* RelA requires oligomerization of the C-terminal domain. Journal of bacteriology 183: 570–579. https://doi.org/10.1128/JB.183.2.570-579.2001 PMID: 11133950

32. Avarbock A, Avarbock D, Teh JS, Buckstein M, Wang ZM, et al. (2005) Functional regulation of the opposing (p)ppGpp synthetase/hydrolase activities of RelMtb from *Mycobacterium tuberculosis*. Biochemistry 44: 9913–9923. https://doi.org/10.1021/bi0505316 PMID: 16026164

33. Yang X, Ishiguro EE (2001) Dimerization of the RelA protein of *Escherichia coli*. Biochem Cell Biol 79: 729–736. PMID: 11800013

34. Geiger T, Francois P, Liebeke M, Fraunholz M, Goerke C, et al. (2012) The stringent response of *Staphylococcus aureus* and its impact on survival after phagocytosis through the induction of intracellular PSMs expression. PLoS pathogens 8: e1003016. https://doi.org/10.1371/journal.ppat.1003016 PMID: 23209405

35. Geiger T, Kastle B, Gratani FL, Goerke C, Wolz C (2014) Two small (p)ppGpp synthases in Staphylococcus aureus mediate tolerance against cell envelope stress conditions. Journal of bacteriology 196: 894–902. https://doi.org/10.1128/JB.01201-13 PMID: 24336937

36. Steinchen W, Vogt MS, Altegoer F, Giammarinaro PI, Horvatek P, et al. (2018) Structural and mechanistic divergence of the small (p)ppGpp synthetases RelP and RelQ. Scientific reports 8: 2195. https://doi.org/10.1038/s41598-018-20634-4 PMID: 29391580

37. Steinchen W, Schuhmacher JS, Altegoer F, Fage CD, Srinivasan V, et al. (2015) Catalytic mechanism and allosteric regulation of an oligomeric (p)ppGpp synthetase by an alarmone. Proceedings of the National Academy of Sciences of the United States of America 112: 13348–13353. https://doi.org/10.1073/pnas.1505271112 PMID: 26460002

38. Manav MC, Beljantseva J, Bojer MS, Tenson T, Ingmer H, et al. (2018) Structural basis for (p)ppGpp synthesis by the Staphylococcus aureus small alarmone synthetase RelP. The Journal of biological chemistry.

39. Liu K, Bittner AN, Wang JD (2015) Diversity in (p)ppGpp metabolism and effectors. Current opinion in microbiology 24: 72–79. https://doi.org/10.1016/j.mib.2015.01.012 PMID: 25636134

40. Gaca AO, Colomer-Winter C, Lemos JA (2015) Many means to a common end: the intricacies of (p)ppGpp metabolism and its control of bacterial homeostasis. Journal of bacteriology 197: 1146–1156. https://doi.org/10.1128/JB.02577-14 PMID: 25605304

41. Geiger T, Wolz C (2014) Intersection of the stringent response and the CodY regulon in low GC Gram-positive bacteria. International journal of medical microbiology: IJMM 304: 150–155. https://doi.org/10.1016/j.ijmm.2013.11.013 PMID: 24462007

42. Krasny L, Gourse RL (2004) An alternative strategy for bacterial ribosome synthesis: *Bacillus subtilis* rRNA transcription regulation. The EMBO journal 23: 4473–4483. https://doi.org/10.1038/sj.emboj.7600423 PMID: 15496987

43. Kastle B, Geiger T, Gratani FL, Reisinger R, Goerke C, et al. (2015) rRNA regulation during growth and under stringent conditions in Staphylococcus aureus. Environmental microbiology 17: 4394–4405. https://doi.org/10.1111/1462-2920.12867 PMID: 25845735

44. Bag S, Das B, Dasgupta S, Bhadra RK (2014) Mutational analysis of the (p)ppGpp synthetase activity of the Rel enzyme of Mycobacterium tuberculosis. Archives of Microbiology 196: 575–588. https://doi.org/10.1007/s00203-014-0996-9 PMID: 24859914

45. He P, Deng C, Liu B, Zeng L, Zhao W, et al. (2013) Characterization of a bifunctional enzyme with (p)ppGpp-hydrolase/synthase activity in Leptospira interrogans. FEMS microbiology letters 348: 133–142. https://doi.org/10.1111/1574-6968.12279 PMID: 24111633

46. Kudrin P, Dzhygyr I, Ishiguro K, Beljantseva J, Maksimova E, et al. (2018) The ribosomal A-site finger is crucial for binding and activation of the stringent factor RelA. Nucleic acids research 46: 1973–1983. https://doi.org/10.1093/nar/gky023 PMID: 29390134

47. Novick RP (1991) Genetic systems in staphylococci. Methods Enzymol 204: 587–636. PMID: 1658572

48. Neidhardt FC, Bloch PL, Smith DF (1974) Culture Medium for Enterobacteria. Journal of Bacteriology 119: 736–747. PMID: 4604283

49. Liese J, Rooijakkers SH, van Strijp JA, Novick RP, Dustin ML (2013) Intravital two-photon microscopy of host-pathogen interactions in a mouse model of *Staphylococcus aureus* skin abscess formation. Cellular microbiology 15: 891–909. https://doi.org/10.1111/cmi.12085 PMID: 23217115

50. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, 3rd, et al. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature methods 6: 343–345. https://doi.org/10.1038/nmeth.1318 PMID: 19363495

51. Xiao H, Kalman M, Ikehara K, Zemel S, Glaser G, et al. (1991) Residual guanosine 3',5'-bispyrophosphate synthetic activity of *rel*A null mutants can be eliminated by *spo*T null mutations. The Journal of biological chemistry 266: 5980–5990. PMID: 2005134

52. Blattner FR, Plunkett G, 3rd, Bloch CA, Perna NT, Burland V, et al. (1997) The complete genome sequence of Escherichia coli K-12. Science 277: 1453–1462. PMID: 9278503

53. Goerke C, Campana S, Bayer MG, Doring G, Botzenhart K, et al. (2000) Direct quantitative transcript analysis of the agr regulon of Staphylococcus aureus during human infection in comparison to the expression profile in vitro. Infection and immunity 68: 1304–1311. PMID: 10678942

54. Burian M, Velic A, Matic K, Gunther S, Kraft B, et al. (2015) Quantitative proteomics of the human skin secretome reveal a reduction in immune defense mediators in ectodermal dysplasia patients. J Invest Dermatol 135: 759–767. https://doi.org/10.1038/jid.2014.462 PMID: 25347115

55. Carpy A, Krug K, Graf S, Koch A, Popic S, et al. (2014) Absolute proteome and phosphoproteome dynamics during the cell cycle of Schizosaccharomyces pombe (Fission Yeast). Mol Cell Proteomics 13: 1925–1936. https://doi.org/10.1074/mcp.M113.035824 PMID: 24763107

56. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nature biotechnology 26: 1367–1372. https://doi.org/10.1038/nbt.1511 PMID: 19029910

57. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, et al. (2011) Andromeda: a peptide search engine integrated into the MaxQuant environment. Journal of proteome research 10: 1794–1805. https://doi.org/10.1021/pr101065j PMID: 21254760

58. Luber CA, Cox J, Lauterbach H, Fancke B, Selbach M, et al. (2010) Quantitative proteomics reveals subset-specific viral recognition in dendritic cells. Immunity 32: 279–289. https://doi.org/10.1016/j.immuni.2010.01.013 PMID: 20171123

8 Additional research

8.1 RelQ-mediated (pp)pGpp synthesis increased expression of genes involved in cell wall biosynthesis and vancomycin tolerance

To compare the global effect of (pp)pGpp, transcriptional induced RSH-Syn and RelQ were compared applying RNA-Seq. The transcriptome analysis revealed more genes were affected by RSH-Syn than by RelQ. Nevertheless, 61 genes were significantly affected by RelQ but not by RSH (table 2). Analyzing these genes more in detail showed increased expression of amino acids involved in cell wall biosynthesis (*dapD*, d*apL*, *alr2*, *lysA*) (Kullik, Jenni, & Berger-Bachi, 1998). The glycopeptide antibiotic vancomycin targets the di-peptide D-ala-lipid II (Nagarajan, 1991). I hypothesized RelQ-mediated increased expression of *dapD*, d*apL* and *alr2* results in additional cell wall stabilization and tolerance to vancomycin. Therefore, wild type and a Δr*elPQ* mutant were grown in CDM until an OD_{600}=0.3 and challenged with 6µg/ml vancomycin and growth was monitored over time. After 14 hours strains slowly started to grow, indicating either consumed vancomycin or stabilization of the cell wall. Second supplementation of vancomycin did not inhibit growth in the wild type strain and resulted in vancomycin tolerance while in contrast the Δ*relPQ* mutant grew very slow and was less tolerant (Fig.8). These results clearly indicate, that RelQ and maybe RelP lead to increased tolerance to vancomycin by stabilizing or repairing the cell wall through targeted induction of genes involved in cell wall biosynthesis.

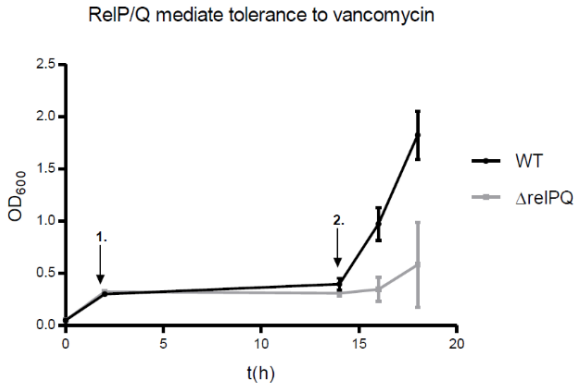

Fig.8: RelP/Q mediate tolerance to vancomycin. Strains were grown in CDM until an OD_{600}=0.3 and challenged (1.) with 6µg/ml vancomycin. Second addition of 6µg/ml vancomycin occurred after initiated growth (2.).

Table 2: Significant up- or down-regulation of RelQ-mediated genes

Name	locus tag	relQ vs. control - Log_2 fold change	relQ vs. control - Fold change
relQ	SAOUHSC_00942	6,747891155	107,4775221
ilvA2	SAOUHSC_02289	4,715287585	26,26896738
S136	S136	4,04050149	16,45554029
SAOUHSC_03018	SAOUHSC_03018	3,764481762	13,59007735
SAOUHSC_03017	SAOUHSC_03017	3,723806412	13,21226963
dapD	SAOUHSC_01398	3,723471429	13,20920219
S302	S302	3,643574177	12,49755679
S1180	S1180	3,583821302	11,99051153
SAOUHSC_00170	SAOUHSC_00170	3,361031836	10,2747532
SAOUHSC_03016	SAOUHSC_03016	3,340154398	10,1271365
dapL	SAOUHSC_01399	3,173016078	9,019303815
S394	S394	3,07671668	8,436921512
alr2	SAOUHSC_01400	3,027372365	8,153233679
S585	S585	2,999767109	7,998708685
SAOUHSC_03012	SAOUHSC_03012	2,772924226	6,834918931
SAOUHSC_01109	SAOUHSC_01109	2,754512227	6,748244413
fadE	SAOUHSC_00198	2,523429083	5,749470448
SAOUHSC_02593	SAOUHSC_02593	2,494941224	5,63705336
SAOUHSC_02594	SAOUHSC_02594	2,421810453	5,358430351
hisB	SAOUHSC_03011	2,415364424	5,334542039
SAOUHSC_00969	SAOUHSC_00969	2,202291395	4,60209701
pycA	SAOUHSC_01064	2,107897828	4,310627289
ssuC	SAOUHSC_00138	2,076007663	4,216388087
SAOUHSC_02875	SAOUHSC_02875	2,019575384	4,054644375
trpF	SAOUHSC_01370	1,962504094	3,897378623
S1042	S1042	1,938847757	3,833993143
trpC	SAOUHSC_01369	1,907233811	3,750892226
hisH	SAOUHSC_03010	1,896957905	3,724270611
pdxS	SAOUHSC_00499	1,840722137	3,581892742
SAOUHSC_01326	SAOUHSC_01326	1,763387197	3,394942634
S303	S303	1,744871098	3,351649062
trpB	SAOUHSC_01371	1,73136027	3,320407411
hisF	SAOUHSC_03008	1,730021305	3,317327172
argC	SAOUHSC_00149	1,72986386	3,316965164
SAOUHSC_00538	SAOUHSC_00538	1,714027119	3,280753315
lysA	SAOUHSC_01401	1,711423639	3,274838223
SAOUHSC_01603	SAOUHSC_01603	1,651848028	3,142359037
S369	S369	1,641655394	3,120236523
SAOUHSC_00970	SAOUHSC_00970	1,626732879	3,088128699
sspA	SAOUHSC_00988	1,609426975	3,051306228
S577	S577	1,587779726	3,005863978
fnbB	SAOUHSC_02802	-1,587643811	-3,005580812
SAOUHSC_02841	SAOUHSC_02841	-1,604140272	-3,040145289
SAOUHSC_01460	SAOUHSC_01460	-1,60995216	-3,052417198
S508	S508	-1,637201991	-3,11061963

SAOUHSC_01077	SAOUHSC_01077	-1,694146271	-3,235853452
SAOUHSC_01233	SAOUHSC_01233	-1,727895151	-3,312441903
S786	S786	-1,795735749	-3,471924917
deoD1	SAOUHSC_00097	-1,831028917	-3,557907288
cntF	SAOUHSC_02763	-1,842289302	-3,585785779
RsaO1	S201	-1,844588933	-3,591506019
rplM	SAOUHSC_02478	-1,879716128	-3,680026429
S61	S61	-1,973979471	-3,928502481
isaA	SAOUHSC_02887	-2,013247466	-4,036898926
argG	SAOUHSC_00899	-2,086578555	-4,247395802
S1077	S1077	-2,189809022	-4,562450865
S894	S894	-2,234178943	-4,704948537
S1182	S1182	-2,676979128	-6,395154121
S989	S989	-2,716002159	-6,57049544
SAOUHSC_02592	SAOUHSC_02592	-3,099218105	-8,569542023
S1106	S1106	-3,147594715	-8,861768969

9 Discussion

Parts of the discussion section have been published in

Regulation of the opposing (p)ppGpp synthetase and hydrolase activities in a bifunctional RelA/SpoT homologue from *Staphylococcus aureus*.
Gratani FL, **Horvatek P**, Geiger T, Borisova M, Mayer C, Grin I, Wagner S, Steinchen W, Bange G, Velic A, Maček B, Wolz C, PLOS Genetics 2018 July 9, 14(7): e1007514.https://doi.org/10.1371/journal.pgen.1007514

and can overlap with parts of the discussion from "**Increasing the cellular (pp)pGpp level is associated with activation of stress response genes in *Staphylococcus aureus*". Horvatek P**, Hanna AMF, Gratani FL, Keinhörster D, Korn N, Mayer-Borisova M, Mayer C, Rejman D, Mäder U, Wolz C.

The stringent response is characterized by the synthesis of the three different alarmones pGpp, ppGpp and pppGpp, collectively called (pp)pGpp. These alarmones are synthesized by different RSH and SAS enzymes (RelA, SpoT, RSH, RelP, RelQ and RelV). However, irrespective of different activation, (pp)pGpp synthesis results in distinct physiological changes such as slow growth, inhibition of replication and transcriptional change. *S. aureus* possess three enzymes responsible for (pp)pGpp synthesis: RSH, RelP and RelQ. In this work we gained more insights in the reprogramming of the transcriptome and the interaction of the stringent response with other stress responses. Furthermore we elucidated the role of the C-terminus and its influence on the hydrolase and synthetase domain and the necessity for sensing amino acid starvation.

9.1 (pp)pGpp reprograms the transcriptome

To analyze the transcriptional consequences of (pp)pGpp synthesis I used a genetic approaches to induce RSH, RelP and RelQ. I was able to show a functional synthesis of (pp)pGpp by RSH, RelP and RelQ under these conditions. (pp)pGpp synthesis showed a profound reprogramming of the transcriptome. Nevertheless, the effects were more striking for RSH than for RelQ. In the following chapter I will discuss the consequences of RSH- and RelQ-mediated (pp)pGpp synthesis.

9.2 RSH-mediated (pp)pGpp synthesis effects a variety of gene categories

In general genes for metabolism and transcription, translation, replication and general metabolic pathways were severely down regulated. Beside inhibition of metabolic genes, many CodY-regulated genes increased. These results are in line with Geiger et.al (Geiger et al., 2012). I mainly focused on genes which showed a significant up-regulation in their transcriptional behavior independent of CodY. These were mainly genes involved in oxidative stress, iron storage and virulence.

9.3 (pp)pGpp-mediated induction of *psms* increases ROS

One of the most prominent effects was the (pp)pGpp-mediated up-regulation of *psmα and psmβ*. This effect has been observed by former microarray analyses by Geiger et.al (Geiger et al., 2012). PSM are amphipathic, alpha-helical peptides which integrate into the membrane and form pores independent of a receptor. PSMs are active against many different eukaryotic cells with special cytotoxicity to polymorph nuclear neutrophils (PMNs) (Cheung et al., 2014; Otto, 2014). Neutrophils are recruited and lysed by PMNs, which partially explains high virulence of community acquired MRSA strains. Interestingly, expression of *psm* did not correlate to the activation of Agr, which positively regulates *psm* expression (Queck et al., 2008). Agr expression was opposing under (pp)pGpp inducing conditions. This is in line with the analysis of (pp)pGpp overproducing clinical isolates which showed (pp)pGpp-dependent *agr* inhibition (W. Gao et al., 2010).

Another prominent effect was the (pp)pGpp-mediated up-regulation of genes for oxidative stress (*dps*), iron storage (*ftnA*) and virulence (*psm*). We questioned, whether the expression of *dps*/*ftnA* is a consequence of increased (pp)pGpp-mediated *psm* expression, since PSM contribute to increased ROS (George et al., 2019). We compared gene expression by applying Northern blot analyses under aerobic and anaerobic conditions to distinguish, whether expression of *psm*, *ftnA* and *dps* is mediated by exogenous ROS or (pp)pGpp. If increased expression of *ftnA* and d*ps* was ROS-dependent, no increased expression would have been observed under anaerobic conditions. Indeed, expression of *psm*, *ftnA* and *dps* is (pp)pGpp-dependent and not a consequence of exogenous ROS formation since increased expression was independent of the availability of oxygen.

Next, ROS was measured in different *psm* and (pp)pGpp^0 mutants to elucidate whether expression of oxidative stress genes is due to (pp)pGpp-mediated PSM expression and consequently ROS formation. ROS measurement indicated a decreased ROS level in the *psm* and (pp)pGpp^0 mutant. These results demonstrate PSM-mediated ROS formation. This is in line with current studies from George et al, who showed enhanced *psm* expression leads to increased ROS (George et al., 2019).

Neutrophils are the first immune cells which migrate to the source of infection to eradicate *S. aureus*. They phagocytize *S. aureus* and keep them trapped in the phagosome. The availability of nutrients within neutrophils is not clear. We hypothesize that stringent response is activated after phagocytosis due to amino acid starvation within the phagosome. (pp)pGpp activates PSMs to escape from the phagosome and protects itself from ROS by simultaneously expressing oxidative stress genes (Fig.9). It has been already shown, (pp)pGpp leads to increased *psm* expression and is necessary for survival after phagocytosis (Geiger et al., 2012). Our results clearly indicate activation of PSMs and oxidative stress genes are consequences of (pp)pGpp synthesis. A correlation of (pp)pGpp dependent *psmα* expression was shown by Mansour et.al. They approved down- regulation of *psmα* is a consequence of inhibition of (pp)pGpp synthesis by the cationic compound DJK-5 (Mansour et al., 2016).

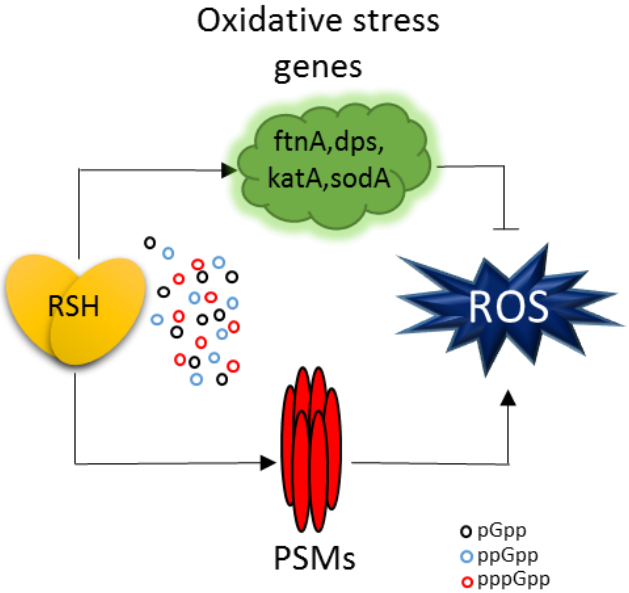

Fig.9 (pp)pGpp-mediated *psm* expression consequently increases ROS. This model proposes stringent response is activated as a consequence of amino acid starvation within the phagosome. (pp)pGpp synthesis results in increased *psm* expression increasing intracellular ROS level. Simultaneously, genes for oxidative stress and iron storage are stronger expressed to sequester cell damaging ROS.

9.4 (pp)pGpp influences gene expression of oxidative stress and iron storage independent of CodY, PerR, Fur and SarA

RNA-Seq data revealed up-regulation of *ftnA* (198 fold), *dps* (48 fold), *sodA* (19 fold), *ahpC* (5 fold) and *perR* (18 fold). All genes are regulated by PerR, in addition *ftnA* is regulated by Fur. Similar observations have been made in mupirocin treated *S. aureus* strain. Mupirocin inhibits the isoleucyl-tRNA synthetase and prevents loading of the tRNA with isoleucine thereby mimicking amino acid starvation. Proteins belonging to the oxidative stress response (FtnA, AhpC, DPS and KatA) increased after mupirocin treatment (Reiss et al., 2012). Increase of SODs in a (pp)pGpp-dependent manner has been reported in antibiotic treated *P. aeruginosa*. SODs were decreased in a (pp)pGpp^0 mutant, indicating (pp)pGpp is necessary to encounter oxidative stress, induced by antibiotic-formed OH radicals (Kohanski et al., 2007). Iron-overload genes *ftnA* and *dps* were highly expressed after transcriptional induction of RSH-Syn. Iron and ROS lead to the Fenton reaction which in turn results in severe protein and DNA damage. To avoid the Fenton reaction, *ftnA* and *dps* can capture free iron and protect cells from lethal damage. In *Vibrio cholarea* overexpression of (pp)pGpp repressed the Fe(III) ABC transporter substrate binding protein FbpA. Consequently, free intracellular iron decreases, leading to a reduced interaction of antibiotic-induced ROS thereby avoiding a formation of the Fenton reaction (H. Y. Kim et al., 2018).

We performed Northern Blot analyses expressing RSH-Syn in different CodY, PerR, Fur and SarA negative backgrounds to exclude upregulation is mediated by these regulators. As read-out genes we used the most abundant genes *ftnA* and *dps*. First, we were able to show the transcriptional changes observed are an effect of (pp)pGpp and not due to CodY de-repression, although we observed up-regulation of *ftnA* and *dps* was less in the *codY*/(pp)pGpp^0 mutant. Recently, a correlation between (pp)pGpp and iron homeostasis have been observed in *Enterococcus faecalis* (Colomer-Winter et al., 2017). Using a chemical defined medium, lacking either iron (Fe) or manganese (Mn), resulted in accumulation of (pp)pGpp. A (pp)pGpp^0 mutant does not grow properly in serum lacking Fe and Mn. Growth was restored to the wild type level by supplementing serum with either Fe or Mn. To distinguish between a direct effect of (pp)pGpp or an indirect due to de-repression of CodY, a (p)ppGpp^0 *codY* mutant was included.

The (p)ppGpp^0 *codY* mutant could restore growth in Mn-depleted medium but not Fe. This observations indicates, CodY is involved in the regulation of Mn and (pp)pGpp most likely in the regulation of Fe. (Colomer-Winter et al., 2017).

Transcriptional analysis revealed, that genes for iron-overload and oxidative stress were strongly activated by (pp)pGpp. This effect is likely independent of PerR, Fur or SarA and has not been reported before. Since oxidative stress genes are induced by ROS, we speculated, whether gene expression differs under anaerobic conditions. However, no difference in expression pattern was observed and (pp)pGpp led to activation of oxidative stress genes independent of ROS. To understand the physiological aspects of the activation of oxidative stress genes, we challenged the WT and a (pp)pGpp^0 mutant with different H_2O_2 concentrations. The (pp)pGpp^0 mutant was killed in a dose dependent manner whereas the WT was able to survive higher H_2O_2 concentrations. A similar protection of H_2O_2 by (pp)pGpp has been demonstrated in *P. aeruginosa*, showing better survival of the wild type compared to a (pp)pGpp^0 stain (Khakimova, Ahlgren, Harrison, English, & Nguyen, 2013). Furthermore, challenging the wild type with hydrogen peroxide resulted in (pp)pGpp synthesis in *S. mutans*. (Seaton, Ahn, Sagstetter, & Burne, 2011). These results indicate a global protective mechanism by (pp)pGpp in response to H_2O_2. Next, we wanted to elucidate, whether RSH, RelP or RelQ contribute to H_2O_2 protection. Growth analyses revealed, that the Δ*relP/Q* mutant was less resistant compared to the rsh_{syn} mutant. However, some of the Δ*relP/Q* mutants were still able to regrow, indicating presumably being rescued by RSH or activation of catalase by PerR (Fig.7). These results indicate RelP or/and RelQ are mainly, but not exclusively, responsible for H_2O_2 protection. This is supported by Kim et al, who demonstrated (pp)pGpp synthesis by RelP after H_2O_2 treatment in *S. mutans* (J. N. Kim, Ahn, Seaton, Garrett, & Burne, 2012).

Our RNA-seq data showed a 4-fold increased expression of *katA* after transcriptional induction of RSH-Syn. Since catalase is regulated by PerR, one might assume an activation of *katA* by PerR, if rapid adaption by (pp)pGpp is not possible. Similarly this was shown for H_2O_2 resistance in *P. aeruginosa*. A (pp)pGpp^0 strain was more killed by H_2O_2 than the WT, exhibited extremely reduced catalase activity and increased ROS production. (Khakimova et al., 2013).

Northern blot analyses need to be performed, to confirm whether H_2O_2 protection occurs via (pp)pGpp-dependent and PerR independent *katA* expression and

decreased ROS. Therefore, expression pattern of *katA* and *perR* after transcriptional induction of RSH-Syn in a (pp)pGpp^0 mutant has to be analyzed. Increased *katA* and decreased *perR e*xpression would indicate a PerR-independent and (pp)pGpp-dependent expression of *katA.* Beside KatA, PerR autoregulates (Horsburgh, Clements, et al., 2001) its own transcription. An increased *perR* expression after (pp)pGpp synthesis would indicate a de-repression of PerR. This in turn would implicate, increased *katA* expression is indirectly regulated by (pp)pGpp due to de-repression of PerR.

Taken together, these results suggest a global protection mechanism of (pp)pGpp to ROS in *S. aureus* and other bacterial species.

9.5 (pp)pGpp induces *psm* expression independent of the major regulators CodY, PerR, Fur and SarA

Another prominent effect was the increased expression of *psm*s α (up to 48-fold) and β (up to 25-fold) and down-regulation of *agrA*,*C* and *D* (up to 6-fold). Agr-independent *psm* expression has been already observed by Geiger et al, who showed induction of the stringent response induced by amino acid starvation, increases *psmα* and *β* expression (Geiger et al., 2012). We investigated the role of *psm* and *agr* expression after transcriptional induction of RSH-Syn in different *codY*, *perR*, *fur* and *sarA* mutants. Northern blot analyses revealed (pp)pGpp-dependent up-regulation of *psm* and down-regulation of *agr* is independent of CodY, PerR and Fur. Only in the *sarA* mutant *psm* expression was abolished, which was partially rescued by RSH-Syn induction. It has been already published that SarA positively regulates Agr (Heinrichs et al., 1996). Considered under this aspect, down-regulation of *psms* in the *sarA* mutant was not surprising, since Agr positively regulates *psm* expression (Queck et al., 2008). So far, not many alternative regulation pathways of PSMs have been reported. More precisely only one alternative regulator has been identified: MgrA. Jiang et al showed MgrA binds to the PSM promoter to inhibit PSM expression (Jiang et al., 2018). Whether (pp)pGpp leads to increased PSM expression via the de-repression of MgrA remains to be analyzed by Northern Blot analysis in different *mgrA* mutants expressing RSH-Syn. Presumable regulation mechanisms of (pp)pGpp-mediated *psm* and oxidative stress/ iron storage genes, will be discussed in the following chapters.

9.6 Regulatory mechanisms of (pp)pGpp on transcription of oxidative stress/ iron storage genes and *psm*/*agr*

Two possible regulatory mechanisms can be considered for (pp)pGpp dependent alteration of genes such as *ftnA*, *dps* and *psm*. (pp)pGpp could influence gene expression by indirectly affecting transcription depended on their initiation nucleotide, or by directly leading to a riboswitch.

9.6.1 (pp)pGpp may indirectly activates/inhibits transcription depending on the initiation nucleotide on position +1

Our results clearly indicate a link between the stringent response and the activation of the virulence gene *psm* and oxidative stress and iron overload genes *dps* and *ftnA*. Nucleotide measurement *in vitro* and *in vivo* resulted in increased ATP and decreased GTP level. We therefore speculated, whether activation or inhibition of genes is dependent on the initiation nucleotide on position +1 and if this correlates with the increase of ATP or decrease of GTP. The promoter regions were identified by Mäder et al (Mader et al., 2016) and transcription starts sites from available unpublished RNA-Seq data. Our predications suggest an A as an initiation nucleotide for *ftnA*, *dps*, *ahpC*, *katA* and *psm*β1/2 and a G for *agrA*/*C*/*D*. Promoter and initiation nucleotide for *psm*α1-4 were hard to identify, nevertheless we also suggest an A on position +1 (Fig.10). Indeed, the increased or decreased gene expression correlates with the level of intracellular ATP or GTP, except for *sodA*. This is in line with Krasny et al who postulated a gene expression mechanism dependent on the initiation nucleotide on position +1. Higher intracellular ATP level leads to the transcription of genes, which initiation nucleotide starts with an A at position +1. Vice versa this is true for genes starting with a G as an initiation nucleotide and increased intracellular GTP concentration in *Bacillus subtilis (Krasny et al., 2008)* and *Staphylococcus aureus* (Kastle et al., 2015). Nevertheless, this assumption needs to be clarified by further experiments. The transcriptional pattern of indicated genes needs to be analyzed upon increasing concentration of ATP or GTP.

If the initiation nucleotide is the reason for activation or inhibition, increasing the GTP/ATP ratio would lead to enhanced expression of the particular genes. Furthermore amino acid substitution changing the A to a G would show decreased or no difference in expression after (pp)pGpp synthesis.

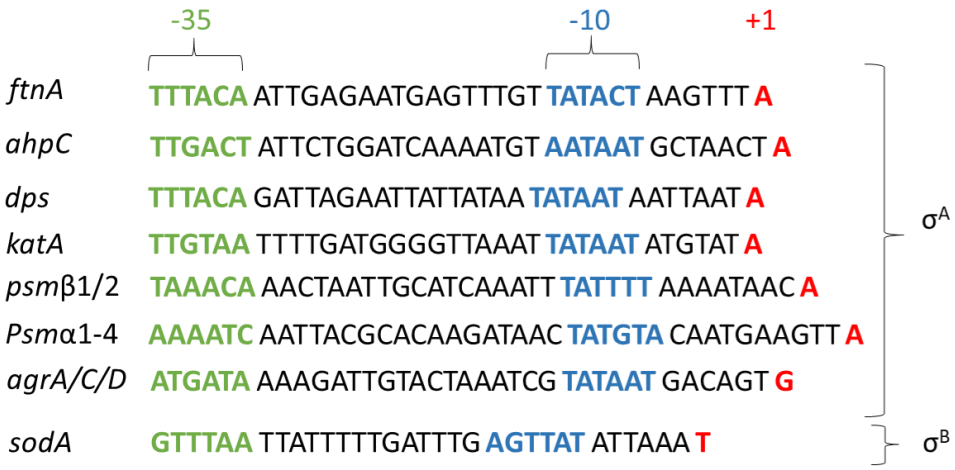

Fig.10 Predicted initiation nucleotides of oxidative stress, iron overload and virulence genes. The -35 region is indicated in green, the -10 in blue and the initiation nucleotide at position on in red. Promoter were identified and adapted by (Mader et al., 2016) and initiation nucleotide predicted from unpublished RNA-Seq data.

9.6.2 (pp)ppGpp might regulate gene expression by riboswitch

Recently, a (pp)pGpp-dependent riboswitch was shown *in vitro*. Sherlock et al showed that *ykkC* Motif RNA bind ppGpp at the anti-terminator stem and transcription of corresponding gene is increased (Sherlock, Sudarsan, & Breaker, 2018). There is evidence of a potential riboswitch for *psm*α by (pp)pGpp. The predicted sequence is based on their analyses and indeed preliminary data show similar riboswitch sequence for *psm*α (ACGGAAGGAGUAUAAUAAAAUGCUUAAUCAAUAUACUGAACAU-CAACCGACAACUUCAAAUAUUAUUAUUUUAUUAUACUCUUUAGGACUCGAAC-GUUAGUAAAUAUUUACUAAACGCUUUAAGUCCTATTTCTGTTTGAAUGGGACU-UGUAAACGUCCCAAUAAUAUUGGGACG) with one exception. The cytosine C71 between P2 and the antiterminator stem is missing. (Sherlock et al., 2018). Riboswitch mechanism is unlikely for *ftnA*, *dps*, *ahpC* and *sodA*, since a similar sequence is missing. This may explain an independent activation of *psmα* by (pp)pGpp. Here we suggest a novel regulatory mechanism of PSMα by (pp)pGpp. Further *in vivo* and *in vitro* experiments have to be performed proving PSMs are directly regulated by (pp)pGpp via riboswitch.

In conclusion, presumably *ftnA*, *dps*, *ahpC*, *kata*, *psm*β1/2 and the Agr system are indirectly regulated by (pp)pGpp through the shift of the nucleotide pool leading to their activation or inhibition depending on the initiation nucleotide +1. In contrast, PSMα are

likely regulated in a direct manner trough riboswitch mechanism by (pp)pGpp binding to the antiterminator stem allowing *psm*α transcription independent of Agr.

9.7 RelQ is less active than RelP *in vivo*

Nucleotide measurements and Northern blot analysis after transcriptional induction of RelQ revealed RelQ is much less active compared to RelP *in vivo*. Recently biochemical structure analyses showed RelQ from *B. subtilis* ($RelQ_{Bsu}$) and *E. faecalis* ($RelQ_{Efa}$) forms homotetramers (Beljantseva et al., 2017; Steinchen et al., 2015) and is inhibited by a single stranded mRNA in *Enterococcus faecalis (Beljantseva et al., 2017)*. This RelQ:RNA complex dissociates by interacting with pppGpp and to a lesser extend ppGpp leading to an activation of RelQ (Beljantseva et al., 2017).We presume low activity of RelQ derives from the mRNA:RelQ complex. Overexpressed RelQ is mainly trapped in the RelQ:RNA complex. Since RelQ was overexpressed in a (pp)$pGpp^0$ background no pppGpp is available for dissolving this complex and RelQ is mainly kept in an inactive state. The weak detectable basal ppGpp signal probably derived from some RelQ homotetramers, which are not inhibited by mRNA and can bind GDP and ATP to synthesize ppGpp. This is in line with Steinchen et al. who showed $RelQ_{Bsu}$ forms homotetramers and synthesizes ppGpp from GDP and ATP *in vitro* (Steinchen et al., 2015; Steinchen et al., 2018). However, this may explain a strong activity of RelQ *in vitro* because inhibiting mRNA is not present in the activity assay.

9.8 RelQ induces genes for cell wall synthesis

Although transcriptional changes were not as drastic in RelQ as for RSH-Syn, there were still some genes which appeared to be specifically regulated by RelQ (Table 2). Interestingly, genes for lysine and alanine synthesis (*dapD, dapL, lysA*) and *alr2* (alanine recemase) (Kullik et al., 1998) are up-regulated. L-lysine and D-alanine are part of the peptidoglycan (PG) and wall teichoic acid (WTA) (Rajagopal & Walker, 2017). The glycopeptide antibiotic vancomycin targets the di-peptide D-ala-lipid II complex thereby inhibiting PG synthesis (McGuinness, Malachowa, & DeLeo, 2017; Nagarajan, 1991). Ampicillin inhibits the transpeptidase and blocks the connection of the penta-glycine peptide bridge between L-lysine and D-alanine leading to an unstable PG. It has been already shown, that RelQ and RelP are transcriptional activated upon cell wall stress by the VraR/S system, which is induced by vancomycin and ampicillin (Geiger et al., 2014).

It is likely, that after vancomycin or ampicillin treatment *S. aureus* needs a rapid way to encounter antibiotic damage. The stringent response is a mechanism known for a rapid change of metabolism in general. The VraR/S system activates RelQ leading to (pp)pGpp synthesis and presumably activation of these genes, to ensure a quick fixation of broken stem peptide, missing trans-peptidation and to thicken the cell wall. It has been reported, a Δ*vraRS* mutant showed a thinner cell wall compared to the wild type (C. Gao et al., 2019). To investigate this hypothesis, I compared a wild type strain with a Δ*relPQ* mutant challenged with vancomycin. Strain were challenged secondly with vancomycin after indicated regrowth. The wild type showed immediate growth, indicating stabilization of the cell wall succeeded, leading to vancomycin tolerance. In contrast, second addition of vancomycin resulted in slow growth and no tolerance in the Δ*relPQ* mutant. These results consolidate the hypothesis, that cell wall stress activates VraS/R followed by transcriptional activation of RelP/Q (Geiger et al., 2014). This in turn activates genes (*dapD, dapL, lysA, alr2*) to repair, thicken and/ or prepare the cell wall for future damage by antibiotics. Nevertheless, further experiments have to be performed, to confirm the hypothesis. Recently, Matsuo et al showed cell wall stress activates RelQ which in turns lead to increased expression of *ehoM* and to vancomycin tolerance (M. Matsuo, N. Yamamoto, et al., 2019). Growth and Northern blot analyses with combinations of *dapD, dapL, lysA* or *alr2* mutants challenged with vancomycin will elucidate whether RelP/Q contribute to tolerance via activation of *dapD, dapL, lysA, alr2* followed by *ehoM* activation or vice versa.

9.9 Synthesis of the new alarmone pGpp

Here I detected the unusual alarmone pGpp using HPLC which could successfully discriminate between GTP and pGpp based on slight differences in retention time. pGpp was detected *in vitro* using purified RelQ and mixture of ATP, GMP, GDP and GTP in equal molar ratio as a substrate. Purified RelP was equally active in synthesizing ppGpp and pppGpp but detection of pGpp failed. This is in line with Gaca et al. and Ruwe et al, who both detected pGpp synthesis by SAS, presumably RelQ, *in vitro* in *Enterococcus faecalis* (Gaca, Kudrin, et al., 2015) and *Corynebacterium glutamicum* (Ruwe et al., 2017) . In contrast, *in vivo* pGpp was detectable only after transcriptional induction of RSH-Syn or by inducing stringent response in the wild type by mupirocin. Not detected pGpp after RelQ induction *in vivo* was presumably due to RelQ's inactivity under our conditions. Thus, RSH but also presumably RelQ

significantly synthesize pGpp under yet unknown conditions. The function of pGpp within the stringent response is largely unknown. Of note, there are some older reports of the accumulation of pGpp in *E. coli* (Pao & Dyess, 1981; Pao & Gallant, 1979), *B. subtilis* (Nishino, Gallant, Shalit, Palmer, & Wehr, 1979) and *S. aureus* (Crosse et al., 2000). Due to technical limitations in separation of GTP, pGpp or ppGp, detection of ppGp cannot be excluded. To clearly separate pGpp from ppGp by comparing the retention time of purified GTP, pGpp and ppGp by HPLC or treating purified nucleotides by alkaline hydrolysis. pGpp is sensitive to alkaline hydrolysis whereas ppGp is not (Pao & Gallant, 1979), Nevertheless, considerably a mixture of both nucleotides is possible, too (Nishino et al., 1979).

Previous studies show ppGp inhibits IMP dehydrogenase, adenylosuccinate synthetase and phosphoenolpyruvate carboxylase in *E. coli* (Pao 1981). Not much is known about the function of pGpp. In *E.faecalis* pGpp inhibits GTPases GMK1 and 2 and in *E. coli* to a lesser extend *rrnB1* P1 promoter (Gaca, Kudrin, et al., 2015). The role of pGpp or ppGp in *S. aureus* remains still unknown and has to be analyzed more in detail. Nevertheless, we demonstrated pGpp synthesis by RSH *in vivo* for the first time.

9.10 RSH mainly functions as a (pp)pGpp hydrolase

RSH_{Sau} shares a similar structure to other RSH enzymes. It can be separated in two major domains. The N-terminus bears a functional hydrolase and synthetase domain, while the C-terminus consists of three conserved motifs (TGS, ACT and the DC domain). Many studies were performed elucidating the function of the N-terminal domain (Hogg et al., 2004; Mechold, Cashel, Steiner, Gentry, & Malke, 1996; Mechold et al., 2002).

These studies do not explain the molecular switch between the two enzymatic activities and role of the C-terminus. Previous studies demonstrated the essentiality of the hydrolase domain in *S. aureus in vivo* under relaxed conditions (Geiger et al., 2014). A conditional RSH hydrolase mutant (rsh_{cond}) is not able to grow due to constitutive (pp)pGpp synthesis by RelQ and RelP (Geiger et al., 2014).

Complementation of rsh_{cond} with full-length or truncated RSH restored growth inhibition due to a functional hydrolase, indicating the C-terminus is not essential for hydrolase activity. Incubation of (pp)pGpp with of full-length or truncated RSH *in vitro* resulted in complete (pp)pGpp degradation, indicating a minor role of the C-terminus on hydrolase

activity *in vitro*. This is in line with the results obtained *in vivo* and demonstrate a strong hydrolase and weak synthetase activity under relaxed conditions.

This is in contrast to recent studies in *Rhodobacter capsulatus*, which showed ACT, which is part of the C-terminus, has an influence on hydrolase activity *in vitro* and *in vivo*. Mutations in the ACT domain lead to enhanced synthetase activity (Fang & Bauer, 2018). However, the C-terminus and especially the ACT domain do unlikely influence hydrolase activity in *S. aureus*. We have not observed a stronger synthetase activity in RSH C-terminus deletion and ACT mutants, which would have indicated an influence on hydrolase activity.

9.11 (pp)pGpp synthesis is dependent on the C-terminus in *S. aureus*

In *S. aureus* RSH combines two enzymatic functions, which can be found in RelA and SpoT from Proteobacteria such as *E. coli*. (pp)pGpp by RSH_{Sau} is essential to trigger changes in cellular process to overcome amino acid starvation by sensing uncharged tRNA at the ribosome, as it has been shown for RelA in *E. coli* (Haseltine & Block, 1973; Wendrich et al., 2002). On the other hand RSH shows strong hydrolase activity, similar to SpoT. This essential to avoid toxic (pp)pGpp accumulation by RelP and RelQ in *S. aureus*. Therefore, a tight regulation between the two enzymatic activities is necessary in order to ensure an appropriate level of (pp)pGpp. Previous studies regarding enzymatic activity of different RSH enzyme were performed by expressing RSH in *E. coli* and indicated a strong synthetase activity of RSH enzymes without a C-terminus (Bag, Das, Dasgupta, & Bhadra, 2014; He et al., 2013; Mechold et al., 2002). We expressed different RSH in an *E. coli relA/spoT* mutant and growth was monitored in/on minimal medium/agar plates.

In line with previous results, RSH w/wo the C-terminus could complement the *relA*/*spoT* mutant, indicating (pp)pGpp synthesis.

However, these results were in contrast to our results performed with the same constructs in the native background of *S. aureus*. RSH lacking the C-terminus is kept in a hydrolase-ON/synthetase-OFF conformation and synthetase activity could be only monitored by mutating the hydrolase domain. These results show two different enzymatic activities using the exact same RSH enzyme. It would be interesting, whether this discrepancy between *E. coli* and the native background appears in other bacteria, too. This discrepancy likely derives from unknown interaction partners with the N-terminus in *E. coli*. So far, our results indicate a minor role of the C-terminus on

hydrolase activity under relaxed condition. Since RSH_{Sau} synthesizes (pp)pGpp as a reaction to amino acid starvation, we compared growth behavior and transcription of stringent response associated genes of the full-length and truncated RSH by mimicking stringent condition with mupirocin. RSH without C-terminus was not able to react to amino acid starvation, indicating no (pp)pGpp synthesis. This was further supported by nucleotide measurement of full-length and truncated RSH *in vitro*. (pp)pGpp synthesis was not detectable in RSH without the C-terminus. Conclusively, the C-terminus is indispensable for the molecular switch from hydrolase-ON/synthetase-OFF to hydrolase-OFF/synthetase-ON under stringent conditions.

9.11.1 (pp)pGpp synthesis is dependent on TGS and DC, but not ACT

However, we further wanted to elucidate which domain of the C-terminus is essential for a correct activation of the synthetase. Therefore we analyzed different RSH enzymes bearing mutations in TGS, DC or ACT and followed growth and transcription of stringent response genes under relaxed and stringent condition. All three mutants showed normal growth behavior and same transcription as the WT under relaxed condition. This led us to the conclusion, that TGS, DC and ACT are not essential and do not have an influence on the hydrolase activity under relaxed conditions.
Under stringent mimicking conditions, the DC and TGS mutants were not able to respond to stringent conditions, indicating no (pp)pGpp synthesis.

9.11.1.1 The DC domain regulates synthetase activity through oligomerization

The DC domain harbors three conserved amino acids, which were proposed to be responsible for oligomerization of RelA. Mutation of these amino acids led to enhanced synthetase activity due to the disassociation from inactive oligomers into active monomers in *E. coli* (Gropp et al., 2001; Yang & Ishiguro, 2001) and *M. tuberculosis* (Avarbock et al., 2005; Jain et al., 2007). Mutation of these amino acids in RSH_{Sau} did not result in activation of the synthetase under relaxed conditions. This would have indicated the disassociation of RSH from inactive oligomers into active monomers. No synthetase activity was observed under stringent conditions. We suggest, that the DC domain in *S. aureus* is likely responsible for oligomerization of free RSH for autoinhibition under relaxed condition but anchors RSH to stalled ribosomes, which

results in (pp)pGpp synthesis, presumably trough disassociation into active monomers. This is supported by our Co-IP studies, which show interaction of DC with a variety of ribosomal proteins. This is line with studies performed in $RelA_{Ec}$, where Cys-612 is important for binding of $RelA_{Ec}$ to the ribosome (A. Brown et al., 2016) resulting in (pp)pGpp synthesis when bound to stalled ribosome (Kudrin et al., 2018).

9.11.1.2 TGS regulates synthetase activity by sensing uncharged tRNAs and interaction with the ribosome

As already mentioned, no synthetase activity can be detected in a TGS mutant. Presumably TGS regulates synthetase activity by sensing uncharged tRNAs and interaction with the ribosome as it has been shown for $RelA_{Ec}$. Since no data are available for RSH_{Sau}, we performed Co-IP to rule out, whether interactions of RSH with the ribosome are also indicated. Indeed we have found several ribosomal proteins, indicating interaction of RSH_{Sau} with the ribosome via TGS, DC and ACT. Here we suggest, the C-terminus interacts with the ribosome and sensing of uncharged tRNAs occurs via TGS in *S. aureus*. This is line with different studies, which has been performed in *E. coli* RelA (Arenz et al., 2016; A. Brown et al., 2016; Loveland et al., 2016).

Furthermore, our presumptions are supported by recent Cryo-EM studies from *E. coli* RelA C-terminus, which binds to stalled ribosome. TGS binds uncharged tRNA (Agirrezabala et al., 2013) (A. Brown et al., 2016) (Loveland et al., 2016) in its center thereby sensing amino acid starvation.

As a consequence, synthetase domain gets closer to the spur of the ribosomal 30S subunit and is activated (Arenz et al., 2016; A. Brown et al., 2016; Loveland et al., 2016) presumably by a conformational change resulting in an open complex and maximal catalytic activity of the synthetase (Arenz et al., 2016). There is evidence TGS recognizes uncharged tRNA before entering the ribosome (Arenz et al., 2016; Kushwaha, Bange, & Bhavesh, 2019) , carrying the tRNA-RSH complex to the ribosome.

9.11.1.3 ACT does not affect synthetase activity but interacts with the ribosome

We could not detect any difference in growth under stringent conditions for the ACT mutant compared to the WT, indicating ACT does not have a regulatory effect on the synthetase activity.

Our Co-IP studies indicated an interaction of ACT with the ribosomal protein L16. This is in line with *E. coli* ACT interacting with the 23S rRNA, ribosomal protein L16 and the elbow region of tRNAs (Arenz et al., 2016; A. Brown et al., 2016; Loveland et al., 2016). The role of ACT in *S. aureus* remains still unclear, although studies showed binding of the branched chain amino acids valine and isoleucine to the ACT domain in *Rhodobacter capsulatus* (Fang & Bauer, 2018). They additionally performed ITC with the C-terminal domain of *S. aureus* which exhibited leucine binding to the C-terminus. Nevertheless, these data do not indicate which domain of the C-terminus binds leucine. However, here we suggest TGS is the major sensor for deacetylated tRNAs and activator of the synthetase and a minor role of ACT for a correct activation of the stringent in *S. aureus*. ACT assumable acts as an anchor between uncharged tRNA and the ribosome (Fig.5).

In conclusion, this clearly demonstrates synthesis and degradation of (pp)pGpp is a tightly regulated system. Under relaxed conditions RSH is in a hydrolase-ON/synthetase-OFF state. The C-terminus switches the hydrolase off and activates the synthetase for a rapid respond to stringent conditions via TGS and DC. The DC domain autoregulates synthetase activity by oligomerization and interacts with the ribosome. The necessity of activating the synthetase is regulated by TGS presumably via sensing uncharged tRNAs at the ribosome. ACT does not affect any of the two enzymatic activities of RSH_{Sau} and the function remains still elusive. Presumably, ACT has a stabilizing function of the RSH-uncharged-tRNA-ribosome complex.

10 Appendix

10.1 Publication 1

Steinchen W, Vogt MS, Altegoer F, Giammarinaro PI, **Horvatek P**, Wolz C, Bange G "Structural and mechanistic divergence of the small (p)ppGpp synthetases RelP and RelQ." Scientific Reports 2018 Feb 1;8(1):2195.doi: 10.1038/s41598-018-20634-4

SCIENTIFIC REPORTS

OPEN

Structural and mechanistic divergence of the small (p)ppGpp synthetases RelP and RelQ

Received: 29 November 2017
Accepted: 22 January 2018
Published online: 01 February 2018

Wieland Steinchen[1], Marian S. Vogt[1], Florian Altegoer[1], Pietro I. Giammarinaro[1], Petra Horvatek[2], Christiane Wolz[2] & Gert Bange[1]

The nutritional alarmones ppGpp and pppGpp (collectively: (p)ppGpp) are nucleotide-based second messengers enabling bacteria to respond to environmental and stress conditions. Several bacterial species contain two highly homologous (p)ppGpp synthetases named RelP (SAS2, YwaC) and RelQ (SAS1, YjbM). It is established that RelQ forms homotetramers that are subject to positive allosteric regulation by pppGpp, but structural and mechanistic insights into RelP lack behind. Here we present a structural and mechanistic characterization of RelP. In stark contrast to RelQ, RelP is not allosterically regulated by pppGpp and displays a different enzyme kinetic behavior. This discrepancy is evoked by different conformational properties of the guanosine-substrate binding site (G-Loop) of both proteins. Our study shows how minor structural divergences between close homologues result in new functional features during the course of molecular evolution.

Microorganisms are able to cope with a broad variety of environmental challenges such as nutrient limitation, antibiotics or changes in abiotic factors like varying pH values or temperatures. To do so, they adapt their metabolism at many different dogmatic processes, e.g. replication, transcription, translation and ribosomal biogenesis[1–3]. The 'stringent response' (SR) is highly conserved among bacteria[4–6] and plant chloroplasts[7–9] and although historically only referring to the adaptation to nutrient depletion[10,11] it has since also been demonstrated to affect virulence[2,12,13], biofilm formation[14], development of cellular heterogeneity[15,16]. Moreover, in some microorganisms the SR has been suggested to affect persister cell formation[17–19]. Central to the stringent response are the two unusual nucleotides ppGpp and pppGpp (collectively (p)ppGpp or alarmones). Proteins of the RelA/SpoT homology (RSH) superfamily[20] catalyze the pyrophosphate transfer from ATP onto the 3′-OH group of GDP or GTP, yielding ppGpp or pppGpp, respectively.

RSH-type synthetases fall into the two classes of 'long' and 'short' RSH (Fig. 1a[1,20]). Long RSH-type synthetases are typically composed of multiple domains and harbor a (p)ppGpp hydrolase followed by a (p)ppGpp synthetase domain in their N-terminal part (NTD). Their C-terminal portion (CTD) is highly variable and comprises domains involved in the binding of ribosomes and regulation of the opposing activities found within the NTD[21–24]. In contrast, short RSH-type alarmone synthetases only contain a synthetase domain and lack the hydrolase domain as well as regulatory domains found within the CTD of long RSH proteins (Fig. 1a). Members of this 'small alarmone synthetase' (SAS) family fall into the RelQ (also: SAS1) and RelP (also: SAS2) subclasses and are found in a wide range of bacteria including *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Listeria monocytogenes*[20,25–30]. Furthermore, there is evidence for a third class of SAS proteins named RelV in *Vibrio cholerae*[31]. Noteworthy, SAS proteins typically occur in pairs (RelP and RelQ) in the same organism. Nevertheless, despite being highly similar on the amino acid sequence level (Fig. 1a), RelP/RelQ proteins seem to exhibit different functional roles as evidenced from disparate transcriptional profiles and their dependence on different stress signals[25,27,32].

So far, only RelQ from *B. subtilis* and *Enterococcus faecalis* have been functionally characterized[29,30,33]. *Bs*RelQ shares the conserved synthetase fold with the long RSH Rel, but in contrast to the monomeric Rel, *Bs*RelQ forms highly symmetric homotetramers. Clarification of the catalytic mechanism of *Bs*RelQ showed that the

[1]Philipps-University Marburg, LOEWE Center for Synthetic Microbiology & Department of Chemistry, Hans-Meerwein-Straße, 35043 Marburg, Germany. [2]University of Tübingen, Interfaculty Institute of Microbiology and Infection Medicine, Elfriede-Aulhorn-Straße 6, 72076 Tübingen, Germany. Correspondence and requests for materials should be addressed to W.S. (email: wieland.steinchen@synmikro.uni-marburg.de) or G.B. (email: gert.bange@synmikro.uni-marburg.de)

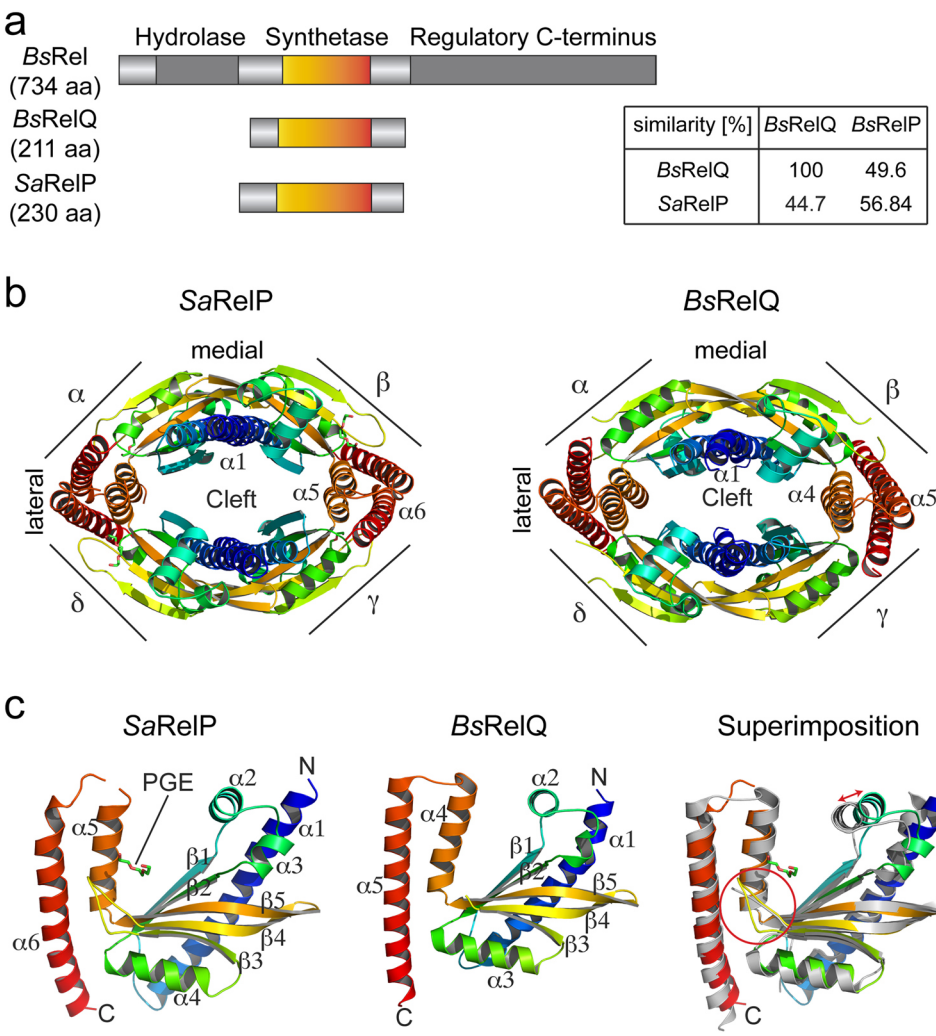

Figure 1. Structural analysis of RelP. (**a**) Domain architecture of the (p)ppGpp synthetases *Bs*Rel, *Bs*RelQ and *Sa*RelP drawn to scale. The inset depicts amino acid similarities between RelP and RelQ proteins from *Bacillus subtilis* (*Bs*) and *Staphylococcus aureus* (*Sa*). (**b**) Cartoon representation of the crystal structures of the *Sa*RelP (*this study*) and *Bs*RelQ (PDB: 5DEC[33]) homotetramers. Each monomer (α-δ) is rainbow-colored from its N- to its C-terminus. (**c**) The (p)ppGpp synthetase monomers of *Sa*RelP (*left; this study*), *Bs*RelQ (*middle*; PDB: 5DEC[33]) and their superimposition (*right*) coloured in rainbow from N- to C-terminus.

enzyme binds ATP and GDP/GTP in a sequential order with ATP being the first substrate and arranges them in a near-attack conformation within the active site to catalyze immediate pyrophosphate transfer. A remarkable feature of the *Bs*RelQ homotetramer is the presence of a pronounced cleft in its center providing the binding site for two allosteric pppGpp molecules that, when present, elevate the (p)ppGpp synthetase activity of *Bs*RelQ[33]. Up to date, no structural characterization of RelP proteins is available. Also, it is unknown whether the (p)ppGpp synthesizing activity of RelP is subject to regulation. Therefore, we set out to provide a structural and biochemical comparison of RelP/RelQ proteins that might explain their divergent functional roles in bacteria.

Results

RelP and RelQ share an equal architecture. To better understand RelP at the molecular level, we determined the crystal structures of RelP homologues from *S. aureus* (*Sa*) and *B. subtilis* (*Bs*) at 2.25 and 3.3 Å resolution, respectively (Table S1). Both, *Sa*RelP and *Bs*RelP form highly symmetrical and oval-shaped homotetramers with a prominent cleft in their centers highly reminiscent of *Bs*RelQ (Figs 1b and S1a). Helix α1 at the N-terminus of each monomer stabilizes the medial sides of the homotetramer interface via hydrogen bonds and salt bridges (buried surface area of ~1200 Å^2). Helices α5 and α6 at the C-terminus of each monomer establish the lateral sides of the homotetramer interface mainly due to polar contacts (buried surface area of ~1200 Å^2). The (p)ppGpp synthetase monomers of *Sa*RelP and *Bs*RelP are highly identical and consist of a mixed β-sheet build by five β-strands (β1–β5) that is surrounded by alpha helices (α1–α6, Figs 1c and S1b).

Structural comparison of RelP and RelQ reveals the architecture of the homotetramer as well as each of the monomers is highly similar (r.m.s.d. of 1.292 over 138 Cα atoms for the RelP and RelQ monomers). However,

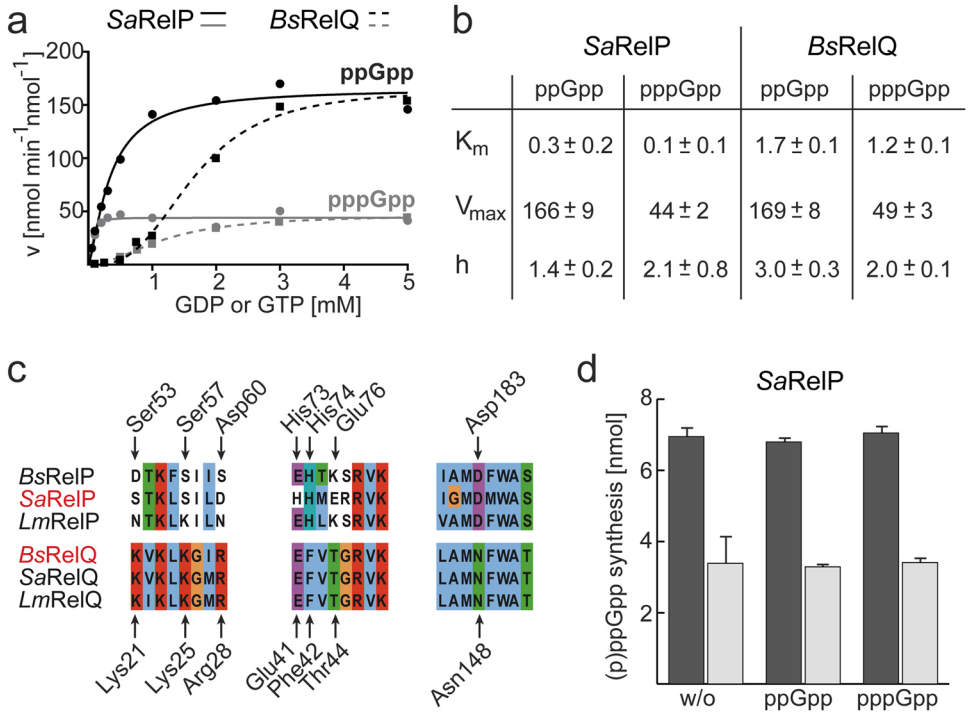

Figure 2. Enzymatic properties of RelP. (**a**) Velocity/substrate (v/S) characteristic of *Sa*RelP (solid lines) and *Bs*RelQ (dashed lines) for ppGpp (black) and pppGpp (grey). Velocity is given in nmol per minute per nmol *Sa*RelP/*Bs*RelQ. The data for *Bs*RelQ have been re-plotted from[33] to enable direct comparison of both enzymatic activities. Data of one representative experiment are shown. (**b**) Kinetic parameters of (p)ppGpp synthesis by *Sa*RelP and *Bs*RelQ. (**c**) Amino acid sequence alignment of residues conferring pppGpp binding to the allosteric cleft of RelQ and their equivalent positions in RelP proteins from *Bacillus subtilis* (*Bs*), *Staphylococcus aureus* (*Sa*) and *Listeria monocytogenes* (*Lm*). Amino acid numberings relate to *Sa*RelP (above) and *Bs*RelQ (below). (**d**) Synthesis of ppGpp (black) and pppGpp (grey) by *Sa*RelP is unaffected by the presence of ppGpp or pppGpp. Error bars indicate the SD of three independent replicates.

RelP and RelQ differ in the orientation of helix α2, which appears to be shifted approximately 3 Å towards the active site center in RelQ when compared to RelP (Fig. 1c; *right panel*). Another interesting observation is that the loop connecting β3 and β4, which is disordered in the structure of *Bs*RelQ, could be resolved in both structures of RelP (Fig. 1c). Taken together, RelP and RelQ share highly conserved ternary and quaternary structures, but also reveal subtle differences that might be of functional relevance (see below).

RelP and RelQ differ in their (p)ppGpp synthetase activity. The most distinguished features of *Bs*RelQ lie in the apparent positive cooperativity of (p)ppGpp synthesis and its susceptibility to allosteric stimulation of by pppGpp but not ppGpp[33]. To test whether both features would also be present in RelP, we performed an *in-depth* kinetic analysis. We used the same buffer composition for characterization of *Sa*RelP as previously for *Bs*RelQ to ensure maximal comparability. *Sa*RelP was incubated together with 5 mM ATP and varying concentrations of GDP or GTP (Of note: *Bs*RelP exhibited no (p)ppGpp synthetase activity under our assay conditions for unclear reasons). *Sa*RelP synthesized ppGpp more efficiently than pppGpp as evidenced from an approximately 4-fold higher V_{max} value (Fig. 2a and b). A similar preference for the product ppGpp was previously observed for *Bs*RelQ[33] and RelQ from other organisms[25,30]. However, the K_m values for (p)ppGpp synthesis drastically differ between both enzymes in that they are significantly lower for *Sa*RelP (i.e. 0.3 ± 0.2 for GDP and 0.1 ± 0.1 for GTP) than for *Bs*RelQ (i.e. 1.7 ± 0.1 for GDP and 1.2 ± 0.1 for GTP; Fig. 2b). It also seemed to us that *Sa*RelP monomers displays less cooperativity within the tetramer than *Bs*RelQ indicated by Hill coefficients closer to 1 (Fig. 2b).

Amino acid sequence analysis of RelP shows that the amino acid residues required for allosteric binding of pppGpp to RelQ are replaced in RelP proteins (Fig. 2c). Indeed, this different set of amino acids found in *Sa*RelP seems incapable to coordinate pppGpp in similar fashion as *Bs*RelQ (Fig. S2) strongly suggesting to us that *Sa*RelP cannot be allosterically stimulated by the alarmone. In agreement with our structural analysis, no change in the enzymatic activity of *Sa*RelP was observed in the absence and the presence of ppGpp or pppGpp (Fig. 2d). Taken together, RelQ and RelP do not differ much in their V_{max} values of (p)ppGpp synthesis, while significantly differing in the in K_m values. Moreover, RelP is not subject to allosteric stimulation by pppGpp.

ATP-binding to RelP and RelQ is identical. To gain further insights into the disparate enzymatic activities of RelQ and RelP, we attempted to solve the structure of *Sa*RelP in presence of the non-hydrolysable ATP analogue AMPCPP (α,β-methyleneadenosine 5′-triphosphate) and GDP or GTP. However, we could only obtain crystals and solve the structure of *Sa*RelP in presence of AMPCPP (Fig. S3a and Table S1). Coordination of

AMPCPP within all the four active sites of *Sa*RelP is guided by π-stacking interactions of the adenine base with the arginine residues 78 and 112 of *Sa*RelP (Fig. S3b). The ribose moiety of the adenosine is coordinated by hydrogen bonding via His190. Interactions with the phosphate moieties of AMPCPP are mainly established by lysine and arginine residues residing in β1 and α2 (i.e. Lys80, Lys88 and Arg91) and Ser84 contacting the 5′ α-phosphate. AMPCPP adopts a kinked conformation that is enforced by a magnesium ion coordinated by Asp107 and Glu174 (Fig. S3b). An identical conformation of AMPCPP is observed in the active site of *Bs*RelP (Fig. S3c). As all ATP-coordinating and catalytic amino acid residues are strictly conserved among RelP/RelQ proteins (Fig. S3d), we suspect a common ATP-binding mode and mode of catalysis.

G-Loop rigidity governs the activity of RelP and RelQ. If binding of ATP to RelP and RelQ is identical (see above), then the different enzymatic properties of both enzymes should originate from differences in binding of GDP/GTP and/or a different susceptibility to allosteric stimulation by pppGpp. As mentioned above, our structural analysis of RelP and RelQ indicated a different conformational flexibility of the loop connecting strands β3 and β4 (Fig. 1c). This loop contains a conserved tyrosine residue (i.e. Tyr151 in *Sa*RelP and Tyr116 in *Bs*RelQ, Fig. 3a) critical to guanosine nucleotide binding in all (p)ppGpp synthetases. Therefore, we decided to term the loop connecting β3 and β4 'G-Loop'. To our surprise, the different configurations of the G-Loop seem to be a common theme among RelP/RelQ proteins. In the apo- and ATP-bound states of *Bs*RelQ, the G-Loop is disordered, and could therefore not be modeled in these structures (Fig. 3b). In stark contrast, the G-Loop of *Sa*RelP was well-ordered and could be unambiguously modeled in its apo- and ATP-bound structures (Fig. 3c). We speculated that the difference in enzymatic activity between RelQ and RelP is founded in the different conformational properties of the G-Loop.

Inspection of the amino acids of the G-Loop reveals the presence of proline in RelP proteins with no correspondent in RelQ (Fig. 3a). We hypothesized that the absence of this proline in RelQ renders the G-Loop less rigid, while its presence in RelP results in a well-ordered G-Loop that might easily facilitate GDP/GTP coordination (Fig. 3d). We challenged this notion by introducing proline into the disordered G-Loop of RelQ (i.e. *Bs*RelQ-H111P). *Bs*RelQ-H111P produces (p)ppGpp as efficient as *Sa*RelP and the V_{max} (i.e. 243 ± 9 and 194 ± 8 nmol min^{-1} $nmol^{-1}$ for ppGpp and pppGpp, respectively), K_m (i.e. 0.4 ± 0.2 for GDP and 1.9 ± 0.2 for GTP) and Hill-coefficient (i.e. 1.6 ± 0.2 for GDP and 1.0 ± 0.1 for GTP) of *Bs*RelQ-H111P more resemble *Sa*RelP than *Bs*RelQ (Fig. 3e and compare to Fig. 2a and b). Moreover and unlike *Bs*RelQ, *Bs*RelQ-H111P is not amenable to allosteric stimulation by pppGpp (Fig. 3f). These results demonstrate a strong dependence of RelP/RelQ activity on the rigidity of the G-Loop.

Allosteric stimulation of RelQ by pppGpp acts via the G-Loop. Our results indicated that RelP proteins synthesize (p)ppGpp more efficiently than RelQ, because RelP can more readily bind the GDP/GTP substrate through increased rigidity of the G-Loop. Moreover, pppGpp stimulates the activity of RelQ, while it does not for RelP (Figs 2d and 3f). Therefore, we hypothesized that binding of pppGpp to the central cleft of RelQ might be translated into an increased (p)ppGpp synthesis via the G-Loop. Superimposition of the crystal structures of apo-*Bs*RelQ and pppGpp-bound *Bs*RelQ (PDB: 5DEC and 5DED[33], respectively) allowed tracing a structurally possible path, which would connect the presence of pppGpp within the allosteric cleft of RelQ with the G-loop (Fig. 4). In short, two opposing subunits of the *Bs*RelQ tetramer are involved in coordination of one allosteric pppGpp in the central cleft[1,33]. Coordination of pppGpp leads to a displacement of Phe42, Thr44 and Asn148 by ~1–2 Å towards the cleft (Figs 4b; S2). Helix α4 comprising Asn148 follows this movement and rotates by approximately 15° in a counterclockwise manner. This movement is relayed onto helix α5 through the hydrophobic core between both helices constituted by Phe149 (α4), Leu183 and Met187 (both α5, Fig. 4c). Rotation of α5 turns Glu178 towards the G-Loop and enables formation of a salt bridge between Glu178 and Arg117 (Fig. 4c). Further contacts between α5 and the G-Loop are established between His111/Glu178 and Glu113/Gln174 (Fig. 4d).

To probe the participation of these amino acids, we replaced them by alanine and measured the (p)ppGpp synthesis of the resulting *Bs*RelQ variants in pppGpp-dependent manner (Figs 4e and S4). Variation of His111 and Glu113 does not affect stimulation of *Bs*RelQ. However, upon replacement of Gln174 and Glu178 the pppGpp-stimulatory effect is decreased and completely abolished when Arg117 is replaced (Figs 4e and S4).

Finally, we tested how the allosteric pppGpp affects the enzyme kinetic behaviour of *Bs*RelQ by determining the (p)ppGpp synthesis *Bs*RelQ in presence of different concentrations of pppGpp (i.e. 0, 2.5, 10, 25, 100, 250 µM). While addition of increasing amounts of pppGpp to *Bs*RelQ does only slightly elevate V_{max} of (p)ppGpp synthesis, the K_m values for the substrates GDP and GTP decrease dramatically (Figs 4f and S5). Also, *Bs*RelQ displays a less cooperative behaviour indicated by a loss of the sigmoidal shape of the v/S characteristic when pppGpp is present. It therefor appears to us that the apparent cooperativity of *Bs*RelQ rather originates from pppGpp produced during the enzymatic reaction rather than from a positive cooperativity between the four active sites of *Bs*RelQ (compare to Fig. 2a and ref.[29]). Noteworthy, at the highest concentration of pppGpp tested (i.e. 250 µM), the enzyme kinetic behavior of *Bs*RelQ is highly similar to *Bs*RelQ-H111P and *Sa*RelP.

These results show that allosteric binding of pppGpp causes structural rearrangements of *Bs*RelQ that are translated into an increased (p)ppGpp synthetase activity via an induced structural rigidity of the G-Loop.

Discussion

Two small alarmone synthetases (i.e. RelP/SAS2 and RelQ/SAS1) are typically found together in members of the Firmicutes phylum e.g. *B. subtilis*, *S. aureus* or *L. monocytogenes*[20]. RelP and RelQ share similarities of ~50 percent on the amino acid sequence level. Our structural analysis shows that RelP and RelQ possess a highly similar (p)ppGpp synthetase domain and both establish highly similar homotetrameric complexes (Fig. 1b and c). Nevertheless, both enzymes decisively differ in their ability to produce (p)ppGpp in that RelP is much more active

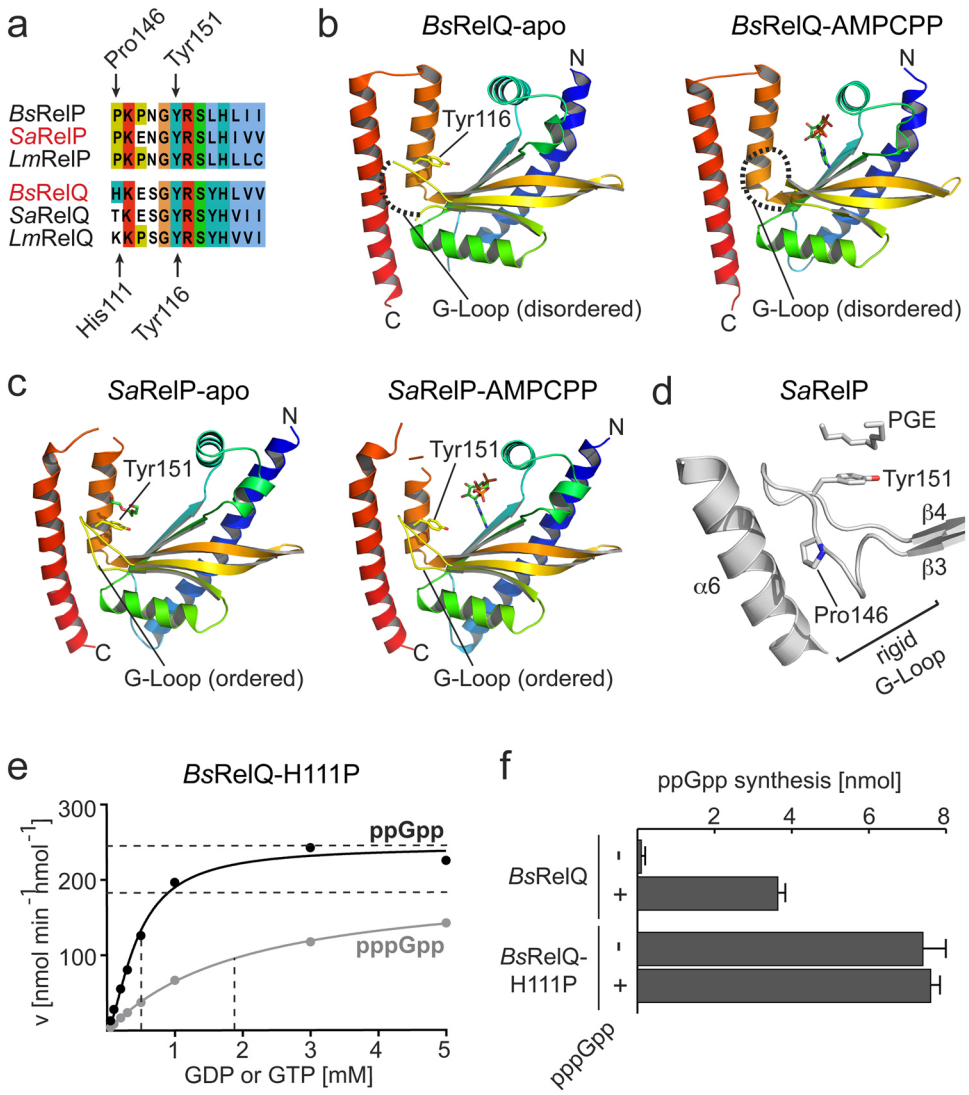

Figure 3. G-loop rigidity dictates the activity of RelP and RelQ. (**a**) Amino acid sequence alignment of the G-Loops found in RelP and RelQ proteins from *Bacillus subtilis* (*Bs*), *Staphylococcus aureus* (*Sa*) and *Listeria monocytogenes* (*Lm*). Amino acid numberings relate to *Sa*RelP (above) and *Bs*RelQ (below). (**b**) Crystal structures of the apo- and AMPCPP-bound state of *Bs*RelQ (PDB: 5DEC and 5F2V[33], respectively) show a disordered G-Loop (dashed line). (**c**) Crystal structures of the apo- and AMPCPP-bound state of *Sa*RelP (this study) show a clearly ordered G-Loop. (**d**) The presence of Pro146 in *Sa*RelP confers a high rigidity of the G-Loop. (**e**) The v/S characteristic of ppGpp (black) and pppGpp (grey) synthesis of the *Bs*RelQ-H111P variant. Velocity is given in nmol per minute per nmol *Bs*RelQ-H111P. Dashed lines indicate the K_m and V_{max} values. Data of one representative experiment are shown. (**f**) ppGpp synthesis of *Bs*RelQ and its variants in absence (−) and presence (+) of pppGpp. Error bars indicate the SD of three independent replicates.

than RelQ (Fig. 2a). Why is that the case? Our analysis demonstrates that binding of ATP proceeds in identical fashion in RelP/RelQ proteins, because both proteins harbor an identical architecture of their ATP-coordination site (Fig. S3). However, RelP and RelQ inherently differ in their ability to coordinate the GDP and GTP substrates. This is caused by a different structural flexibility of their G-Loops. While the G-loop of RelQ is highly disordered, the equivalent region of RelP is highly ordered and can therefore readily coordinate GDP/GTP (Fig. 3). However, the activity of RelQ can be enhanced by coordination of pppGpp within the central cleft[33]. This pppGpp results in a rearrangement of helices α4 and α5 at the lateral sides of the RelQ homotetramer and, by establishing a salt bridge between Glu178 (α5) and Arg117 (G-Loop) (Fig. 4), results in a more ordered (and active) conformation of the G-Loop. The (p)ppGpp synthetase activity of the so-stimulated RelQ resembles RelP. Notably, the K_m values obtained for *Sa*RelP (Fig. 2a and b) and allosterically stimulated *Bs*RelQ (Figs S4 and S5) accord with the intracellular concentrations of GDP and GTP, estimated as 200–500 µM and 1–5 mM, respectively[34,35]. Under these conditions, both enzymes are highly sensitive to small changes in GDP/GTP levels. Non-stimulated *Bs*RelQ, in contrast, appears rather insensitive to changes in GDP/GTP levels because of its high K_m values for both substrates (Fig. 2a and b). In summary, RelP always appears as a highly active alarmone synthetase, while

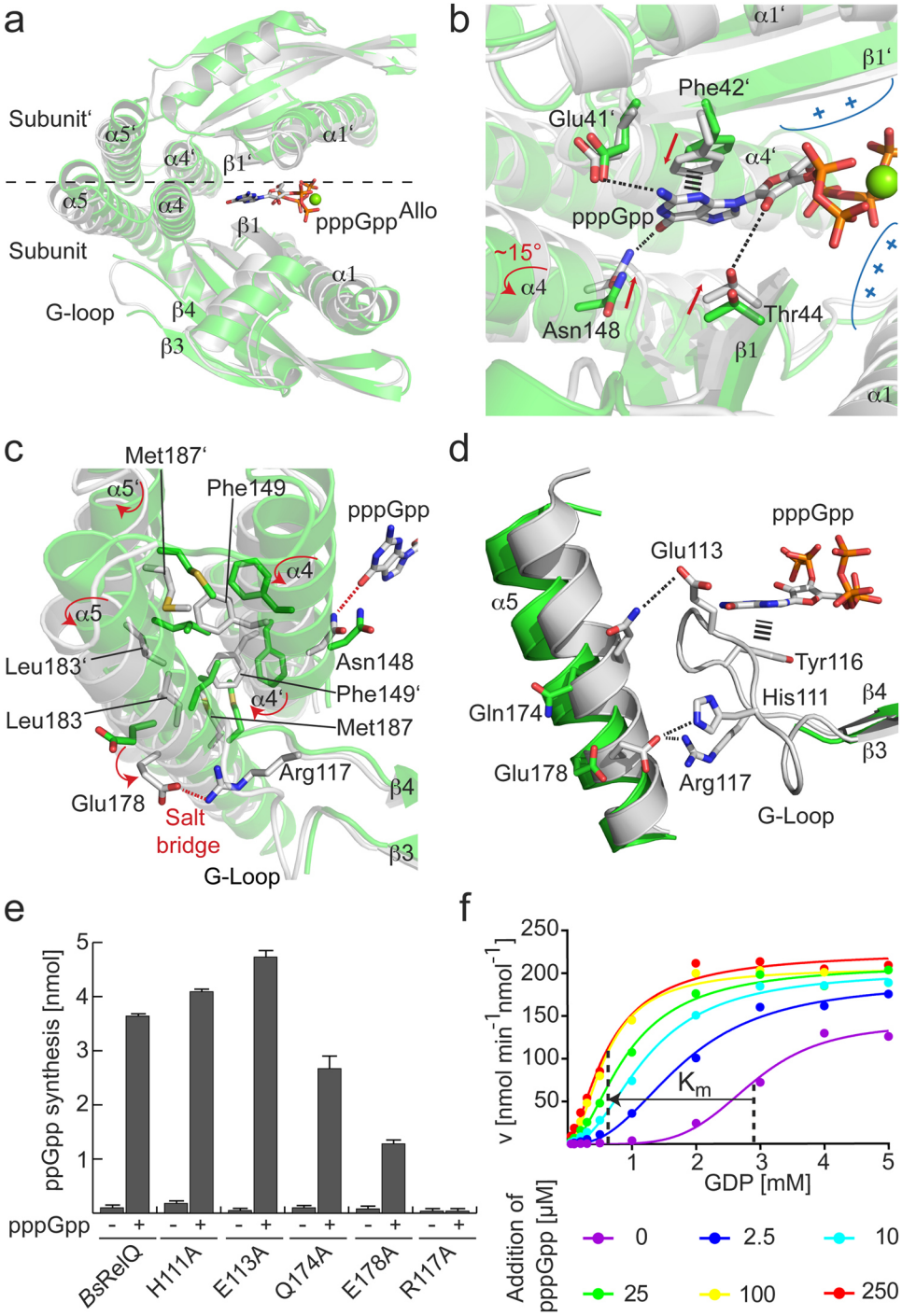

Figure 4. Allosteric binding of pppGpp to RelQ stabilizes the G-Loop. (**a**) Superimposition of one half of the tetramers of *Bs*RelQ (white, PDB: 5DEC[33]) and *Bs*RelQ-pppGpp (green, PDB: 5DED[33]). (**b**) Coordination of pppGpp in the central cleft of *Bs*RelQ by amino acids residing in α1, β1 and α4 results in conformational changes (indicated by red arrows). (**c**) Interaction of Asn148 with pppGpp causes a rotation of α4 that is transmitted onto α5 through the hydrophobic core established by Phe149, Leu183 and Met187 from two subunits of *Bs*RelQ. Concerted rotation of helices α4 and α5 enables formation of a salt bridge between Glu178 and Arg117. (**d**) Interactions between amino acid side chains from α5 and the G-Loop of *Bs*RelQ are only established in presence of pppGpp and result in ordering of the G-Loop. (**e**) ppGpp synthesis by *Bs*RelQ and *Bs*RelQ variants in absence (−) and presence (+) of pppGpp. Error bars indicate the SD of three independent replicates. (**f**) The v/S characteristic of ppGpp synthesis by *Bs*RelQ in presence of different amounts of pppGpp. The velocity is given in nmol per minute per nmol *Bs*RelQ. The K_m values of *Bs*RelQ in absence and presence of 250 μM pppGpp are indicated by dashed lines. Data of one representative experiment are shown.

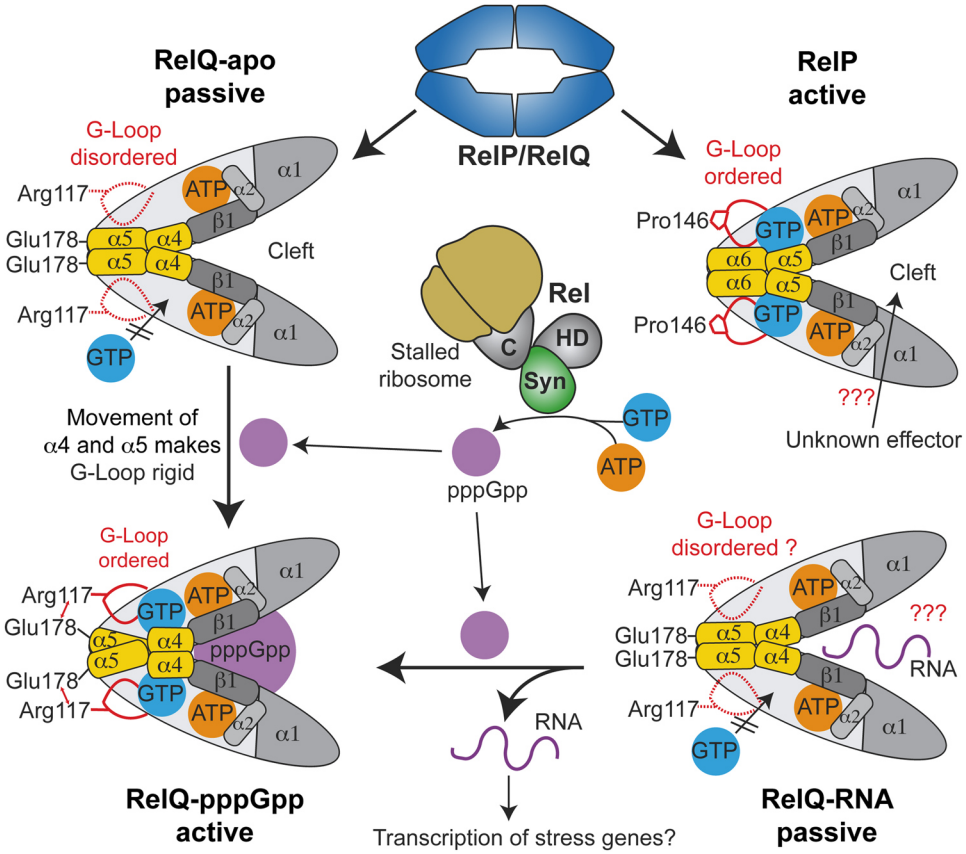

Figure 5. Mechanistic framework of Rel, RelP and RelQ. Three states of RelQ differing in (p)ppGpp synthetase activity are known: apo-RelQ and the RNA-bound RelQ are catalytically passive states, while RelQ bound to the alarmone pppGpp is an active (p)ppGpp synthetase. In both passive states, RelQ readily binds ATP (orange). However, GTP (blue) is only poorly coordinated, because of the disordered nature of the G-Loop. Binding of pppGpp (violet) into the allosteric cleft of RelQ results in a concerted rearrangement of α4 and α5 (yellow) that rigidifies the G-Loop, enables tight coordination of GTP and renders RelQ highly active. Such pppGpp molecules might originate from Rel's (p)ppGpp synthetase activity, which is enhanced under conditions of amino acid starvation. In such a case, pppGpp might bind into to the unoccupied central cleft of apo-RelQ or could competitively replace an RNA molecule from the cleft as shown previously[29]. The G-Loop of RelP is always ordered enforcing the active state of RelP. Whether an RNA or any other unknown effector molecule can bind into the central cleft of RelP is not known.

RelQ can switch between a passive state with low and an active (i.e. pppGpp-stimulated) state with high (p)ppGpp synthetase activity.

Having elucidated the different properties of RelP and RelQ, we wondered how this divergence might be relevant for the bacterial cell. In our current understanding, RelQ can appear in two passive states. In the apo-state, RelQ's central cleft is unoccupied while in the RNA-bound state a so far uncharacterized RNA[29,36] might reside in the central cleft (Fig. 5). We suspect that RelQ is predominantly found in either of those passive states in nutrient-rich conditions, because the (p)ppGpp hydrolytic activity of Rel should keep (p)ppGpp levels below the limit of RelQ stimulation. When the microorganism is suddenly confronted with nutrient limitation, Rel will recognize and bind to stalled ribosomes. When doing so, Rel could provide the pppGpp needed to bring RelQ into its active (i.e. pppGpp-bound) state by the intricate mechanism involving helical rearrangements and loop stabilization (Fig. 5). RelQ would then simply serves as an amplifier of the stress signal given by Rel. Additionally, the RNA bound to RelQ would be outcompeted by pppGpp and might result in the transcription of stress genes. Unfortunately, it is unclear so far, which genes might be differentially regulated, as the 'real' RNA bound by RelQ *in vivo* still remains to be identified[29,36]. Seemingly, RelQ's activity is intensively coupled to Rel (Fig. 5). Although experimental data for this functional link of Rel and RelQ are missing so far, the outlined scenario would provide an elegant way for an instant rise of (p)ppGpp levels dominated by the activity Rel and aided by RelQ.

RelP, in contrast to RelQ, is always a highly active enzyme that possesses all features enabling efficient (p)ppGpp synthesis, mainly an ordered G-Loop (Fig. 5). RelP should therefore not rely on the signal provided by Rel but might rather work independently. The presence of a central cleft within the tetramer of RelP nevertheless allows hypothesizing that an unknown factor might regulate the activity of RelP (Fig. 5). Noteworthy, the different activities of RelP and RelQ seem to be perfectly matched with their disparate transcriptional profiles. The switchable RelQ, predominantly transcribed during logarithmic growth[27], can counteract a sudden nutrient limitation

with the help of the Rel protein. The presence of RelP during logarithmic growth, however, might be detrimental for the microorganism. Consequently, RelP transcripts appear only during early stationary phase and in response to treatment with antibiotics, ethanol, high salt and acidic or alkalic pH stress conditions[25,37,38]. Also, RelP has been implicated in mediating inactivation of ribosomes by forming translation-inactive ribosome dimers thereby providing an elegant and fast shutdown mechanism for the bacterial metabolism[32,39]. In conclusion, our study strengthens the understanding of disparate roles of RelP/RelQ proteins and sets the stage for future investigations on this class of (p)ppGpp synthetases.

Materials and Methods

Cloning and mutagenesis. Genes encoding for RelP (*ywaC* and *SA2297*, respectively) were amplified from *B. subtilis* PY79 and *S. aureus* strain Newman genomic DNA by polymerase chain reaction using Phusion High-Fidelity DNA polymerase (NEB) according to the manufacturer's manual. The *forward* primer for *SA2297* encoded a hexahistidine-tag in frame with the DNA sequence of *relP*. The *forward* primer for *ywaC* encoded a strep-tag in frame with the DNA sequence. The resulting PCR fragments were cloned into the pET24d(+) vector (Novagen) at the *Nco*I/*Xho*I restriction sites. Mutations within RelP were generated by overlapping PCR.

Protein Production and Purification. *Escherichia coli* BL21 (DE3) (NEB) carrying the plasmids for His-tagged proteins were grown in lysogeny broth (LB)-medium supplemented with 50 µg/ml kanamycin and 12.5 g/l D(+)-lactose-monohydrate for 20 h at 30 °C. Cells were harvested by centrifugation (3500 × *g*, 20 min, 4 °C), resuspended in lysis buffer (20 mM of HEPES-Na pH 8.0, 250 mM NaCl, 40 mM imidazole, 20 mM $MgCl_2$, 20 mM KCl) and lysed by two passages through the M-110L Microfluidizer (Microfluidics). After centrifugation (47850 × *g*, 20 min, 4 °C), the clear supernatant was loaded on a 1-ml HisTrap column (GE Healthcare) equilibrated with 10 column volumes (CV) lysis buffer. After washing with 10 CV of lysis buffer, the protein was eluted with 5 CV elution buffer (lysis buffer containing 500 mM imidazole). The protein was concentrated (Amicon Ultracel-10K (Millipore)) and applied to size-exclusion chromatography (SEC) on a HiLoad 26/600 Superdex 200 pg column (GE Healthcare) equilibrated in SEC buffer (20 mM of HEPES-Na, pH 7.5, 200 mM NaCl 20 mM $MgCl_2$, 20 mM KCl). Protein containing fractions were pooled, concentrated (Amicon Ultracel-10K (Millipore)), deep-frozen in liquid nitrogen and stored at −80 °C. Protein concentration was determined by a spectrophotometer (NanoDrop Lite, Thermo Scientific).

*Bs*RelP was purified by a similar procedure using a 1-ml StrepTrap column (GE Healthcare). Lysis buffer without imidazole was employed for cell lysis, column equilibration and washing and elution from the column was conducted with 5 CV of SEC buffer containing 2.5 mM desthiobiotin.

Preparation of ppGpp and pppGpp. (p)ppGpp was produced essentially as described previously[33]. In brief, 5 µM SAS1 were incubated in SEC buffer together with 10 mM ATP and 10 mM GDP for 30 min at 37 °C to produce ppGpp or together with 10 mM ATP and 10 mM GTP for 2 h at 37 °C to produce pppGpp. Afterwards, the reaction was mixed with the same volume of chloroform and centrifuged (17300 × *g*, 5 min, 4 °C). The aqueous phase was removed and the organic phase mixed with one volume of double-destilled water and centrifuged (17300 × *g*, 5 min, 4 °C). The combined aqueous phases were subjected to anion-exchange chromatography using a ResourceQ. 6-ml column (GE Healthcare) at a flow rate of 6 ml/min and the nucleotides eluted with a gradient of NaCl. Fractions containing ppGpp or pppGpp were pooled followed by addition of lithium chloride with a concentration of 1 M and four volumes of ethanol. The suspension was then incubated at −20 °C for 20 min and centrifuged (5000 × *g*, 20 min, 4 °C). The resulting pellets were washed with absolute ethanol, dried and stored at −20 °C. Quality of the so-prepared alarmones was controlled by HPLC and yielded ppGpp and pppGpp in purities of 98% and 95%, respectively.

Kinetic analysis of RelP/RelQ. The enzyme kinetic behavior of RelP and RelQ (compare to Figs 2a, 3e, 4f and S5), were monitored by HPLC. Reactions were prepared in SEC buffer supplemented with 100 mM HEPES-Na pH 7.5 by incubating 0.2 µM protein together with 5 mM ATP and varying concentrations of GDP or GTP (i.e. 0.05, 0.1, 0.2, 0.3, 0.5, 1, 3 and 5 mM; 2 and 4 mM were included where necessary). For the analysis of pppGpp affecting the kinetic behavior of *Bs*RelQ, pppGpp was also added to the reaction in concentrations of 0/2.5/10/25/100/250 µM. Samples were taken after different time points (i.e. 2, 4, 6, 8 and 10 minutes) and stopped as follows: two volume parts of chloroform were added to the sample, thoroughly mixed for 15 seconds, kept at 95 °C for 15 seconds and flash-frozen in liquid nitrogen. While thawing, the samples were centrifuged (17300 × *g*, 30 min, 4 °C) and the aqueous phase used for analysis. HPLC measurements were conducted on an Agilent 1100 Series system (Agilent technologies) equipped with a C18 column (EC 250/4.6 Nucleodur HTec 3 µM; Macherey-Nagel). Nucleotides were eluted isocratically with a buffer containing 50 mM KH_2PO_4, 50 mM K_2HPO_4, 10 mM TPAB (tetrapentylammonium bromide) and 20% (v/v) acetonitrile and detected at 260 nm wavelength in agreement with standards. Analysis of enzymatic measurements was performed with GraphPad Prism version 6.04 for Windows, (GraphPad Software, San Diego, California, USA). The velocity of (p)ppGpp synthesis was obtained by linear regression of the amount of AMP quantified after different incubation times. Kinetic parameters (K_m, V_{max} and the Hill coefficient (h) ± standard deviation) were obtained from the fit of the v/S characteristic according to the equation $v = V_{max} S^h/(K_m{}^h + S^h)$.

Stimulation of RelP/RelQ by (p)ppGpp. In experiments probing the stimulatory effect of (p)ppGpp (compare to Figs 2d, 3f, 4e and S4), 0.2 µM RelP/RelQ were incubated together with 5 mM ATP and 0.25 mM GDP/GTP in presence or absence of 200 µM (p)ppGpp for 10 minutes at 37 °C. The reactions were stopped and analyzed as described above.

Crystallization and structure determination. Crystallization was carried out at room temperature by sitting drop vapor diffusion in SWISSCI MRC 2-well plates (Jena Bioscience) with a reservoir volume of 50 µl and the drop containing 0.5 µl of protein and crystallization solution each. Crystals of *Bs*RelP were obtained from a 10 mg/ml solution after 1 week from 0.1 M CHES pH 9.5 and 30% (w/v) PEG 3000. Crystals of *Sa*RelP were obtained from a 15 mg/ml solution after 1 week in 0.1 M CHES pH 9.5 and 40% (v/v) PEG600. For crystallization of *Sa*RelP-AMPCPP, a 15 mg/ml concentrated protein solution was incubated together with 5 mM AMPCPP for 30 minutes on ice. Crystals of *Sa*RelP-AMPCPP were obtained after 2 days from 0.1 M Tris pH 8.5, 0.2 M lithium sulfate and 30% (w/v) PEG4000.

To harvest crystals, 0.5 µl of a cryo-protecting solution containing mother liquor supplemented with 20% (v/v) glycerol was added to the drop, crystals looped and flash-frozen in liquid nitrogen. Diffraction data were collected at the European Synchrotron Radiation Facility (ESRF) Grenoble, France, at beamlines ID23-1 and ID29 under laminar nitrogen flow at 100 K (Oxford Cryostream 700 Series) with a DECTRIS PILATUS 6M detector. Data were processed with XDS[40] and CCP4-implemented SCALA[41]. Crystal structures were determined by molecular replacement (MR) employing BsRelQ (PDB: 5DEC[33]) as search model using the CCP4-implemented PHASER[41]. Structures were manually built in COOT[42] and refined with PHENIX[43]. Figures were prepared with PYMOL (www.pymol.org).

Accession Codes

Atomic coordinates and structure factors were deposited in the Protein Data Bank (PDB) under 6FGJ (apo-*Sa*RelP), 6FGK (apo-*Bs*RelP) and 6FGX (AMPCPP-bound *Sa*RelP).

References

1. Steinchen, W. & Bange, G. The magic dance of the alarmones (p)ppGpp. *Molecular microbiology* **101**, 531–544, https://doi.org/10.1111/mmi.13412 (2016).
2. Dalebroux, Z. D. & Swanson, M. S. ppGpp: magic beyond RNA polymerase. *Nature reviews. Microbiology* **10**, 203–212, https://doi.org/10.1038/nrmicro2720 (2012).
3. Kanjee, U., Ogata, K. & Houry, W. A. Direct binding targets of the stringent response alarmone (p)ppGpp. *Molecular microbiology* **85**, 1029–1043, https://doi.org/10.1111/j.1365-2958.2012.08177.x (2012).
4. Boutte, C. C. & Crosson, S. Bacterial lifestyle shapes stringent response activation. *Trends in microbiology* **21**, 174–180, https://doi.org/10.1016/j.tim.2013.01.002 (2013).
5. Potrykus, K. & Cashel, M. (p)ppGpp: still magical? *Annual review of microbiology* **62**, 35–51, https://doi.org/10.1146/annurev.micro.62.081307.162903 (2008).
6. Liu, K., Bittner, A. N. & Wang, J. D. Diversity in (p)ppGpp metabolism and effectors. *Current opinion in microbiology* **24**, 72–79, https://doi.org/10.1016/j.mib.2015.01.012 (2015).
7. Masuda, S. *et al. The bacterial stringent re*sponse, conserved in chloroplasts, controls plant fertilization. *Plant & cell physiology* **49**, 135–141, https://doi.org/10.1093/pcp/pcm177 (2008).
8. van der Biezen, E. A., Sun, J., Coleman, M. J., Bibb, M. J. & Jones, J. D. Arabidopsis RelA/SpoT homologs implicate (p)ppGpp in plant signaling. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 3747–3752, https://doi.org/10.1073/pnas.060392397 (2000).
9. Takahashi, K., Kasai, K. & Ochi, K. Identification of the bacterial alarmone guanosine 5′-diphosphate 3′-diphosphate (ppGpp) in plants. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 4320–4324, https://doi.org/10.1073/pnas.0308555101 (2004).
10. Stent, G. S. & Brenner, S. A genetic locus for the regulation of ribonucleic acid synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **47**, 2005–2014 (1961).
11. Cashel, M. The control of ribonucleic acid synthesis in Escherichia coli. IV. Relevance of unusual phosphorylated compounds from amino acid-starved stringent strains. *The Journal of biological chemistry* **244**, 3133–3141 (1969).
12. Weiss, L. A. & Stallings, C. L. Essential roles for Mycobacterium tuberculosis Rel beyond the production of (p)ppGpp. *Journal of bacteriology* **195**, 5629–5638, https://doi.org/10.1128/JB.00759-13 (2013).
13. Vogt, S. L. *et al.* The stringent response is essential for Pseudomonas aeruginosa virulence in the rat lung agar bead and Drosophila melanogaster feeding models of infection. *Infection and immunity* **79**, 4094–4104, https://doi.org/10.1128/IAI.00193-11 (2011).
14. He, H., Cooper, J. N., Mishra, A. & Raskin, D. M. Stringent response regulation of biofilm formation in Vibrio cholerae. *Journal of bacteriology* **194**, 2962–2972, https://doi.org/10.1128/JB.00014-12 (2012).
15. Ababneh, Q. O. & Herman, J. K. RelA inhibits Bacillus subtilis motility and chaining. *Journal of bacteriology* **197**, 128–137, https://doi.org/10.1128/JB.02063-14 (2015).
16. Ababneh, Q. O. & Herman, J. K. CodY Regulates SigD Levels and Activity by Binding to Three Sites in the fla/che Operon. *Journal of bacteriology* **197**, 2999–3006, https://doi.org/10.1128/JB.00288-15 (2015).
17. Lewis, K. Persister cells. *Annual review of microbiology* **64**, 357–372, https://doi.org/10.1146/annurev.micro.112408.134306 (2010).
18. Amato, S. M., Orman, M. A. & Brynildsen, M. P. Metabolic control of persister formation in Escherichia coli. *Molecular cell* **50**, 475–487, https://doi.org/10.1016/j.molcel.2013.04.002 (2013).
19. Conlon, B. P. *et al.* Persister formation in Staphylococcus aureus is associated with ATP depletion. *Nature microbiology* **1**, 16051, https://doi.org/10.1038/nmicrobiol.2016.51 (2016).
20. Atkinson, G. C., Tenson, T. & Hauryliuk, V. The RelA/SpoT homolog (RSH) superfamily: distribution and functional evolution of ppGpp synthetases and hydrolases across the tree of life. *PloS one* **6**, e23479, https://doi.org/10.1371/journal.pone.0023479 (2011).
21. Brown, A., Fernandez, I. S., Gordiyenko, Y. & Ramakrishnan, V. Ribosome-dependent activation of stringent control. *Nature* **534**, 277–280, https://doi.org/10.1038/nature17675 (2016).
22. Arenz, S. *et al.* The stringent factor RelA adopts an open conformation on the ribosome to stimulate ppGpp synthesis. *Nucleic acids research* **44**, 6471–6481, https://doi.org/10.1093/nar/gkw470 (2016).
23. Loveland, A. B. *et al.* Ribosome*RelA structures reveal the mechanism of stringent response activation. *eLife* **5**, https://doi.org/10.7554/eLife.17029 (2016).
24. Mechold, U., Murphy, H., Brown, L. & Cashel, M. Intramolecular regulation of the opposing (p)ppGpp catalytic activities of Rel(Seq), the Rel/Spo enzyme from Streptococcus equisimilis. *Journal of bacteriology* **184**, 2878–2888 (2002).
25. Geiger, T., Kastle, B., Gratani, F. L., Goerke, C. & Wolz, C. Two small (p)ppGpp synthases in Staphylococcus aureus mediate tolerance against cell envelope stress conditions. *Journal of bacteriology* **196**, 894–902, https://doi.org/10.1128/JB.01201-13 (2014).
26. Lemos, J. A., Lin, V. K., Nascimento, M. M., Abranches, J. & Burne, R. A. Three gene products govern (p)ppGpp production by Streptococcus mutans. *Molecular microbiology* **65**, 1568–1581, https://doi.org/10.1111/j.1365-2958.2007.05897.x (2007).
27. Nanamiya, H. *et al.* Identification and functional analysis of novel (p)ppGpp synthetase genes in Bacillus subtilis. *Molecular microbiology* **67**, 291–304, https://doi.org/10.1111/j.1365-2958.2007.06018.x (2008).

28. Srivatsan, A. *et al*. High-precision, whole-genome sequencing of laboratory strains facilitates genetic studies. *PLoS genetics* **4**, e1000139, https://doi.org/10.1371/journal.pgen.1000139 (2008).
29. Beljantseva, J. *et al*. Negative allosteric regulation of Enterococcus faecalis small alarmone synthetase RelQ by single-stranded RNA. *Proceedings of the National Academy of Sciences of the United States of America* **114**, 3726–3731, https://doi.org/10.1073/pnas.1617868114 (2017).
30. Gaca, A. O. *et al*. From (p)ppGpp to (pp)pGpp: Characterization of Regulatory Effects of pGpp Synthesized by the Small Alarmone Synthetase of Enterococcus faecalis. *Journal of bacteriology* **197**, 2908–2919, https://doi.org/10.1128/JB.00324-15 (2015).
31. Das, B., Pal, R. R., Bag, S. & Bhadra, R. K. Stringent response in Vibrio cholerae: genetic analysis of spoT gene function and identification of a novel (p)ppGpp synthetase gene. *Molecular microbiology* **72**, 380–398, https://doi.org/10.1111/j.1365-2958.2009.06653.x (2009).
32. Tagami, K. *et al*. Expression of a small (p)ppGpp synthetase, YwaC, in the (p)ppGpp(0) mutant of Bacillus subtilis triggers YvyD-dependent dimerization of ribosome. *Microbiology Open* **1**, 115–134, https://doi.org/10.1002/mbo3.16 (2012).
33. Steinchen, W. *et al*. Catalytic mechanism and allosteric regulation of an oligomeric (p)ppGpp synthetase by an alarmone. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 13348–13353, https://doi.org/10.1073/pnas.1505271112 (2015).
34. Varik, V., Oliveira, S. R. A., Hauryliuk, V. & Tenson, T. HPLC-based quantification of bacterial housekeeping nucleotides and alarmone messengers ppGpp and pppGpp. *Scientific reports* **7**, 11022, https://doi.org/10.1038/s41598-017-10988-6 (2017).
35. Bennett, B. D. *et al*. Absolute metabolite concentrations and implied enzyme active site occupancy in Escherichia coli. *Nature chemical biology* **5**, 593–599, https://doi.org/10.1038/nchembio.186 (2009).
36. Hauryliuk, V. & Atkinson, G. C. Small Alarmone Synthetases as novel bacterial RNA-binding proteins. *RNA biology* **14**, 1695–1699, https://doi.org/10.1080/15476286.2017.1367889 (2017).
37. Thackray, P. D. & Moir, A. SigM, an extracytoplasmic function sigma factor of Bacillus subtilis, is activated in response to cell wall antibiotics, ethanol, heat, acid, and superoxide stress. *Journal of bacteriology* **185**, 3491–3498 (2003).
38. Zweers, J. C., Nicolas, P., Wiegert, T., van Dijl, J. M. & Denham, E. L. Definition of the sigma(W) regulon of Bacillus subtilis in the absence of stress. *PloS one* **7**, e48471, https://doi.org/10.1371/journal.pone.0048471 (2012).
39. Beckert, B. *et al*. Structure of the Bacillus subtilis hibernating 100 S ribosome reveals the basis for 70 S dimerization. *The EMBO journal* **36**, 2061–2072, https://doi.org/10.15252/embj.201696189 (2017).
40. Kabsch, W. X. *Acta crystallographica. Section D, Biological crystallography* **66**, 125–132, https://doi.org/10.1107/S0907444909047337 (2010).
41. Winn, M. D. *et al*. Overview of the CCP4 suite and current developments. *Acta crystallographica. Section D, Biological crystallography* **67**, 235–242, https://doi.org/10.1107/S0907444910045749 (2011).
42. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta crystallographica. Section D, Biological crystallography* **60**, 2126–2132, https://doi.org/10.1107/S0907444904019158 (2004).
43. Adams, P. D. *et al*. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta crystallographica. Section D, Biological crystallography* **66**, 213–221, https://doi.org/10.1107/S0907444909052925 (2010).

Acknowledgements

We thank the European Synchrotron Radiation Facility (ESRF), Grenoble, France for support during data collection. We thank the SFB987 of the Deutsche Forschungsgemeinschaft (DFG) for financial support (to G.B.). We are grateful to Uwe Linne (Marburg) for advice.

Author Contributions

W.S. and G.B. designed research. W.S., M.S.V., F.A., P.I.G. and P.H. performed experiments. W.S., F.A., C.W. and G.B. analyzed data. W.S. and G.B. prepared the manuscript. All authors commented on the manuscript.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-20634-4.

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

11 References

Abranches, J., Martinez, A. R., Kajfasz, J. K., Chavez, V., Garsin, D. A., & Lemos, J. A. (2009). The molecular alarmone (p)ppGpp mediates stress responses, vancomycin tolerance, and virulence in Enterococcus faecalis. *J Bacteriol, 191*(7), 2248-2256. doi:10.1128/JB.01726-08

Aedo, S., & Tomasz, A. (2016). Role of the Stringent Stress Response in the Antibiotic Resistance Phenotype of Methicillin-Resistant Staphylococcus aureus. *Antimicrob Agents Chemother, 60*(4), 2311-2317. doi:10.1128/AAC.02697-15

Agirrezabala, X., Fernandez, I. S., Kelley, A. C., Carton, D. G., Ramakrishnan, V., & Valle, M. (2013). The ribosome triggers the stringent response by RelA via a highly distorted tRNA. *EMBO Rep, 14*(9), 811-816. doi:10.1038/embor.2013.106

Amato, S. M., Orman, M. A., & Brynildsen, M. P. (2013). Metabolic control of persister formation in Escherichia coli. *Mol Cell, 50*(4), 475-487. doi:10.1016/j.molcel.2013.04.002

Andresen, L., Tenson, T., & Hauryliuk, V. (2016). Cationic bactericidal peptide 1018 does not specifically target the stringent response alarmone (p)ppGpp. *Sci Rep, 6*, 36549. doi:10.1038/srep36549

Aravind, L., & Koonin, E. V. (1998). The HD domain defines a new superfamily of metal-dependent phosphohydrolases. *Trends Biochem Sci, 23*(12), 469-472.

Arenz, S., Abdelshahid, M., Sohmen, D., Payoe, R., Starosta, A. L., Berninghausen, O., . . . Wilson, D. N. (2016). The stringent factor RelA adopts an open conformation on the ribosome to stimulate ppGpp synthesis. *Nucleic Acids Res, 44*(13), 6471-6481. doi:10.1093/nar/gkw470

Atkinson, G. C., Tenson, T., & Hauryliuk, V. (2011). The RelA/SpoT homolog (RSH) superfamily: distribution and functional evolution of ppGpp synthetases and hydrolases across the tree of life. *PLoS One, 6*(8), e23479. doi:10.1371/journal.pone.0023479

Avarbock, A., Avarbock, D., Teh, J. S., Buckstein, M., Wang, Z. M., & Rubin, H. (2005). Functional regulation of the opposing (p)ppGpp synthetase/hydrolase activities of RelMtb from Mycobacterium tuberculosis. *Biochemistry, 44*(29), 9913-9923. doi:10.1021/bi0505316

Bae, T., & Schneewind, O. (2006). Allelic replacement in Staphylococcus aureus with inducible counter-selection. *Plasmid, 55*(1), 58-63. doi:10.1016/j.plasmid.2005.05.005

Bag, S., Das, B., Dasgupta, S., & Bhadra, R. K. (2014). Mutational analysis of the (p)ppGpp synthetase activity of the Rel enzyme of Mycobacterium tuberculosis. *Arch Microbiol, 196*(8), 575-588. doi:10.1007/s00203-014-0996-9

Balaban, N. Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D. I., . . . Zinkernagel, A. (2019). Definitions and guidelines for research on antibiotic persistence. *Nat Rev Microbiol, 17*(7), 441-448. doi:10.1038/s41579-019-0196-3

Ballal, A., & Manna, A. C. (2010). Control of thioredoxin reductase gene (trxB) transcription by SarA in Staphylococcus aureus. *J Bacteriol, 192*(1), 336-345. doi:10.1128/JB.01202-09

Battesti, A., & Bouveret, E. (2006). Acyl carrier protein/SpoT interaction, the switch linking SpoT-dependent stress response to fatty acid metabolism. *Mol Microbiol, 62*(4), 1048-1063. doi:10.1111/j.1365-2958.2006.05442.x

Battesti, A., & Bouveret, E. (2009). Bacteria possessing two RelA/SpoT-like proteins have evolved a specific stringent response involving the acyl carrier protein-SpoT interaction. *J Bacteriol, 191*(2), 616-624. doi:10.1128/JB.01195-08

Beasley, F. C., & Heinrichs, D. E. (2010). Siderophore-mediated iron acquisition in the staphylococci. *J Inorg Biochem, 104*(3), 282-288. doi:10.1016/j.jinorgbio.2009.09.011

Beavers, W. N., & Skaar, E. P. (2016). Neutrophil-generated oxidative stress and protein damage in Staphylococcus aureus. *Pathog Dis, 74*(6). doi:10.1093/femspd/ftw060

Belcheva, A., & Golemi-Kotra, D. (2008). A close-up view of the VraSR two-component system. A mediator of Staphylococcus aureus response to cell wall damage. *J Biol Chem, 283*(18), 12354-12364. doi:10.1074/jbc.M710010200

Beljantseva, J., Kudrin, P., Andresen, L., Shingler, V., Atkinson, G. C., Tenson, T., & Hauryliuk, V. (2017). Negative allosteric regulation of Enterococcus faecalis small alarmone synthetase

RelQ by single-stranded RNA. *Proc Natl Acad Sci U S A, 114*(14), 3726-3731. doi:10.1073/pnas.1617868114

Berditsch, M., Lux, H., Babii, O., Afonin, S., & Ulrich, A. S. (2016). Therapeutic Potential of Gramicidin S in the Treatment of Root Canal Infections. *Pharmaceuticals (Basel), 9*(3). doi:10.3390/ph9030056

Bhawini, A., Pandey, P., Dubey, A. P., Zehra, A., Nath, G., & Mishra, M. N. (2019). RelQ Mediates the Expression of beta-Lactam Resistance in Methicillin-Resistant Staphylococcus aureus. *Front Microbiol, 10*, 339. doi:10.3389/fmicb.2019.00339

Bigger, J. W. (1944). <1944 Beschreibung persister in Saures.pdf>. *The Lancet*.

Bojer, M. S., Lindemose, S., Vestergaard, M., & Ingmer, H. (2018). Quorum Sensing-Regulated Phenol-Soluble Modulins Limit Persister Cell Populations in Staphylococcus aureus. *Front Microbiol, 9*, 255. doi:10.3389/fmicb.2018.00255

Brauner, A., Fridman, O., Gefen, O., & Balaban, N. Q. (2016). Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nat Rev Microbiol, 14*(5), 320-330. doi:10.1038/nrmicro.2016.34

Brinsmade, S. R. (2017). CodY, a master integrator of metabolism and virulence in Gram-positive bacteria. *Curr Genet, 63*(3), 417-425. doi:10.1007/s00294-016-0656-5

Brown, A., Fernandez, I. S., Gordiyenko, Y., & Ramakrishnan, V. (2016). Ribosome-dependent activation of stringent control. *Nature, 534*(7606), 277-280. doi:10.1038/nature17675

Brown, S. A., Palmer, K. L., & Whiteley, M. (2008). Revisiting the host as a growth medium. *Nat Rev Microbiol, 6*(9), 657-666. doi:10.1038/nrmicro1955

Buglino, J., Shen, V., Hakimian, P., & Lima, C. D. (2002). Structural and biochemical analysis of the Obg GTP binding protein. *Structure, 10*(11), 1581-1592.

Cashel, M., & Kalbacher, B. (1970). The control of ribonucleic acid synthesis in Escherichia coli. V. Characterization of a nucleotide associated with the stringent response. *J Biol Chem, 245*(9), 2309-2318.

Chávez de Paz, L. E., Lemos, J. A., Wickström, C., & Sedgley, C. M. (2012). Role of (p)ppGpp in Biofilm Formation by Enterococcus faecalis. *Applied and Environmental Microbiology, 78*(5), 1627-1630. doi:10.1128/aem.07036-11

Cheung, G. Y., Joo, H. S., Chatterjee, S. S., & Otto, M. (2014). Phenol-soluble modulins--critical determinants of staphylococcal virulence. *FEMS Microbiol Rev, 38*(4), 698-719. doi:10.1111/1574-6976.12057

Chipman, D. M., & Shaanan, B. (2001). The ACT domain family. *Curr Opin Struct Biol, 11*(6), 694-700.

Colomer-Winter, C., Flores-Mireles, A. L., Kundra, S., Hultgren, S. J., & Lemos, J. A. (2019). (p)ppGpp and CodY Promote Enterococcus faecalis Virulence in a Murine Model of Catheter-Associated Urinary Tract Infection. *mSphere, 4*(4). doi:10.1128/mSphere.00392-19

Colomer-Winter, C., Gaca, A. O., Chuang-Smith, O. N., Lemos, J. A., & Frank, K. L. (2018). Basal levels of (p)ppGpp differentially affect the pathogenesis of infective endocarditis in Enterococcus faecalis. *Microbiology, 164*(10), 1254-1265. doi:10.1099/mic.0.000703

Colomer-Winter, C., Gaca, A. O., & Lemos, J. A. (2017). Association of Metal Homeostasis and (p)ppGpp Regulation in the Pathophysiology of Enterococcus faecalis. *Infect Immun, 85*(7). doi:10.1128/IAI.00260-17

Conlon, B. P., Rowe, S. E., Gandt, A. B., Nuxoll, A. S., Donegan, N. P., Zalis, E. A., . . . Lewis, K. (2016). Persister formation in Staphylococcus aureus is associated with ATP depletion. *Nature Microbiology, 1*(5). doi:10.1038/nmicrobiol.2016.51

Corrigan, R. M., Bellows, L. E., Wood, A., & Grundling, A. (2016). ppGpp negatively impacts ribosome assembly affecting growth and antimicrobial tolerance in Gram-positive bacteria. *Proc Natl Acad Sci U S A, 113*(12), E1710-1719. doi:10.1073/pnas.1522179113

Corrigan, R. M., Bellows, L. E., Wood, A., & Gründling, A. (2016). ppGpp negatively impacts ribosome assembly affecting growth and antimicrobial tolerance in Gram-positive bacteria. *113*(12), E1710-1719. doi:10.1073/pnas.1522179113

Cosgrove, K., Coutts, G., Jonsson, I. M., Tarkowski, A., Kokai-Kun, J. F., Mond, J. J., & Foster, S. J. (2007). Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) have compensatory roles in peroxide stress resistance and are required for survival, persistence, and nasal colonization in Staphylococcus aureus. *J Bacteriol, 189*(3), 1025-1035. doi:10.1128/JB.01524-06

Crosse, A. M., Greenway, D. L., & England, R. R. (2000). Accumulation of ppGpp and ppGp in Staphylococcus aureus 8325-4 following nutrient starvation. *Lett Appl Microbiol, 31*(4), 332-337.

Dalebroux, Z. D., Svensson, S. L., Gaynor, E. C., & Swanson, M. S. (2010). ppGpp conjures bacterial virulence. *Microbiol Mol Biol Rev, 74*(2), 171-199. doi:10.1128/MMBR.00046-09

Dalebroux, Z. D., & Swanson, M. S. (2012). ppGpp: magic beyond RNA polymerase. *Nat Rev Microbiol, 10*(3), 203-212. doi:10.1038/nrmicro2720

Das, B., Pal, R. R., Bag, S., & Bhadra, R. K. (2009). Stringent response in Vibrio cholerae: genetic analysis of spoT gene function and identification of a novel (p)ppGpp synthetase gene. *Mol Microbiol, 72*(2), 380-398. doi:10.1111/j.1365-2958.2009.06653.x

Das, T., Paino, D., Manoharan, A., Farrell, J., Whiteley, G., Kriel, F. H., . . . Manos, J. (2019). Conditions Under Which Glutathione Disrupts the Biofilms and Improves Antibiotic Efficacy of Both ESKAPE and Non-ESKAPE Species. *Front Microbiol, 10*, 2000. doi:10.3389/fmicb.2019.02000

de la Fuente-Nunez, C., Reffuveille, F., Haney, E. F., Straus, S. K., & Hancock, R. E. (2014). Broad-spectrum anti-biofilm peptide that targets a cellular stress response. *PLoS Pathog, 10*(5), e1004152. doi:10.1371/journal.ppat.1004152

de la Fuente-Nunez, C., Reffuveille, F., Mansour, S. C., Reckseidler-Zenteno, S. L., Hernandez, D., Brackman, G., . . . Hancock, R. E. (2015). D-enantiomeric peptides that eradicate wild-type and multidrug-resistant biofilms and protect against lethal Pseudomonas aeruginosa infections. *Chem Biol, 22*(2), 196-205. doi:10.1016/j.chembiol.2015.01.002

Defraine, V., Fauvart, M., & Michiels, J. (2018). Fighting bacterial persistence: Current and emerging anti-persister strategies and therapeutics. *Drug Resist Updat, 38*, 12-26. doi:10.1016/j.drup.2018.03.002

Dordel, J., Kim, C., Chung, M., Pardos de la Gandara, M., Holden, M. T., Parkhill, J., . . . Tomasz, A. (2014). Novel determinants of antibiotic resistance: identification of mutated loci in highly methicillin-resistant subpopulations of methicillin-resistant Staphylococcus aureus. *MBio, 5*(2), e01000. doi:10.1128/mBio.01000-13

Dozot, M., Boigegrain, R. A., Delrue, R. M., Hallez, R., Ouahrani-Bettache, S., Danese, I., . . . Kohler, S. (2006). The stringent response mediator Rsh is required for *Brucella melitensis* and *Brucella suis* virulence, and for expression of the type IV secretion system *vir*B. *Cell Microbiol, 8*(11), 1791-1802. doi:CMI749 [pii]

10.1111/j.1462-5822.2006.00749.x

English, B. P., Hauryliuk, V., Sanamrad, A., Tankov, S., Dekker, N. H., & Elf, J. (2011). Single-molecule investigations of the stringent response machinery in living bacterial cells. *Proc Natl Acad Sci U S A, 108*(31), E365-373. doi:10.1073/pnas.1102255108

Eymann, C., Homuth, G., Scharf, C., & Hecker, M. (2002). *Bacillus subtilis* functional genomics: global characterization of the stringent response by proteome and transcriptome analysis. *J Bacteriol, 184*(9), 2500-2520.

Fang, M., & Bauer, C. E. (2018). Regulation of stringent factor by branched-chain amino acids. *Proc Natl Acad Sci U S A, 115*(25), 6446-6451. doi:10.1073/pnas.1803220115

Fuchs, S., Mehlan, H., Bernhardt, J., Hennig, A., Michalik, S., Surmann, K., . . . Mader, U. (2018). AureoWiki The repository of the Staphylococcus aureus research and annotation community. *Int J Med Microbiol, 308*(6), 558-568. doi:10.1016/j.ijmm.2017.11.011

Gaca, A. O., Abranches, J., Kajfasz, J. K., & Lemos, J. A. (2012). Global transcriptional analysis of the stringent response in Enterococcus faecalis. *Microbiology, 158*(Pt 8), 1994-2004. doi:10.1099/mic.0.060236-0

Gaca, A. O., Colomer-Winter, C., & Lemos, J. A. (2015). Many means to a common end: the intricacies of (p)ppGpp metabolism and its control of bacterial homeostasis. *J Bacteriol, 197*(7), 1146-1156. doi:10.1128/jb.02577-14

Gaca, A. O., Kajfasz, J. K., Miller, J. H., Liu, K., Wang, J. D., Abranches, J., & Lemos, J. A. (2013). Basal levels of (p)ppGpp in Enterococcus faecalis: the magic beyond the stringent response. *MBio, 4*(5), e00646-00613. doi:10.1128/mBio.00646-13

Gaca, A. O., Kudrin, P., Colomer-Winter, C., Beljantseva, J., Liu, K., Anderson, B., . . . Lemos, J. A. (2015). From (p)ppGpp to (pp)pGpp: Characterization of Regulatory Effects of pGpp Synthesized by the Small Alarmone Synthetase of Enterococcus faecalis. *J Bacteriol, 197*(18), 2908-2919. doi:10.1128/JB.00324-15

Gao, C., Dai, Y., Chang, W., Fang, C., Wang, Z., & Ma, X. (2019). VraSR has an important role in immune evasion of Staphylococcus aureus with low level vancomycin resistance. *Microbes Infect*. doi:10.1016/j.micinf.2019.04.003

Gao, W., Chua, K., Davies, J. K., Newton, H. J., Seemann, T., Harrison, P. F., . . . Howden, B. P. (2010). Two novel point mutations in clinical Staphylococcus aureus reduce linezolid susceptibility and switch on the stringent response to promote persistent infection. *PLoS Pathog, 6*(6), e1000944. doi:10.1371/journal.ppat.1000944

Gaupp, R., Ledala, N., & Somerville, G. A. (2012). Staphylococcal response to oxidative stress. *Front Cell Infect Microbiol, 2*, 33. doi:10.3389/fcimb.2012.00033

Ge, Y., Borne, E., Stewart, S., Hansen, M. R., Arturo, E. C., Jaffe, E. K., & Voelz, V. A. (2018). Simulations of the regulatory ACT domain of human phenylalanine hydroxylase (PAH) unveil its mechanism of phenylalanine binding. *J Biol Chem, 293*(51), 19532-19543. doi:10.1074/jbc.RA118.004909

Geiger, T., Francois, P., Liebeke, M., Fraunholz, M., Goerke, C., Krismer, B., . . . Wolz, C. (2012). The stringent response of Staphylococcus aureus and its impact on survival after phagocytosis through the induction of intracellular PSMs expression. *PLoS Pathog, 8*(11), e1003016. doi:10.1371/journal.ppat.1003016

Geiger, T., Goerke, C., Fritz, M., Schafer, T., Ohlsen, K., Liebeke, M., . . . Wolz, C. (2010). Role of the (p)ppGpp synthase RSH, a RelA/SpoT homolog, in stringent response and virulence of Staphylococcus aureus. *Infect Immun, 78*(5), 1873-1883. doi:10.1128/IAI.01439-09

Geiger, T., Kastle, B., Gratani, F. L., Goerke, C., & Wolz, C. (2014). Two small (p)ppGpp synthases in Staphylococcus aureus mediate tolerance against cell envelope stress conditions. *J Bacteriol, 196*(4), 894-902. doi:10.1128/jb.01201-13

Geiger, T., & Wolz, C. (2014). Intersection of the stringent response and the CodY regulon in low GC Gram-positive bacteria. *Int J Med Microbiol, 304*(2), 150-155. doi:10.1016/j.ijmm.2013.11.013

George, S. E., Hrubesch, J., Breuing, I., Vetter, N., Korn, N., Hennemann, K., . . . Wolz, C. (2019). Oxidative stress drives the selection of quorum sensing mutants in the Staphylococcus aureus population. *Proc Natl Acad Sci U S A, 116*(38), 19145-19154. doi:10.1073/pnas.1902752116

Germain, E., Castro-Roa, D., Zenkin, N., & Gerdes, K. (2013). Molecular mechanism of bacterial persistence by HipA. *Mol Cell, 52*(2), 248-254. doi:10.1016/j.molcel.2013.08.045

Germain, E., Roghanian, M., Gerdes, K., & Maisonneuve, E. (2015). Stochastic induction of persister cells by HipA through (p)ppGpp-mediated activation of mRNA endonucleases. *Proc Natl Acad Sci U S A, 112*(16), 5171-5176. doi:10.1073/pnas.1423536112

Goerke, C., Campana, S., Bayer, M. G., Doring, G., Botzenhart, K., & Wolz, C. (2000). Direct quantitative transcript analysis of the agr regulon of Staphylococcus aureus during human infection in comparison to the expression profile in vitro. *Infect Immun, 68*(3), 1304-1311.

Gratani, F. L., Horvatek, P., Geiger, T., Borisova, M., Mayer, C., Grin, I., . . . Wolz, C. (2018). Regulation of the opposing (p)ppGpp synthetase and hydrolase activities in a bifunctional RelA/SpoT homologue from Staphylococcus aureus. *PLoS Genet, 14*(7), e1007514. doi:10.1371/journal.pgen.1007514

Gropp, M., Strausz, Y., Gross, M., & Glaser, G. (2001). Regulation of Escherichia coli RelA requires oligomerization of the C-terminal domain. *J Bacteriol, 183*(2), 570-579. doi:10.1128/JB.183.2.570-579.2001

Grosser, M. R., Weiss, A., Shaw, L. N., & Richardson, A. R. (2016). Regulatory Requirements for Staphylococcus aureus Nitric Oxide Resistance. *J Bacteriol, 198*(15), 2043-2055. doi:10.1128/jb.00229-16

Handke, L. D., Shivers, R. P., & Sonenshein, A. L. (2008). Interaction of Bacillus subtilis CodY with GTP. *J Bacteriol, 190*(3), 798-806. doi:10.1128/JB.01115-07

Hanna, N., Ouahrani-Bettache, S., Drake, K. L., Adams, L. G., Kohler, S., & Occhialini, A. (2013). Global Rsh-dependent transcription profile of Brucella suis during stringent response unravels adaptation to nutrient starvation and cross-talk with other stress responses. *BMC Genomics, 14*, 459. doi:10.1186/1471-2164-14-459

Harms, A., Maisonneuve, E., & Gerdes, K. (2016). Mechanisms of bacterial persistence during stress and antibiotic exposure. *Science, 354*(6318). doi:10.1126/science.aaf4268

Haseltine, W. A., & Block, R. (1973). Synthesis of guanosine tetra- and pentaphosphate requires the presence of a codon-specific, uncharged transfer ribonucleic acid in the acceptor site of ribosomes. *Proc Natl Acad Sci U S A, 70*(5), 1564-1568. doi:10.1073/pnas.70.5.1564

Haseltine, W. A., Block, R., Gilbert, W., & Weber, K. (1972). MSI and MSII made on ribosome in idling step of protein synthesis. *Nature, 238*(5364), 381-384. doi:10.1038/238381a0

Hauryliuk, V., Atkinson, G. C., Murakami, K. S., Tenson, T., & Gerdes, K. (2015). Recent functional insights into the role of (p)ppGpp in bacterial physiology. *Nat Rev Microbiol, 13*(5), 298-309. doi:10.1038/nrmicro3448

He, P., Deng, C., Liu, B., Zeng, L., Zhao, W., Zhang, Y., . . . Qin, J. (2013). Characterization of a bifunctional enzyme with (p)ppGpp-hydrolase/synthase activity in Leptospira interrogans. *FEMS Microbiol Lett, 348*(2), 133-142. doi:10.1111/1574-6968.12279

Heinrichs, J. H., Bayer, M. G., & Cheung, A. L. (1996). Characterization of the sar locus and its interaction with agr in Staphylococcus aureus. *J Bacteriol, 178*(2), 418-423. doi:10.1128/jb.178.2.418-423.1996

Helle, L., Kull, M., Mayer, S., Marincola, G., Zelder, M. E., Goerke, C., . . . Bertram, R. (2011). Vectors for improved Tet repressor-dependent gradual gene induction or silencing in Staphylococcus aureus. *Microbiology, 157*(Pt 12), 3314-3323. doi:10.1099/mic.0.052548-0

Herbert, S., Ziebandt, A. K., Ohlsen, K., Schafer, T., Hecker, M., Albrecht, D., . . . Gotz, F. (2010). Repair of global regulators in Staphylococcus aureus 8325 and comparative analysis with other clinical isolates. *Infect Immun, 78*(6), 2877-2889. doi:10.1128/iai.00088-10

Hobbs, J. K., & Boraston, A. B. (2019a). (p)ppGpp and the Stringent Response: An Emerging Threat to Antibiotic Therapy. *ACS Infect Dis*. doi:10.1021/acsinfecdis.9b00204

Hobbs, J. K., & Boraston, A. B. (2019b). (p)ppGpp and the Stringent Response: An Emerging Threat to Antibiotic Therapy. *ACS Infect Dis, 5*(9), 1505-1517. doi:10.1021/acsinfecdis.9b00204

Hogg, T., Mechold, U., Malke, H., Cashel, M., & Hilgenfeld, R. (2004). Conformational antagonism between opposing active sites in a bifunctional RelA/SpoT homolog modulates (p)ppGpp metabolism during the stringent response [corrected]. *Cell, 117*(1), 57-68. doi:10.1016/s0092-8674(04)00260-0

Holley, C., Gangaiah, D., Li, W., Fortney, K. R., Janowicz, D. M., Ellinger, S., . . . Spinola, S. M. (2014). A (p)ppGpp-null mutant of Haemophilus ducreyi is partially attenuated in humans due to multiple conflicting phenotypes. *Infect Immun, 82*(8), 3492-3502. doi:10.1128/iai.01994-14

Honsa, E. S., Cooper, V. S., Mhaissen, M. N., Frank, M., Shaker, J., Iverson, A., . . . Rosch, J. W. (2017). RelA Mutant Enterococcus faecium with Multiantibiotic Tolerance Arising in an Immunocompromised Host. *MBio, 8*(1). doi:10.1128/mBio.02124-16

Hooven, T. A., Catomeris, A. J., Bonakdar, M., Tallon, L. J., Santana-Cruz, I., Ott, S., . . . Ratner, A. J. (2018). The Streptococcus agalactiae Stringent Response Enhances Virulence and Persistence in Human Blood. *Infect Immun, 86*(1). doi:10.1128/IAI.00612-17

Horsburgh, M. J., Clements, M. O., Crossley, H., Ingham, E., & Foster, S. J. (2001). PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in Staphylococcus aureus. *Infect Immun, 69*(6), 3744-3754. doi:10.1128/IAI.69.6.3744-3754.2001

Horsburgh, M. J., Ingham, E., & Foster, S. J. (2001). In Staphylococcus aureus, fur is an interactive regulator with PerR, contributes to virulence, and Is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis. *J Bacteriol, 183*(2), 468-475. doi:10.1128/JB.183.2.468-475.2001

Hsieh, M. H., & Goodman, H. M. (2002). Molecular characterization of a novel gene family encoding ACT domain repeat proteins in Arabidopsis. *Plant Physiol, 130*(4), 1797-1806. doi:10.1104/pp.007484

Hussain, R. M., Abdullah, N. F., & Amom, Z. (2016). Killing of Staphylococcus aureus by allylpyrocatechol is potentiated by induction of intracellular oxidative stress and inhibition of catalase activity. *J Integr Med, 14*(6), 456-464. doi:10.1016/s2095-4964(16)60279-0

Imlay, J. A., Chin, S. M., & Linn, S. (1988). Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science, 240*(4852), 640-642. doi:10.1126/science.2834821

Irving, S. E., & Corrigan, R. M. (2018). Triggering the stringent response: signals responsible for activating (p)ppGpp synthesis in bacteria. *Microbiology, 164*(3), 268-276. doi:10.1099/mic.0.000621

Jain, V., Saleem-Batcha, R., & Chatterji, D. (2007). Synthesis and hydrolysis of pppGpp in mycobacteria: a ligand mediated conformational switch in Rel. *Biophys Chem, 127*(1-2), 41-50. doi:10.1016/j.bpc.2006.12.003

Jang, S., & Imlay, J. A. (2007). Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes. *J Biol Chem, 282*(2), 929-937. doi:10.1074/jbc.M607646200

Ji, C. J., Kim, J. H., Won, Y. B., Lee, Y. E., Choi, T. W., Ju, S. Y., . . . Lee, J. W. (2015). Staphylococcus aureus PerR Is a Hypersensitive Hydrogen Peroxide Sensor using Iron-mediated Histidine Oxidation. *J Biol Chem, 290*(33), 20374-20386. doi:10.1074/jbc.M115.664961

Jiang, Q., Jin, Z., & Sun, B. (2018). MgrA Negatively Regulates Biofilm Formation and Detachment by Repressing the Expression of psm Operons in Staphylococcus aureus. *Appl Environ Microbiol, 84*(16). doi:10.1128/AEM.01008-18

Jin, W., Ryu, Y. G., Kang, S. G., Kim, S. K., Saito, N., Ochi, K., . . . Lee, K. J. (2004). Two relA/spoT homologous genes are involved in the morphological and physiological differentiation of Streptomyces clavuligerus. *Microbiology, 150*(Pt 5), 1485-1493. doi:10.1099/mic.0.26811-0

Kastle, B., Geiger, T., Gratani, F. L., Reisinger, R., Goerke, C., Borisova, M., . . . Wolz, C. (2015). rRNA regulation during growth and under stringent conditions in Staphylococcus aureus. *Environ Microbiol, 17*(11), 4394-4405. doi:10.1111/1462-2920.12867

Katayama, Y., Azechi, T., Miyazaki, M., Takata, T., Sekine, M., Matsui, H., . . . Chambers, H. F. (2017). Prevalence of Slow-Growth Vancomycin Nonsusceptibility in Methicillin-Resistant Staphylococcus aureus. *Antimicrob Agents Chemother, 61*(11). doi:10.1128/aac.00452-17

Kazmierczak, K. M., Wayne, K. J., Rechtsteiner, A., & Winkler, M. E. (2009). Roles of rel(Spn) in stringent response, global regulation and virulence of serotype 2 Streptococcus pneumoniae D39. *Mol Microbiol, 72*(3), 590-611. doi:10.1111/j.1365-2958.2009.06669.x

Keyer, K., & Imlay, J. A. (1996). Superoxide accelerates DNA damage by elevating free-iron levels. *Proc Natl Acad Sci U S A, 93*(24), 13635-13640.

Khakimova, M., Ahlgren, H. G., Harrison, J. J., English, A. M., & Nguyen, D. (2013). The stringent response controls catalases in Pseudomonas aeruginosa and is required for hydrogen peroxide and antibiotic tolerance. *J Bacteriol, 195*(9), 2011-2020. doi:10.1128/jb.02061-12

Kim, C., Mwangi, M., Chung, M., Milheirico, C., de Lencastre, H., & Tomasz, A. (2013). The mechanism of heterogeneous beta-lactam resistance in MRSA: key role of the stringent stress response. *PLoS One, 8*(12), e82814. doi:10.1371/journal.pone.0082814

Kim, C. K., Milheirico, C., de Lencastre, H., & Tomasz, A. (2017). Antibiotic Resistance as a Stress Response: Recovery of High-Level Oxacillin Resistance in Methicillin-Resistant Staphylococcus aureus "Auxiliary" (fem) Mutants by Induction of the Stringent Stress Response. *Antimicrob Agents Chemother, 61*(8). doi:10.1128/AAC.00313-17

Kim, H. Y., Go, J., Lee, K. M., Oh, Y. T., & Yoon, S. S. (2018). Guanosine tetra- and pentaphosphate increase antibiotic tolerance by reducing reactive oxygen species production in Vibrio cholerae. *J Biol Chem, 293*(15), 5679-5694. doi:10.1074/jbc.RA117.000383

Kim, J. N., Ahn, S. J., Seaton, K., Garrett, S., & Burne, R. A. (2012). Transcriptional organization and physiological contributions of the relQ operon of Streptococcus mutans. *J Bacteriol, 194*(8), 1968-1978. doi:10.1128/JB.00037-12

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A., & Collins, J. J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell, 130*(5), 797-810. doi:10.1016/j.cell.2007.06.049

Korch, S. B., Henderson, T. A., & Hill, T. M. (2003). Characterization of the hipA7 allele of Escherichia coli and evidence that high persistence is governed by (p)ppGpp synthesis. *Mol Microbiol, 50*(4), 1199-1213. doi:10.1046/j.1365-2958.2003.03779.x

Krasny, L., & Gourse, R. L. (2004). An alternative strategy for bacterial ribosome synthesis: Bacillus subtilis rRNA transcription regulation. *Embo j, 23*(22), 4473-4483. doi:10.1038/sj.emboj.7600423

Krasny, L., Tiserova, H., Jonak, J., Rejman, D., & Sanderova, H. (2008). The identity of the transcription +1 position is crucial for changes in gene expression in response to amino acid starvation in Bacillus subtilis. *Mol Microbiol, 69*(1), 42-54. doi:10.1111/j.1365-2958.2008.06256.x

Kreiswirth, B. N., Lofdahl, S., Betley, M. J., O'Reilly, M., Schlievert, P. M., Bergdoll, M. S., & Novick, R. P. (1983). The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature, 305*(5936), 709-712. doi:10.1038/305709a0

Kriel, A., Bittner, A. N., Kim, S. H., Liu, K., Tehranchi, A. K., Zou, W. Y., . . . Wang, J. D. (2012a). Direct regulation of GTP homeostasis by (p)ppGpp: a critical component of viability and stress resistance. *Mol Cell, 48*(2), 231-241. doi:10.1016/j.molcel.2012.08.009

Kriel, A., Bittner, A. N., Kim, S. H., Liu, K., Tehranchi, A. K., Zou, W. Y., . . . Wang, J. D. (2012b). Direct regulation of GTP homeostasis by (p)ppGpp: a critical component of viability and stress resistance. *Molecular Cell, 48*(2), 231-241. doi:10.1016/j.molcel.2012.08.009

Krismer, B., Liebeke, M., Janek, D., Nega, M., Rautenberg, M., Hornig, G., . . . Peschel, A. (2014). Nutrient limitation governs Staphylococcus aureus metabolism and niche adaptation in the human nose. *PLoS Pathog, 10*(1), e1003862. doi:10.1371/journal.ppat.1003862

Kudrin, P., Dzhygyr, I., Ishiguro, K., Beljantseva, J., Maksimova, E., Oliveira, S. R. A., . . . Hauryliuk, V. (2018). The ribosomal A-site finger is crucial for binding and activation of the stringent factor RelA. *Nucleic Acids Res, 46*(4), 1973-1983. doi:10.1093/nar/gky023

Kullik, I., Jenni, R., & Berger-Bachi, B. (1998). Sequence of the putative alanine racemase operon in Staphylococcus aureus: insertional interruption of this operon reduces D-alanine substitution of lipoteichoic acid and autolysis. *Gene, 219*(1-2), 9-17. doi:10.1016/s0378-1119(98)00404-1

Kuroda, M., Kuroda, H., Oshima, T., Takeuchi, F., Mori, H., & Hiramatsu, K. (2003). Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in Staphylococcus aureus. *Mol Microbiol, 49*(3), 807-821. doi:10.1046/j.1365-2958.2003.03599.x

Kushwaha, G. S., Bange, G., & Bhavesh, N. S. (2019). Interaction studies on bacterial stringent response protein RelA with uncharged tRNA provide evidence for its prerequisite complex for ribosome binding. *Curr Genet*. doi:10.1007/s00294-019-00966-y

Kushwaha, G. S., Oyeyemi, B. F., & Bhavesh, N. S. (2019). Stringent response protein as a potential target to intervene persistent bacterial infection. *Biochimie, 165*, 67-75. doi:10.1016/j.biochi.2019.07.006

Lemos, J. A., Brown, T. A., Jr., & Burne, R. A. (2004). Effects of RelA on key virulence properties of planktonic and biofilm populations of Streptococcus mutans. *Infect Immun, 72*(3), 1431-1440. doi:10.1128/iai.72.3.1431-1440.2004

Lemos, J. A., Lin, V. K., Nascimento, M. M., Abranches, J., & Burne, R. A. (2007). Three gene products govern (p)ppGpp production by Streptococcus mutans. *Mol Microbiol, 65*(6), 1568-1581. doi:10.1111/j.1365-2958.2007.05897.x

Lemos, J. A., Nascimento, M. M., Lin, V. K., Abranches, J., & Burne, R. A. (2008). Global regulation by (p)ppGpp and CodY in Streptococcus mutans. *J Bacteriol, 190*(15), 5291-5299. doi:10.1128/JB.00288-08

Lister, J. L., & Horswill, A. R. (2014). Staphylococcus aureus biofilms: recent developments in biofilm dispersal. *Front Cell Infect Microbiol, 4*, 178. doi:10.3389/fcimb.2014.00178

Liu, K., Bittner, A. N., & Wang, J. D. (2015). Diversity in (p)ppGpp metabolism and effectors. *Curr Opin Microbiol, 24*, 72-79. doi:10.1016/j.mib.2015.01.012

Loveland, A. B., Bah, E., Madireddy, R., Zhang, Y., Brilot, A. F., & Grigorieff, N. (2016). Ribosome*RelA structures reveal the mechanism of stringent response activation. *5*. doi:10.7554/eLife.17029

Lowy, F. D. (1998). Staphylococcus aureus infections. *N Engl J Med, 339*(8), 520-532. doi:10.1056/nejm199808203390806

Mader, U., Nicolas, P., Depke, M., Pane-Farre, J., Debarbouille, M., van der Kooi-Pol, M. M., . . . van Dijl, J. M. (2016). Staphylococcus aureus Transcriptome Architecture: From Laboratory to Infection-Mimicking Conditions. *PLoS Genet, 12*(4), e1005962. doi:10.1371/journal.pgen.1005962

Maisonneuve, E., Castro-Camargo, M., & Gerdes, K. (2013). (p)ppGpp controls bacterial persistence by stochastic induction of toxin-antitoxin activity. *Cell, 154*(5), 1140-1150. doi:10.1016/j.cell.2013.07.048

Majerczyk, C. D., Dunman, P. M., Luong, T. T., Lee, C. Y., Sadykov, M. R., Somerville, G. A., . . . Sonenshein, A. L. (2010). Direct targets of CodY in *Staphylococcus aureus*. *J Bacteriol, 192*(11), 2861-2877. doi:10.1128/JB.00220-10

Manav, M. C., Beljantseva, J., Bojer, M. S., Tenson, T., Ingmer, H., Hauryliuk, V., & Brodersen, D. E. (2018). Structural basis for (p)ppGpp synthesis by the Staphylococcus aureus small alarmone synthetase RelP. *J Biol Chem, 293*(9), 3254-3264. doi:10.1074/jbc.RA117.001374

Mandell, G. L. (1975). Catalase, superoxide dismutase, and virulence of Staphylococcus aureus. In vitro and in vivo studies with emphasis on staphylococcal--leukocyte interaction. *J Clin Invest, 55*(3), 561-566. doi:10.1172/jci107963

Mansour, S. C., Pletzer, D., de la Fuente-Nunez, C., Kim, P., Cheung, G. Y. C., Joo, H. S., . . . Hancock, R. E. W. (2016). Bacterial Abscess Formation Is Controlled by the Stringent Stress Response and Can Be Targeted Therapeutically. *EBioMedicine, 12*, 219-226. doi:10.1016/j.ebiom.2016.09.015

Martins, D., McKay, G., Sampathkumar, G., Khakimova, M., English, A. M., & Nguyen, D. (2018). Superoxide dismutase activity confers (p)ppGpp-mediated antibiotic tolerance to stationary-phase Pseudomonas aeruginosa. *Proc Natl Acad Sci U S A, 115*(39), 9797-9802. doi:10.1073/pnas.1804525115

Massey, V., Strickland, S., Mayhew, S. G., Howell, L. G., Engel, P. C., Matthews, R. G., . . . Sullivan, P. A. (1969). The production of superoxide anion radicals in the reaction of reduced flavins and flavoproteins with molecular oxygen. *Biochem Biophys Res Commun, 36*(6), 891-897.

Matsuo, M., Hiramatsu, M., Singh, M., Sasaki, T., Hishinuma, T., Yamamoto, N., . . . Hiramatsu, K. (2019). Genetic and Transcriptomic Analyses of Ciprofloxacin-Tolerant Staphylococcus aureus Isolated by the Replica Plating Tolerance Isolation System (REPTIS). *Antimicrob Agents Chemother, 63*(2), e02019-02018. doi:10.1128/aac.02019-18

Matsuo, M., Hiramatsu, M., Singh, M., Sasaki, T., Hishinuma, T., Yamamoto, N., . . . Hiramatsu, K. (2019). Genetic and Transcriptomic Analyses of Ciprofloxacin-Tolerant Staphylococcus aureus Isolated by the Replica Plating Tolerance Isolation System (REPTIS). *Antimicrob Agents Chemother, 63*(2). doi:10.1128/aac.02019-18

Matsuo, M., Yamamoto, N., Hishinuma, T., & Hiramatsu, K. (2019). Identification of a Novel Gene Associated with High-Level beta-Lactam Resistance in Heterogeneous Vancomycin-Intermediate Staphylococcus aureus Strain Mu3 and Methicillin-Resistant S. aureus Strain N315. *Antimicrob Agents Chemother, 63*(2). doi:10.1128/aac.00712-18

McCallum, N., Meier, P. S., Heusser, R., & Berger-Bächi, B. (2011). Mutational Analyses of Open Reading Frames within thevraSROperon and Their Roles in the Cell Wall Stress Response ofStaphylococcus aureus. *Antimicrobial Agents and Chemotherapy, 55*(4), 1391-1402. doi:10.1128/aac.01213-10

McGuinness, W. A., Malachowa, N., & DeLeo, F. R. (2017). Vancomycin Resistance in Staphylococcus aureus. *Yale J Biol Med, 90*(2), 269-281.

Mechold, U., Cashel, M., Steiner, K., Gentry, D., & Malke, H. (1996). Functional analysis of a relA/spoT gene homolog from Streptococcus equisimilis. *J Bacteriol, 178*(5), 1401-1411. doi:10.1128/jb.178.5.1401-1411.1996

Mechold, U., Murphy, H., Brown, L., & Cashel, M. (2002). Intramolecular regulation of the opposing (p)ppGpp catalytic activities of Rel(Seq), the Rel/Spo enzyme from Streptococcus equisimilis. *J Bacteriol, 184*(11), 2878-2888. doi:10.1128/jb.184.11.2878-2888.2002

Mechold, U., Potrykus, K., Murphy, H., Murakami, K. S., & Cashel, M. (2013). Differential regulation by ppGpp versus pppGpp in *Escherichia coli*. *Nucleic Acids Res, 41*(12), 6175-6189. doi:10.1093/nar/gkt302

Mittenhuber, G. (2001). Comparative genomics and evolution of genes encoding bacterial (p)ppGpp synthetases/hydrolases (the Rel, RelA and SpoT proteins). *J Mol Microbiol Biotechnol, 3*(4), 585-600.

Modun, B., Evans, R. W., Joannou, C. L., & Williams, P. (1998). Receptor-mediated recognition and uptake of iron from human transferrin by Staphylococcus aureus and Staphylococcus epidermidis. *Infect Immun, 66*(8), 3591-3596.

Morikawa, K., Ohniwa, R. L., Kim, J., Maruyama, A., Ohta, T., & Takeyasu, K. (2006). Bacterial nucleoid dynamics: oxidative stress response in Staphylococcus aureus. *Genes Cells, 11*(4), 409-423. doi:10.1111/j.1365-2443.2006.00949.x

Morrissey, J. A., Cockayne, A., Brummell, K., & Williams, P. (2004). The Staphylococcal Ferritins Are Differentially Regulated in Response to Iron and Manganese and via PerR and Fur. *Infect Immun, 72*(2), 972-979. doi:10.1128/iai.72.2.972-979.2004

Mwangi, M. M., Kim, C., Chung, M., Tsai, J., Vijayadamodar, G., Benitez, M., . . . Tomasz, A. (2013). Whole-genome sequencing reveals a link between beta-lactam resistance and synthetases of the alarmone (p)ppGpp in Staphylococcus aureus. *Microb Drug Resist, 19*(3), 153-159. doi:10.1089/mdr.2013.0053

Nagarajan, R. (1991). Antibacterial activities and modes of action of vancomycin and related glycopeptides. *Antimicrob Agents Chemother, 35*(4), 605-609. doi:10.1128/aac.35.4.605

Nascimento, M. M., Lemos, J. A., Abranches, J., Lin, V. K., & Burne, R. A. (2008). Role of RelA of Streptococcus mutans in global control of gene expression. *J Bacteriol, 190*(1), 28-36. doi:10.1128/jb.01395-07

Nishino, T., Gallant, J., Shalit, P., Palmer, L., & Wehr, T. (1979). Regulatory nucleotides involved in the Rel function of Bacillus subtilis. *J Bacteriol, 140*(2), 671-679.

Novichkov, P. S., Kazakov, A. E., Ravcheev, D. A., Leyn, S. A., Kovaleva, G. Y., Sutormin, R. A., . . . Rodionov, D. A. (2013). RegPrecise 3.0--a resource for genome-scale exploration of transcriptional regulation in bacteria. *BMC Genomics, 14*, 745. doi:10.1186/1471-2164-14-745

Otto, M. (2014). Phenol-soluble modulins. *Int J Med Microbiol, 304*(2), 164-169. doi:10.1016/j.ijmm.2013.11.019

P. Skaar, E., & Schneewind, O. (2004). Iron-regulated surface determinants (Isd) of Staphylococcus aureus: stealing iron from heme. *Microbes and Infection, 6*(4), 390-397. doi:10.1016/j.micinf.2003.12.008

Pando, J. M., Pfeltz, R. F., Cuaron, J. A., Nagarajan, V., Mishra, M. N., Torres, N. J., . . . Gustafson, J. E. (2017). Ethanol-induced stress response of Staphylococcus aureus. *Can J Microbiol, 63*(9), 745-757. doi:10.1139/cjm-2017-0221

Pao, C. C., & Dyess, B. T. (1981). Effect of unusual guanosine nucleotides on the activities of some Escherichia coli cellular enzymes. *Biochim Biophys Acta, 677*(3-4), 358-362. doi:10.1016/0304-4165(81)90247-6

Pao, C. C., & Gallant, J. (1979). A new nucleotide involved in the stringent response in Escherichia coli. Guanosine 5'-diphosphate-3'-monophosphate. *J Biol Chem, 254*(3), 688-692.

Peschel, A., & Otto, M. (2013). Phenol-soluble modulins and staphylococcal infection. *Nat Rev Microbiol, 11*(10), 667-673. doi:10.1038/nrmicro3110

Pohl, K., Francois, P., Stenz, L., Schlink, F., Geiger, T., Herbert, S., . . . Wolz, C. (2009). CodY in Staphylococcus aureus: a regulatory link between metabolism and virulence gene expression. *J Bacteriol, 191*(9), 2953-2963. doi:10.1128/JB.01492-08

Potrykus, K., & Cashel, M. (2008). (p)ppGpp: still magical? *Annu Rev Microbiol, 62*, 35-51. doi:10.1146/annurev.micro.62.081307.162903

Pynnonen, M., Stephenson, R. E., Schwartz, K., Hernandez, M., & Boles, B. R. (2011). Hemoglobin promotes Staphylococcus aureus nasal colonization. *PLoS Pathog, 7*(7), e1002104. doi:10.1371/journal.ppat.1002104

Queck, S. Y., Jameson-Lee, M., Villaruz, A. E., Bach, T. H., Khan, B. A., Sturdevant, D. E., . . . Otto, M. (2008). RNAIII-independent target gene control by the agr quorum-sensing system: insight into the evolution of virulence regulation in Staphylococcus aureus. *Mol Cell, 32*(1), 150-158. doi:10.1016/j.molcel.2008.08.005

Rajagopal, M., & Walker, S. (2017). Envelope Structures of Gram-Positive Bacteria. *Curr Top Microbiol Immunol, 404*, 1-44. doi:10.1007/82_2015_5021

Reiss, S., Pane-Farre, J., Fuchs, S., Francois, P., Liebeke, M., Schrenzel, J., . . . Engelmann, S. (2012). Global analysis of the Staphylococcus aureus response to mupirocin. *Antimicrob Agents Chemother, 56*(2), 787-804. doi:10.1128/AAC.05363-11

Repine, J. E., Fox, R. B., Berger, E. M., & Harada, R. N. (1981). Effect of staphylococcal iron content on the killing of Staphylococcus aureus by polymorphonuclear leukocytes. *Infect Immun, 32*(1), 407-410.

Romling, U., & Balsalobre, C. (2012). Biofilm infections, their resilience to therapy and innovative treatment strategies. *J Intern Med, 272*(6), 541-561. doi:10.1111/joim.12004

Ruwe, M., Kalinowski, J., & Persicke, M. (2017). Identification and Functional Characterization of Small Alarmone Synthetases in Corynebacterium glutamicum. *Front Microbiol, 8*, 1601. doi:10.3389/fmicb.2017.01601

Sakr, A., Bregeon, F., Mege, J. L., Rolain, J. M., & Blin, O. (2018). Staphylococcus aureus Nasal Colonization: An Update on Mechanisms, Epidemiology, Risk Factors, and Subsequent Infections. *Front Microbiol, 9*, 2419. doi:10.3389/fmicb.2018.02419

Sanchez-Vazquez, P., Dewey, C. N., Kitten, N., Ross, W., & Gourse, R. L. (2019). Genome-wide effects on Escherichia coli transcription from ppGpp binding to its two sites on RNA polymerase. *Proc Natl Acad Sci U S A, 116*(17), 8310-8319. doi:10.1073/pnas.1819682116

Santajit, S., & Indrawattana, N. (2016). Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. *Biomed Res Int, 2016*, 2475067. doi:10.1155/2016/2475067

Schlatterer, K., Beck, C., Hanzelmann, D., Lebtig, M., Fehrenbacher, B., Schaller, M., . . . Peschel, A. (2018). The Mechanism behind Bacterial Lipoprotein Release: Phenol-Soluble Modulins Mediate Toll-Like Receptor 2 Activation via Extracellular Vesicle Release from Staphylococcus aureus. *MBio, 9*(6). doi:10.1128/mBio.01851-18

Schroder, W., Goerke, C., & Wolz, C. (2013). Opposing effects of aminocoumarins and fluoroquinolones on the SOS response and adaptability in Staphylococcus aureus. *J Antimicrob Chemother, 68*(3), 529-538. doi:10.1093/jac/dks456

Seaton, K., Ahn, S. J., Sagstetter, A. M., & Burne, R. A. (2011). A transcriptional regulator and ABC transporters link stress tolerance, (p)ppGpp, and genetic competence in Streptococcus mutans. *J Bacteriol, 193*(4), 862-874. doi:10.1128/JB.01257-10

Sheldon, J. R., & Heinrichs, D. E. (2012). The iron-regulated staphylococcal lipoproteins. *Front Cell Infect Microbiol, 2*, 41. doi:10.3389/fcimb.2012.00041

Sherlock, M. E., Sudarsan, N., & Breaker, R. R. (2018). Riboswitches for the alarmone ppGpp expand the collection of RNA-based signaling systems. *Proc Natl Acad Sci U S A, 115*(23), 6052-6057. doi:10.1073/pnas.1720406115

Singh, M., Matsuo, M., Sasaki, T., Morimoto, Y., Hishinuma, T., & Hiramatsu, K. (2017). In Vitro Tolerance of Drug-Naive Staphylococcus aureus Strain FDA209P to Vancomycin. *Antimicrob Agents Chemother, 61*(2). doi:10.1128/AAC.01154-16

Sojka, L., Kouba, T., Barvik, I., Sanderova, H., Maderova, Z., Jonak, J., & Krasny, L. (2011). Rapid changes in gene expression: DNA determinants of promoter regulation by the concentration of the transcription initiating NTP in Bacillus subtilis. *Nucleic Acids Res, 39*(11), 4598-4611. doi:10.1093/nar/gkr032

Steinchen, W., & Bange, G. (2016). The magic dance of the alarmones (p)ppGpp. *Mol Microbiol, 101*(4), 531-544. doi:10.1111/mmi.13412

Steinchen, W., Schuhmacher, J. S., Altegoer, F., Fage, C. D., Srinivasan, V., Linne, U., . . . Bange, G. (2015). Catalytic mechanism and allosteric regulation of an oligomeric (p)ppGpp synthetase by an alarmone. *Proc Natl Acad Sci U S A, 112*(43), 13348-13353. doi:10.1073/pnas.1505271112

Steinchen, W., Vogt, M. S., Altegoer, F., Giammarinaro, P. I., Horvatek, P., Wolz, C., & Bange, G. (2018). Structural and mechanistic divergence of the small (p)ppGpp synthetases RelP and RelQ. *Sci Rep, 8*(1), 2195. doi:10.1038/s41598-018-20634-4

Sun, F., Ding, Y., Ji, Q., Liang, Z., Deng, X., Wong, C. C., . . . He, C. (2012). Protein cysteine phosphorylation of SarA/MgrA family transcriptional regulators mediates bacterial virulence and antibiotic resistance. *Proc Natl Acad Sci U S A, 109*(38), 15461-15466. doi:10.1073/pnas.1205952109

Surewaard, B. G., de Haas, C. J., Vervoort, F., Rigby, K. M., DeLeo, F. R., Otto, M., . . . Nijland, R. (2013). Staphylococcal alpha-phenol soluble modulins contribute to neutrophil lysis after phagocytosis. *Cell Microbiol, 15*(8), 1427-1437. doi:10.1111/cmi.12130

Syal, K., Flentie, K., Bhardwaj, N., Maiti, K., Jayaraman, N., Stallings, C. L., & Chatterji, D. (2017). Synthetic (p)ppGpp Analogue Is an Inhibitor of Stringent Response in Mycobacteria. *Antimicrob Agents Chemother, 61*(6). doi:10.1128/AAC.00443-17

Tian, C., Roghanian, M., Jorgensen, M. G., Sneppen, K., Sorensen, M. A., Gerdes, K., & Mitarai, N. (2016). Rapid Curtailing of the Stringent Response by Toxin-Antitoxin Module-Encoded mRNases. *J Bacteriol, 198*(14), 1918-1926. doi:10.1128/JB.00062-16

VandenBerg, K. E., Ahn, S., & Visick, J. E. (2016). (p)ppGpp-Dependent Persisters Increase the Fitness of Escherichia coli Bacteria Deficient in Isoaspartyl Protein Repair. *Appl Environ Microbiol, 82*(17), 5444-5454. doi:10.1128/AEM.00623-16

Wang, J., Tian, Y., Zhou, Z., Zhang, L., Zhang, W., Lin, M., & Chen, M. (2016). Identification and Functional Analysis of RelA/SpoT Homolog (RSH) Genes in Deinococcus radiodurans. *J Microbiol Biotechnol, 26*(12), 2106-2115. doi:10.4014/jmb.1601.01017

Wang, J. D., Sanders, G. M., & Grossman, A. D. (2007). Nutritional control of elongation of DNA replication by (p)ppGpp. *Cell, 128*(5), 865-875. doi:10.1016/j.cell.2006.12.043

Wang, X., Thompson, C. D., Weidenmaier, C., & Lee, J. C. (2018). Release of Staphylococcus aureus extracellular vesicles and their application as a vaccine platform. *Nat Commun, 9*(1), 1379. doi:10.1038/s41467-018-03847-z

Wendrich, T. M., Blaha, G., Wilson, D. N., Marahiel, M. A., & Nierhaus, K. H. (2002). Dissection of the mechanism for the stringent factor RelA. *Mol Cell, 10*(4), 779-788.

Wexselblatt, E., Kaspy, I., Glaser, G., Katzhendler, J., & Yavin, E. (2013). Design, synthesis and structure-activity relationship of novel Relacin analogs as inhibitors of Rel proteins. *Eur J Med Chem, 70*, 497-504. doi:10.1016/j.ejmech.2013.10.036

Wexselblatt, E., Oppenheimer-Shaanan, Y., Kaspy, I., London, N., Schueler-Furman, O., Yavin, E., . . . Ben-Yehuda, S. (2012). Relacin, a novel antibacterial agent targeting the Stringent Response. *PLoS Pathog, 8*(9), e1002925. doi:10.1371/journal.ppat.1002925

Winther, K. S., Roghanian, M., & Gerdes, K. (2018). Activation of the Stringent Response by Loading of RelA-tRNA Complexes at the Ribosomal A-Site. *Mol Cell, 70*(1), 95-105 e104. doi:10.1016/j.molcel.2018.02.033

Wolz, C., Geiger, T., & Goerke, C. (2010). The synthesis and function of the alarmone (p)ppGpp in firmicutes. *Int J Med Microbiol, 300*(2-3), 142-147. doi:10.1016/j.ijmm.2009.08.017

Wu, J., & Xie, J. (2009). Magic spot: (p) ppGpp. *J Cell Physiol, 220*(2), 297-302. doi:10.1002/jcp.21797

Xu, T., Wang, X. Y., Cui, P., Zhang, Y. M., Zhang, W. H., & Zhang, Y. (2017). The Agr Quorum Sensing System Represses Persister Formation through Regulation of Phenol Soluble Modulins in Staphylococcus aureus. *Front Microbiol, 8*, 2189. doi:10.3389/fmicb.2017.02189

Yan, X., Zhao, C., Budin-Verneuil, A., Hartke, A., Rince, A., Gilmore, M. S., . . . Pichereau, V. (2009). The (p)ppGpp synthetase RelA contributes to stress adaptation and virulence in Enterococcus faecalis V583. *Microbiology, 155*(Pt 10), 3226-3237. doi:10.1099/mic.0.026146-0

Yang, X., & Ishiguro, E. E. (2001). Dimerization of the RelA protein of Escherichia coli. *Biochem Cell Biol, 79*(6), 729-736. doi:10.1139/o01-144

Zapf, R. L., Wiemels, R. E., Keogh, R. A., Holzschu, D. L., Howell, K. M., Trzeciak, E., . . . Carroll, R. K. (2019). The Small RNA Teg41 Regulates Expression of the Alpha Phenol-Soluble Modulins and Is Required for Virulence in Staphylococcus aureus. *MBio, 10*(1). doi:10.1128/mBio.02484-18

Zhang, K., Du, Y., Si, Z., Liu, Y., Turvey, M. E., Raju, C., . . . Chan-Park, M. B. (2019). Enantiomeric glycosylated cationic block co-beta-peptides eradicate Staphylococcus aureus biofilms and antibiotic-tolerant persisters. *Nat Commun, 10*(1), 4792. doi:10.1038/s41467-019-12702-8

Zhu, J., Zhang, T., Su, Z., Li, L., Wang, D., Xiao, R., . . . Zhou, R. (2016). (p)ppGpp synthetases regulate the pathogenesis of zoonotic Streptococcus suis. *Microbiol Res, 191*, 1-11. doi:10.1016/j.micres.2016.05.007

Zhu, M., Pan, Y., & Dai, X. (2019). (p)ppGpp: the magic governor of bacterial growth economy. *Curr Genet*. doi:10.1007/s00294-019-00973-z

Zielinska, A. K., Beenken, K. E., Joo, H. S., Mrak, L. N., Griffin, L. M., Luong, T. T., . . . Smeltzer, M. S. (2011). Defining the strain-dependent impact of the Staphylococcal accessory regulator (sarA) on the alpha-toxin phenotype of Staphylococcus aureus. *J Bacteriol, 193*(12), 2948-2958. doi:10.1128/jb.01517-10

12 Curriculum vitae

13 Acknowledgements

Zunächst möchte ich mich bei meiner Doktormutter **Prof. Dr. Christiane Wolz** von ganzen Herzen dafür bedanken, dass sie mir die Möglichkeit gegeben hat bei ihr die Doktorarbeit zu machen. Christiane, du warst und bist eine großartige Chefin und standest mir immer zur Seite. Ich möchte dir von Herzen danken, dass du mich auf diesem Weg begleitet und unterstützt hast. Du hattest immer ein offenes Ohr, sei es in wissenschaftlicher oder auch privater Hinsicht gewesen. Ich danke dir für die vielen tollen Anregungen, die Möglichkeit für den Freiraum wissenschaftliche Ideen zu entwickeln und durchzuführen und für deine konstruktive Kritik. Ich habe in all den Jahren so viel gelernt und mich durch deine Hilfe, Unterstützung und Wertschätzung weiterentwickeln können. Du bist als „Chefin“ einzigartig und großartig.

Ich möchte mich auch herzlichst bei Prof. Dr. Heike Brötz-Oesterhelt für die Erstellung des Zweitgutachtens bedanken.

Meine Labgirls und Labboys! Wie kann ich mich in so wenigen Sätzen für die ganze Zeit bei euch nur bedanken? Die Zeit mit euch wird immer unvergesslich und der Wahnsinn bleiben. Ihr seid der Grund, weshalb die AG Wolz einfach der Hammer war. Wir haben so viel erlebt, gelacht, geweint und zusammen durch gestanden. Ihr seid nicht nur einfach Kollegen, sondern ihr seid zur Freunden und Teil meines Herzens geworden. Ihr standet mir nicht nur in guten, sondern auch in schlechten Zeiten stets mit offenen Armen und Ohren zur Seite und habt mir in vielen Hinsichten Kraft gegeben. **Fabio**, mein Čokolino. Ich habe an dir immer stets deine herzliche, offene italienische Art geschätzt. Vielen Dank für die vielen tollen Gespräche. Du warst ein guter Kollege und bist mir ein guter Freund geworden. Ich danke dir, dass du mir viel beigebracht hast und auch für unsere „stringent response“ Meetings. Danke, dass wir unsere gegenseitige Musik ertragen haben ;). **Shilpa**, you are a brilliant person and thank always giving me advice and discussing scientific problems. Thank you for all the fun and long/late lab days we could spent together. And thank you for all the spontaneity and support! You became a big part of my life and I am grateful for this. Thank you for all the laughter and fun. And thank you for supporting and pushing me in my darkest times. You are my sister from another mister =). **Mein liebes Lieschen**! Wir haben so einiges vom Bachelor bis zur Promotion zusammen durch gemacht. Danke für die all die tolle Zeit mit dir im Studium und später in der Promotion.

Danke für das gegenseitige Anspornen und unterstützen. Es ist so schön, dass du mich auf diesem Weg begleitet hast und auch immer für mich da warst. **Dani**, auch dir möchte ich für deine Freundschaft von ganzem Herzen danken. Du warst immer für mich da und hast mich auch aufgefangen und stets bestärkt. Danke für all den Spaß und die „nach Hause"-Wege und all die lustigen Freizeitaktivitäten. Meine zwei Mäuschens **Carina** und **Esther**. Ihr beide seid von Herzen einfach gute und hilfsbereite Menschen. Es hat mir immer sehr viel Freude bereitet mit euch im Lab zu stehen. Danke für all die verrückten und bekloppten Momenten, die ich mich euch haben durfte. Ihr seid einfach klasse! Ein besonderer Dank geht auch **Andrew**, der eine klasse Arbeit geleistet hat und stets motiviert mitgearbeitet hat. Ich möchte mich auch ganz herzliche bei **Isa** und **Vitty** für ihre Hilfe und super Leistungen im Labor. Aber besonders möchte ich mich bei meiner Labormama **Naty** und ihre magischen Händen bedanken. Diese Hände sind aus Gold :D. **Naty**, ich danke dir auch für dein offenes Ohr und die besondere Freundschaft, welche sich zwischen uns entwickelt hat. Du wirst immer meine Labormama und meine Freundin sein. Du bist ein ganz besonderer Mensch und nimmst einen ganz besonderen Platz in meinem Herzen ein. Ach und danke, dass du mich immer beschuldigst hast, auch wenn ich es nicht war :p. Auch bei allen ehemaligen und aktuellen AG Wolz Mitarbeiter und Studenten (Jessy, Naisa, Andrea, Janina, Andres, Jana, Alex, Julie, Valentin), möchte ich mich dafür bedanken, dass ihr den Laboralltag mit so viel Leben, Liebe und Freude gefüllt habt!

Als nächstes möchte ich mich bei zwei ganz besonderen Menschen bedanken, welche mich all die Jahre unterstützt und in allen Lebenslagen begleitet haben; meinen zwei besten Freundinnen und „Schwestern" **Madi** und **Rose**! Um Marie von Ebner-Eschenbach zu zitieren „Wirklich gute Freunde sind Menschen, die uns ganz genau kennen und trotzdem zu uns halten". Ich möchte mich bei euch für eure unerschütterliche Liebe und ausnahmslose Unterstützung und Ehrlichkeit bedanken. Vielen Dank, dass ihr mir immer interessiert zugehört habt und mich immer darin bestärkt habt, nicht von meinem Weg abzukommen und immer an mich geglaubt habt. Ich bin stolz, solche wunderbaren Menschen meine Freunde nennen zu dürfen! Ich weiß, egal was kommt, ihr seid da und ich für euch. Ihr seid der Spiegel meiner Seele und meines Herzens!

Ich möchte mich auch bei meiner Chaosgruppe **Helmut**, **Rafa**, **Rene**, **Patrick**, **Chris**, **Nadine, Karin**, **Stella, Silvi** und **Nicky** bedanken. Obwohl ich einen anderen beruflichen bzw. akademischen Weg gegangen bin, sind wir trotzdem immer alle immer gemeinsam den gleichen Weg der Freundschaft gegangen. Ich danke euch dafür, dass ihr mich nie habt vergessen lassen, wo meine Wurzeln liegen. Ohne euch wäre mein Leben nur halb so aufregend.

Ganz besonders möchte ich mich bei meiner **Familie** bedanken.

Danke an:

Meinen Bruder Kiki, welcher mich immer unterstützt hat und mir versucht hat so viele Lasten wie möglich von den Schultern zu nehmen und mir stets ein liebevoller Bruder gewesen ist und sich mein „Fachchinesisch“ angehört hat.

Meine „Baka“, dass du immer an mich geglaubt hast und mich mit all deinen möglichen Mitteln unterstützt hast und immer an mich gedacht hast.

Meinen Vater (Tata), welcher das alles nicht mehr miterleben konnte. Tata, ich möchte mich bei dir von ganzen Herzen bedanken, dass du dich zu deinen Lebzeiten so für mich aufgeopfert hast und alles gegeben hast, um mir den Weg zu ebnen.

Meiner Mutter (Majko). Mama, ich weiß viele Kinder behaupten von ihren Eltern, dass sie die besten der Welt haben. Aber ich habe definitiv die allerbeste Mutter dieser Welt. Ich möchte dir mehr als nur vom tiefsten Herzen für die jahrelange Unterstützung und Aufopferung danken. Du hast alles Mögliche getan, um mir das Leben zu erleichtern und mir Sorgen und Lasten abzunehmen. Du hast mir immer zugehört und mir mit deiner bedingungslosen Liebe Kraft gegeben. Du warst nicht nur in guten, sondern auch in schweren Zeiten für mich eine der größten Stützen. Egal was war, egal was kommt, ich weiß, du bist immer für mich da. Mama, danke für deine Liebe und Fürsorge. Danke, dass du es mir ermöglich hast diesen Weg zu gehen. Ich danke dir dafür, dass du mich zu dem Menschen gemacht hast, der ich heute bin. Du motivierst und inspirierst mich jeden Tag aufs Neue. Es gibt nicht genügend Worte auf dieser Welt, um dir zu sagen wie sehr ich dir für alles danke und dich liebe. Volim te Majko.