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

Petra Horvatek1, Andrea Salzer1, Andrew Magdy Fekry Hanna1, Fabio Lino Gratani1,2, Daniela Keinhörster1, Natalya Korn1, Marina Borisova1, Christoph Mayer1, Dominik Rejman3, Ulrike Mäder4, Christiane Wolz1,*

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

* christiane.wolz@uni-tuebingen.de

**Abstract**

The stringent response is characterized by the synthesis of the messenger molecules pppGpp, ppGpp or pGpp (here collectively designated (pp)pGpp). The phenotypic consequences resulting from (pp)pGpp accumulation vary among species and can be mediated by different underlying mechanisms. Most genome-wide analyses have been performed under stress conditions, which often mask the immediate effects of (pp)pGpp-mediated regulatory circuits. In *Staphylococcus aureus*, (pp)pGpp can be synthesized via the RelA-SpoT-homolog, RelSau upon amino acid limitation or via one of the two small (pp)pGpp synthetases RelP or RelQ upon cell wall stress. We used RNA-Seq to compare the global effects in response to induction of the synthetase of *rel-Syn* (coding for the enzymatic region of *RelSau*), or *relQ* without the need to apply additional stress conditions. Induction of *rel-Syn* resulted in changes in the nucleotide pool similar to induction of the stringent response via the tRNA synthetase inhibitor mupirocin: a reduction in the GTP pool, an increase in the ATP pool and synthesis of pppGpp, ppGpp and pGpp. Induction of all three enzymes resulted in similar changes in the transcriptome. However, RelQ was less active than Rel-Syn and RelP, indicating strong restriction of its (pp)pGpp-synthesis activity in vivo.

PPP induction resulted in the downregulation of many genes involved in protein and RNA/DNA metabolism. Many of the (pp)pGpp upregulated genes are part of the GTP sensitive CodY regulon and thus likely regulated through lowering of the GTP pool. New CodY independent transcriptional changes were detected including genes involved in the SOS response, iron storage (e.g., *ftnA*, *dps*), oxidative stress response (e.g., *perR*, *katA*, *sodA*) and the *psma1–4* and *psmβ1–2* operons coding for cytotoxic, phenol soluble modulins (PSMs). Analyses of the *ftnA*, *dps* and *psm* genes in different regulatory mutants revealed that their (pp)pGpp-dependent regulation can occur independent of the regulators PerR, Fur, SarA or CodY. Moreover, *psm* expression is uncoupled from expression of the quorum sensing system Agr, the main known *psm* activator. The expression of central genes of the oxidative stress response protects the bacteria from anticipated ROS stress derived from...
PSMs or exogenous sources. Thus, we identified a new link between the stringent response and oxidative stress in *S. aureus* that is likely crucial for survival upon phagocytosis.

**Author summary**

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

**Introduction**

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

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

RelP and RelQ are part of the VraRS cell-wall stress regulon [15] and are thus transcriptionally induced, e.g., after vancomycin treatment [16]. Thereby, they contribute to tolerance towards cell-wall active antibiotics such as ampicillin or vancomycin. Recently, structural and mechanistic characterization revealed that RelQ from *Bacillus subtilis* and *Enterococcus faecalis* form tetramers [17,18]. RelQ activity is strongly inhibited through the binding of single-stranded RNA. pppGpp binding leads to disassociation of the RelQ-RNA complex and its activation [18]. In contrast, RelP activity is inhibited by both pppGpp and ppGpp, activated by Zn²⁺ and insensitive to inhibition by RNA [19,20]. For the *S. aureus* enzymes it could be
shown that RelQ, but not RelP are allosterically stimulated by the addition of pppGpp, ppGpp or pGpp [21]. Thus, although highly homologous, RelP and RelQ seem to have different functions within the cell. One can assume that different post-translational regulatory mechanisms are in play to fine-tune (pp)pGpp synthesis under different growth conditions.

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

Here, we aimed to compare the Rel-, RelQ- and RelP-mediated effects on nucleotide pools, transcription and functional consequences without imposing nutrient starvation or stress. Therefore, the Rel synthetase (Rel-Syn, N-Terminal region of Rel with inactivated hydrolase domain [14] designated as Rel-Syn. Rel-Syn or relQ were expressed using an ATc-inducible promoter in a (pp)pGpp strain in which the enzymatic domains of all three synthetases were mutated. Strain (pp)pGpp is unable to synthesize (pp)pGpp due to mutations in all three (pp)pGpp synthetases (full deletion of rel, synthetase mutations in relP and relQ [14]). Strains were grown to an early exponential growth phase and gene expression was induced by ATc for 30 min. Sub-inhibitory concentration of ATc resulted in similar induction of rel-Syn or relQ, (7.0 and 6.7 log₂ fold increase compared

**Results**

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

We first compared the stringent response imposed by mupirocin (isoleucyl-tRNA synthase inhibitor [39]) with the genetic induction of (pp)pGpp synthetases. To analyse Rel dependent (pp)pGpp synthesis without stress, we cloned the N-terminal region of Rel with inactivated hydrolase, RelP and RelQ were expressed from an anhydrotetracycline (ATc)-inducible promoter in a (pp)pGpp strain in which the enzymatic domains of all three synthetases were mutated. Through RNA-Seq analyses, we identified new (pp)pGpp-regulated genes, many of which are involved in the oxidative stress response, iron storage and the synthesis of phenol-soluble modulins (PSMs). Thus, (pp)pGpp synthesis contributes not only to PSM-derived ROS production but also to protection from these toxic molecules.
to un-induced, respectively, S1 Data). ATc treatment did not influence gene expression in strains containing the empty control plasmid pCG248 (S1 Fig).

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

Fig 1. Changes in the nucleotide pool after mupirocin treatment or transcriptional induction of *rel-Syn* or *relQ*. Strain HG001 and derivatives were grown to OD<sub>600</sub> = 0.3 and treated for 30 min with or without 0.125 μg/ml mupirocin (light grey) or 0.1 μg/ml ATc (dark grey). Nucleotide analyses were performed using mass spectrometry (ESI-TOF) in negative ion mode. Error bars represent SEM (n = 3) from three biological replicates. The adenylate energy charge was calculated by [ATP] + 0.5 [ADP]/[ATP] + [ADP] + [AMP]. Statistical significance was determined by two-tailed Student’s T-test, \( *p \leq 0.05, **p \leq 0.01, ***p \leq 0.001 \) and \( ****p \leq 0.0001 \).

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

**Impact of (pp)pGpp synthesis on the transcriptome**

We next analysed the impact of (pp)pGpp synthesis after induction of rel-syn or relQ on mRNA abundance. RNA-Seq data revealed that 1074 genes or sRNAs were significantly affected by either Rel-Syn (total: 478 up, 551 down) or RelQ (total: 155 up, 97 down) with a large overlap in genes affected by Rel-Syn and RelQ (Fig 2A and S1 Data). However, consistent with the nucleotide measurements (Fig 1), the effect of relQ induction was less prominent (S1 Table). We compared the RNA-Seq data with previous microarray analyses obtained after stringent response imposed by transferring bacteria to amino acid limiting conditions (-Leu, -Val) [22]. Most of the previously identified stringent response genes were confirmed by the RNA-Seq analysis (Fig 2A). Of note, in the present analysis, only genes with at least three-fold differences and a significance level of p < 0.001 were included in the analysis shown in Fig 2 and S2 Data. Despite the higher stringency in the analysis, the present analysis revealed far more (pp)pGpp-regulated genes. Genes were classified into functional categories using the SEED annotation (http://pubseed.theseed.org). (pp)pGpp induction resulted in the downregulation of many genes involved in protein synthesis (e.g ribosomal proteins RplA-T) and RNA/DNA (e.g. purine biosynthesis, gyrase) metabolism consistent with previous results that the stringent response mainly leads to the shutdown of translation and replication [22] (Fig 2B). Many of the genes upregulated by Rel-Syn and RelQ are part of the CodY regulon (S1 Data) and thus likely regulated by lowering of the GTP pool. The sRNA, RsaD was recently described to be directly repressed by CodY [40]. Concordantly, rsaD was found strongly up-regulated upon rel-Syn and relQ induction (8.5 and 6.0 log₂ fold change, respectively) and used as read-out for a prototypic CodY target is subsequent experiments. AlsS was identified as RsaD repressed target gene [40] and as expected downregulated upon rel-Syn expression. Thus, the genetic approach proved to gain reliable, physiologically relevant results as validated by comparison with previous studies on (pp)pGpp mediated transcriptional changes. We further focused on so far unknown genes/phenotypes which were induced during stringent conditions. Therefore, we concentrated on genes that were strongly affected by rel-syn induction (Fig 2C and S1 Data) but not known to be under the control of CodY. Interestingly, many of these genes were assigned to iron acquisition/metabolism (upregulation of genes involved in iron storage; downregulation of genes involved in siderophore biosynthesis and iron transport), stress response (dps, sodA, katA ahpC, uspA1/2, asp23, ptpA, and msrA2), and virulence (upregulation of psmαβ; downregulation of agr). Phage-encoded genes were also upregulated, indicating phage-inducing conditions. This is in line with the upregulation of recA and lexA. For further analysis, we selected ftnA, dps, agr and psmα as read-out for (pp)pGpp-mediated CodY independent activities.

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

We compared the expression of the selected genes after induction of the stringent response via mupirocin and after rel-Syn induction by Northern blot analysis (Fig 3A) and qRT-PCR (Fig 3B).
Fig 2. Global changes in gene expression upon transcriptional induction of rel-Syn or relQ. (pp)pGpp\(^6\) with inducible rel-Syn or relQ was grown to \(OD_{600} = 0.3\) and treated for 30 min without or with 0.1 \(\mu\)g/ml ATc. A. Venn diagrams showing genes or sRNAs upregulated (yellow) or downregulated (blue) after induction in comparison to uninduced cultures (\(< 3\)-fold difference, \(p < 0.001\)). Previously described stringent genes [22] are indicated as Rel-Leu/Val. B. Genes with significant changes after induction of rel-syn (\(< 3\)-fold difference, \(p < 0.001\)) according to functional categories. C. Heatmap representing Rel-Syn-dependent up- and downregulated genes assigned to the functional categories iron acquisition and metabolism, stress response and Agr-related genes.

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

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

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

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

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

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

To confirm that the observed gene expression pattern is not restricted to strain HG001 and not due to potential second site mutations in the (pp)pGpp0 strain we repeated key experiments in strain USA300. Therefore a (pp)pGpp0 strain was constructed by sequential mutation of relP, relQ and rel. rel-Syn, relQ and relP were induced from the ATc inducible expression vectors as described. The transcriptional changes imposed by rel-Syn induction recapitulated the findings of rel-Syn induction in strain HG001 (Fig 4). We also verified that induction of relQ only slightly effects marker gene expression (Fig 4). However, induction of relP was highly effective, resulting in an expression pattern comparable to induction of rel-Syn. The strong effect of relP induction could be due to the fact that in contrast to relP it does not require pppGpp activation [20].

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

Some of the prominent (pp)pGpp-activated genes are known to be under the control of other global regulators, such as PerR, Fur and SarA [42]. We speculated that (pp)pGpp dependent
gene expression may somehow be mediated via these global regulators. Therefore, we analysed rel-Syn induction in per, fur and sarA mutants (Figs 5A and S3A).

The (pp)pGpp controlled genes finA, dps, ahpC, katA and perR (S1 Data) are likely controlled via binding of PerR to a conserved PerR-binding motif (based on the public databases RegPrecise [43] and Aureowiki [44]). As expected, finA and dps were both upregulated in the perR mutants. Inducing rel-Syn in perR/(pp)pGpp0 strain showed further increase in finA, supporting that (pp)pGpp acts in addition and independent of PerR. For dps, the perR mutation also resulted in high expression, which was slightly decreased by (pp)pGpp. PerR deletion resulted in a slight decrease in psm expression, which was compensated by rel-syn induction. Thus, (pp)pGpp also affects gene expression in a per-negative background.

We found that many of the genes affected by Rel-Syn are indicative of iron overload conditions (e.g. upregulation of finA and dps (Fig 2D). We induced rel-Syn in a fur/(pp)pGpp0 background under low and high iron conditions (Figs 5B and S3). Independent of the availability of iron, finA, dps and psm were upregulated and agr was downregulated after rel-Syn induction in the fur-negative background indicating that (pp)pGpp regulation is not determined by iron availability or fur regulation.

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

Recently, it was shown that PSMs lead to the production of reactive oxygen species (ROS) [50]. One might speculate that under stringent conditions PSM-mediated ROS production triggers the expression of the oxidative stress genes. In this case, induction of rel-Syn should not result in the induction of these genes under anaerobic conditions, where ROS cannot be produced. However, rel-Syn induction resulted in the same transcriptional pattern regardless of whether bacteria were grown with or without oxygen (Fig 6A). We also analysed whether ROS would result in activation of the stringent response. However, H$_2$O$_2$ treatment did not affect transcription of stringent response genes such as ftnA, dps, psm or rpsl (S5 Fig). Thus, (pp)pGpp-mediated gene alterations of the selected marker genes are not a consequence of ROS formation.

These data indicate that (pp)pGpp simultaneously activates ROS-producing PSMs as well as ROS defence systems to prepare cells to withstand oxidative stress. To verify this hypothesis, we challenged wild type and mutant strains deficient in (pp)pGpp synthesis with H$_2$O$_2$. The (pp)pGpp$^0$ strain was indeed more sensitive to oxidative stress. The minimal inhibitor concentration (MIC) of H$_2$O$_2$ to inhibit growth of the wild type was 6.4 mM and 3.2 mM for the (pp)pGpp$^0$ strain. Moreover, the (pp)pGpp$^0$ strain was more efficiently killed after 1 h or 2 h incubation with H$_2$O$_2$ (S4 Fig). Under the assay conditions, the basal (pp)pGpp might be derived from any of the (pp)pGpp synthetases. We analyzed a relPQ mutant and a rel-Syn mutant in which the synthetase domain of Rel was mutated. Both strains showed an intermittent phenotype in which the MIC varied between 3.2 and 6.4 mM when biological replicates were analyzed. To follow up on these ambiguities, we monitored growth after addition of H$_2$O$_2$ (Fig 6C). There was high variation in the lag time between biological replicates. Replicates of the relPQ or rel-Syn mutant showed a delayed lag phase, and some of the replicates could not grow. In cases where no growth was detectable the cultures were sterile as determined by colony counting. The delay of the lag phase was more prominent for the relPQ mutant than for the rel-Syn mutant. Nevertheless, none of the (pp)pGpp$^0$ replicates could resume growth, and this result was consistent with the reproducible lowered MIC of this strain, indicating that (pp)pGpp indeed protects against oxidative stress.
We next speculated that under H$_2$O$_2$ conditions may induce stringent response in wild type bacteria. However, treatment of bacteria with H$_2$O$_2$ even at MIC concentrations did not induce stringent marker genes (S5 Fig). This indicates that the basal level of (pp)pGpp produced in wild type bacteria is sufficient to confer resistance.

**Discussion**

We chose a genetic approach to define the early transcriptional response upon (pp)pGpp synthesis without the need to apply additional stress conditions. As expected from previous studies, [22,51] (pp)pGpp synthesis resulted in severe downregulation of the translational machinery and de-repression of CodY target genes. Many additional (pp)pGpp-regulated genes that are presumably important for the survival of *S. aureus* during starvation conditions were identified. Regulation of these genes might occur indirectly through other so far ill-defined regulatory circuits or via changes in the nucleotide pool. Here, we focused mainly on genes that were found to be activated upon (pp)pGpp synthesis in a CodY-independent manner, particularly *psm, finA* and *dps*. The (pp)pGpp-dependent activation of these genes can also occur in strains missing the prototypic proteinaceous transcriptional regulators PerR, Fur, or SarA. These regulators are well known to be involved in the regulation of the selected genes. However, they need to be activated through oxidative stress and/or iron [42]. The RNaseq data revealed that transcription of the repressor *perR* is also upregulated upon (pp)pGpp synthesis indicating a feedback regulation to dampen the response. In summary, (pp)pGpp functions as a complementary, immediate message, allowing cells to react to adverse conditions such as amino acid starvation or cell wall stress. Under these conditions, upcoming oxidative stress seems to be anticipated, and (pp)pGpp prepares the cells for survival, e.g. ROS challenges (Fig 7). Indeed, a (pp)pGpp$^0$ strain is more sensitive to H$_2$O$_2$. Both Rel and RelP/RelQ contribute to the protective effect.

**(pp)pGpp leads to *psm* activation**

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

![Fig 7. (pp)pGpp protects from oxidative stress.](https://doi.org/10.1371/journal.pgen.1009282.g007)
However, PSMs also interact with the membrane of the producer, promote the release of membrane vesicles from the cytoplasmic membrane via an increase in membrane fluidity [55,56], reduce persister formation [57,58] and are involved in self-toxicity via ROS formation [50]. Interestingly, (pp)pGpp-dependent psm activation was not correlated with the activation of the quorum sensing Agr system, the main regulator required for psm expression [41]. Agr was even repressed under (pp)pGpp-inducing conditions. Previously, analysis of a clinical isolate overproducing (pp)pGpp also indicated that (pp)pGpp leads to agr inhibition [24]. Thus, (pp)pGpp-mediated psm activation is clearly uncoupled from agr expression. Previously, analysis of a clinical isolate overproducing (pp)pGpp also indicated that (pp)pGpp leads to agr inhibition [24]. Thus, (pp)pGpp-mediated psm activation is clearly uncoupled from agr expression. Recently, the sRNA Teg41 (S131) [59] and the transcriptional regulator MgrA [60], Rsp [61] or Rbf [62] were found to interfere with psm expression. However, it is unlikely that they mediate the (pp)pGpp regulatory effect because transcription of these regulators was unaltered based on our RNA-Seq analysis (S1 Data). Thus, the molecular mechanism by which (pp)pGpp leads to psm activation has to be elucidated. It is likely that the accompanying changes in the ATP/GTP ratios are crucial for this activation pattern. psm promoters might be sensitive to the concentration of the initiating nucleoside triphosphate (iNTP). The +1 position (e.g., G or A) of sensitive genes dictates whether transcriptional initiation/elongation requires high GTP or ATP levels, respectively [29]. Various sequence combinations determine whether a promoter is sensitive to iNTP [63]. Such sequence motifs are hard to predict within psm promoters. However, both the psmα and psmß operons start with an A at the +1 position [41], which might explain the higher expression due to the increased ATP levels following (p)ppGpp synthesis.

(pp)pGpp and oxidative stress response

Genes whose expression is indicative of iron overload conditions were also highly affected by (pp)pGpp. We recently, could confirm that the (pp)pGpp⁰ strain of strain USA300 has elevated free iron levels contributing to oxidative stress and increased ROS production [64]. A similar effect was reported for Vibrio cholera [65]. Here, the expression of the iron transporter FbpA was repressed via (pp)pGpp, resulting in a reduction of intracellular free iron required for the ROS-generating Fenton reaction. This contributed to reducing antibiotic-induced oxidative stress and thus tolerance, and this is also true for S. aureus [64]. In addition to interfering with iron metabolism, other genes involved in oxidative stress were activated by (pp)pGpp. A link between the stringent response and oxidative stress response has been observed in different organisms, although the underlying mechanisms and outcome might be highly diverse. (pp)pGpp-dependent upregulation of superoxide dismutase (SOD) was described in B. suis [66] and P. aeruginosa [67]. SOD was shown to be the key factor responsible for (pp)pGpp-mediated multidrug tolerance in P. aeruginosa [67]. Moreover, (pp)pGpp-deficient strains are often found to be more sensitive to oxidative stress [68,69,70]. However, in E. faecalis, a (pp)pGpp⁰ strain grew faster and to a higher growth yield than its parent in the presence of H₂O₂ [71].

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

Comparison of Rel-Syn, RelQ and RelP activity

We compared the in vivo activity of Rel-Syn, RelQ and RelP. RelP showed similar effect on gene expression as Rel-Syn, whereas RelQ was far less active (Fig 4). Thus, under our growth conditions RelQ activity seems to be restricted in vivo. Comparison of RelP and RelQ from
other organisms revealed that RelQ is inhibited through RNA binding and auto-activated by (pp)pGpp [19,20]. We analyzed RelQ activity in an (pp)pGpp background under non-stress conditions where RelQ activity is likely restricted via RNA binding and/or the missing basal (pp)pGpp provided by other synthetases. The conditions that would relieve this restriction remain to be determined. A recent biochemical analysis of purified RelP and RelQ from *S. aureus* confirmed that RelQ, but not RelP, was allosterically-stimulated by the addition of pppGpp, ppGpp or pGpp [21]. Moreover, in vitro both enzymes were shown to efficiently synthesize pGpp in addition to pppGpp and ppGpp. In our *in vivo* analysis we could detect considerable quantities of pGpp derived from Rel activity (Fig 1). However, *in vitro* Rel seems to preferentially synthesize pppGpp [21]. One may speculate that pGpp detected *in vivo* might be enzymatically derived from NuDiX hydrolase, NahA, an enzyme that in *B. subtilis* efficiently produces pGpp by hydrolyzing (p)ppGpp [73]. In this organism, pGpp potently regulates the purine biosynthesis pathway but in contrast to (p)ppGpp does not interact with the GTPases. It was proposed that the different nucleotides may fulfill different roles through fine-tuning in signal transduction. Moreover, the different levels of (pp)pGpp in the cell may also dictate different outcomes. There is now growing evidence that basal level of (pp)pGpp (often below detection limit) are involved to maintain balanced growth whereas high levels are indicative for stress conditions resulting in severe reprogramming of the cell and growth arrest [74,75]. In this context, the induction of *rel-Syn* likely mirrors stress conditions, whereas *relQ* induction with low detectable (pp)pGpp synthesis more resembles the proposed basal level. From the RNA-Seq analysis we found no clear quantitative difference between both conditions. However, many of the genes affected by RelQ could be assigned to the CodY regulon. This illustrates the high sensitivity of CodY towards subtle changes of the GTP pool imposed by (pp)pGpp synthesis.

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

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

**Materials and methods**

**Strains and growth conditions**

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

**Generation of (pp)pGpp<sup>0</sup> mutant in USA300 JE2**

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

**Generation of *perR*, *fur*, *sarA* and *psmA/β (pp)pGpp<sup>0</sup>** mutants in HG001

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

**RNA isolation, Northern Blot analysis and qRT-PCR analysis**

RNA isolation and Northern blot analysis were performed as described previously [78]. Briefly, bacteria were pelleted and resuspended in 1 ml TRIzol (Thermo Fisher Scientific) and lysed using zirconia/silica beads (0.1mm diameter) and a high speed homogenizer. RNA was isolated following the recommended procedure by TRIzol manufacturer. For RNA-Seq analysis RNA from the aqueous phase was further purified following the RNA-isolation protocol by Amp Tech ExpressArt RNA ready. Transcripts on the Northern blot were detected by dioxigenin-labeled probes, which were generated by a DNA-labelling PCR-Kit (Roche Life Science). Relative quantification of *ftnA*, *dps*, *agrA*, *α-type psms*, *rsaD* and *rpsl* transcripts by qRT-PCR was performed using the Quantstudio3 (Applied Biosystems) and the QuantiFast SYBR Green RT-PCR Kit (Qia-gen). Briefly, 5 μg of total RNA were DNase-treated and diluted 1:10 for qRT-PCR. Relative quantities of transcripts were calculated by a standard curve for each gene generated using 6-fold serial dilution of HG001 wild type RNA. Primers for qRT-PCR are listed in S2 Table.

**In vivo nucleotide extraction**

Nucleotides were isolated based on published protocol [79]. Briefly, strains were grown in CDM overnight, diluted to an OD<sub>600</sub> = 0.05 and grown in CDM until an OD<sub>600</sub> = 0.3. Strains
were split and treated with or without 0.1 μg/ml ATc for 30 min at 37˚C and 220 rpm shaking. 100 ml bacterial cultures were harvested and transferred into 50 ml centrifuge tubes half filled with ice and centrifuged (5 min, 5000 x g, 4˚C). Pellets were immediately frozen in liquid nitrogen and stored at -80˚C until usage. Samples were thawed on ice and resuspended in 2M formic acid and incubated for 30 min. Resuspended bacteria were lysed by high speed homogenizer using zirconia/silica beads (0.1 mm diameter) and kept on ice for 30 min. The aqueous phase was collected and mixed with 3 ml 50 mM NH₄OAc (pH 4.5), loaded on columns (OASIS Wax cartridge 3xcc) and centrifuged (5000 x g, 5 min, 4˚C). Columns were pre-treated first with pure 3 ml methanol and then with 3 ml 50 mM NH₄OAc (pH 4.5). Samples were washed first with 3 ml 50 mM NH₄OAc (pH 4.5) followed by a washing step with 3 ml methanol. Elution was performed with 1 ml of 20% methanol, 10% NH₄OH. Eluted nucleotides were flash frozen in liquid nitrogen and lyophilized overnight. Lyophilized nucleotides were resuspended in 100 μl ddH₂O and analyzed via HPLC-MS. pppGpp and ppGpp standard molecule were purchased Jena Biosciences. pGpp was synthesized starting from conveniently protected guanosine and employing both phosphoramidite and phosphotriester methods (S1 Methods).

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

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

I₁ = \sum_{k \mid k \leq L_1} \hat{I}_k. The second component was expressed as I₂ = 1−I₁.

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

**H₂O₂ killing assay**

Strains were grown over night in CDM, diluted in fresh CDM to an OD₆0₀ = 0.1 and growth followed for 24 hours with different H₂O₂ concentrations in a microplate reader (Infinite M200, Tecan). H₂O₂ killing was determined by incubation of bacteria grown from an overnight culture to OD₆0₀ = 0.3 followed by incubation with 80 mM H₂O₂ for 1 or 2 h. MIC determination was performed according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines using CDM medium (at least three biological replicates for each strain).
RNA-Seq analysis

Strains were grown in triplicates to OD₆₀₀ = 0.3, split into treated (ATc, 0.1 μg/ml) and untreated control and grown for 30 min, 37˚C. Purified RNA was sent to Vertis Biotechnologie AG Freiburg for RNA Sequencing based on Illumina Next Seq 500 system. RNA was examined by a capillary electrophoresis on a Shimadzu MultiNA microchip followed by rRNA depletion using Ribo-Zero rRNA removal Kit from Illumina. RNA was converted to cDNA by fragmenting RNA samples by ultrasound and ligating an oligonucleotide adapter to the 3’end of the RNA. Using M-MLV reverse transcriptase first strand cDNA was created using 3’ adapter as primer. The 5’Illumina TruSeq sequencing adapter was ligated to the 3’end of the purified (Agencourt AMPure XP kit) cDNA and PCR was performed. Samples were pooled in equimolar amounts and fractionated in a size range of 200–500 bp using a preparative agarose gel and Illumina sequencing was performed using 75 bp reads. RNA-Seq analysis was performed using CLC Genomic Workbench (Qiagen). Reads were trimmed (TrueSeq-Antinsense Primer AGATCGGAAGAGCACACGTCTGAACTCCAGTCA) and mapped to the reference genome of HG001 (NZ_CP018205.1). Differential gene expression was performed comparing Rel-Syn or RelQ versus the (pp)pGpp⁰ mutant. Venn diagrams were performed comparing Rel-Syn vs. control and RelQ vs. control. Genes with at least 3-fold difference and a p-value ≥0.001 were defined as differentially regulated compared to the untreated control. Annotation of genes are according to recent “Aureowiki” annotation of strain 8325 (http://aureowiki.med.uni-greifswald.de/Main_Page) [44]. Of the previously identified RNA segments [81] those annotated as indep, s-indep, and indep-NT were regarded as potential regulatory RNAs (sRNAs) and included in the analysis.

Supporting information

S1 Fig. ATc treatment of strains containing empty vector pCG248. Strain HG001 and derivatives were grown to OD₆₀₀ = 0.3 and treated for 30 min with or without 0.1 μg/ml ATc (mutant strains with inducible rel-Syn, relQ or empty vector). For Northern blot analysis, RNA was hybridized with digoxigenin-labelled probes specific for sodA, ftnA, dps, per, agrA or psm. The 16S rRNA detected in ethidium bromide-stained gels is indicated as a loading control. (TIF)

S2 Fig. Rel-Syn induction in HG001 WT. Gene expression following rel-Syn induction in HG001 wild type. HG001 was grown to OD₆₀₀ = 0.3 and treated for 30 min without or with 0.1 μg/ml ATc. Transcript were quantified by qRT-PCR on equal amount of total RNA. Statistical significance was determined by two-tailed Student’s T-test, "p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001 (TIF)

S3 Fig. Rel-Syn induction in fur, per and sar mutants qRT-PCR results accompanying Fig 5. Quantification of mRNA by qRT-PCR based on three biological replicates. Statistical significance was determined by two-tailed Student’s T-test, "p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001. (TIF)

S4 Fig. H₂O₂ kill curves. Strains were grown to OD₆₀₀ = 0.3 and then treated with 80mM H₂O₂ for 1 or 2 h. (TIF)

S5 Fig. H₂O₂ does not induce stringent response in S. aureus. Strains were grown to OD₆₀₀ = 0.3 and treated with mupirocin or H₂O₂ for 30 min. RNA was hybridized with digoxigenin-
labelled probes specific for fnA, dps, psm, agrA or rpsl. The 16S rRNA detected in ethidium bromide-stained gels is indicated as a loading control in the bottom lane.

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

S1 Table. Strains and plasmids.

S2 Table. Oligonucleotides.

S1 Methods. Synthesis of guanosine 3'-O-diphosphate 5'-O-phosphate pGpp.

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

S2 Data. Expression profile of all genes upon induction of rel-Syn or relQ.

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Author Contributions
Conceptualization: Christiane Wolz.
Data curation: Petra Horvatek, Andrea Salzer, Ulrike Mäder.
Formal analysis: Petra Horvatek, Andrea Salzer, Andrew Magdy Fekry Hanna.
Funding acquisition: Christiane Wolz.
Investigation: Petra Horvatek, Andrea Salzer, Fabio Lino Gratani, Daniela Keinhoerster.
Methodology: Petra Horvatek, Andrea Salzer, Andrew Magdy Fekry Hanna, Daniela Keinhoerster, Natalya Korn, Marina Borisova, Dominik Rejman.
Software: Ulrike Mäder.
Supervision: Fabio Lino Gratani, Marina Borisova, Christoph Mayer, Ulrike Mäder, Christiane Wolz.
Validation: Christoph Mayer.
Visualization: Andrea Salzer.
Writing – original draft: Petra Horvatek, Ulrike Mäder, Christiane Wolz.
Writing – review & editing: Andrea Salzer, Christiane Wolz.
References

1. Irving SE, Corrigan RM. Triggering the stringent response: signals responsible for activating (p)ppGpp synthesis in bacteria. Microbiology. 2018; 164(3):268–76. https://doi.org/10.1099/mic.0.000621 PMID: 29493495

2. Dozot M, Boigegrain RA, Delrue RM, Hailez R, Ouaharni-Bettache S, Danese I, et al. The stringent response mediator Rsh is required for Brucella melitensis and Brucella suis virulence, and for expression of the type IV secretion system viB. Cell Microbiol. 2006; 8(11):1791–802. https://doi.org/10.1111/j.1462-5822.2006.00749.x PMID: 16803581

3. Steinchen W, Bange G. The magic dance of the alarmones (p)ppGpp. Mol Microbiol. 2016; 101(4):531–44. https://doi.org/10.1111/mmi.13412 PMID: 27149325

4. Wu J, Xie J. Magic spot: (p)ppGpp. J Cell Physiol. 2009; 220(2):297–302. https://doi.org/10.1002/jcp.21797 PMID: 19391118

5. Zhu M, Pan Y, Dai X. (p)ppGpp: the magic governor of bacterial growth economy. Curr Genet. 2019. https://doi.org/10.1007/s00294-019-00973-z PMID: 30993414

6. Gaca AO, Colomer-Winter C, Lemos JA. Many means to a common end: the intricacies of (p)ppGpp metabolism and its control of bacterial homeostasis. J Bacteriol. 2015; 197(7):1146–56. https://doi.org/10.1128/JB.00046-14 PMID: 25605304

7. Liu K, Bittner AN, Wang JD. Diversity in (p)ppGpp metabolism and effectors. Curr Opin Microbiol. 2015; 24:72–9. https://doi.org/10.1016/j.mib.2015.01.012 PMID: 25636134

8. Hobbs JK, Boraston AB. (p)ppGpp and the Stringent Response: An Emerging Threat to Antibiotic Therapy. ACS Infect Dis. 2019; 5(9):1505–17. https://doi.org/10.1021/acsinfecdis.9b00204 PMID: 31287287

9. Geiger T, Goerke C, Fritz M, Schafer T, Ohlsen K, Liebeke M, et al. Role of the (p)ppGpp synthase RSH, a RelA/SpoT homolog, in stringent response and virulence of Staphylococcus aureus. Infect Immun. 2010; 78(5):1873–83. https://doi.org/10.1128/IAI.01439-09 PMID: 20212088

10. Kuroda M, Kuroda H, Oshima T, Takeuchi F, Mori H, Hiramatsu K. Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in Staphylococcus aureus. Mol Microbiol. 2003; 49(3):807–21. https://doi.org/10.1046/j.1365-2958.2003.03599.x PMID: 12864861

11. Geiger T, Kast B, Gratani FL, Horvatek P, Goerke C, Wolz C. Two small (p)ppGpp synthases in Staphylococcus aureus mediate tolerance against cell envelope stress conditions. J Bacteriol. 2014; 196(4):894–902. https://doi.org/10.1128/JB.01201-13 PMID: 24336937

12. Geiger T, Kast B, Gratani FL, Horvatek P, Goerke C, Wolz C. Two small (p)ppGpp synthases in Staphylococcus aureus mediate tolerance against cell envelope stress conditions. J Bacteriol. 2014; 196(4):894–902. https://doi.org/10.1128/JB.01201-13 PMID: 24336937

13. Kuroda M, Kuroda H, Oshima T, Takeuchi F, Mori H, Hiramatsu K. Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in Staphylococcus aureus. Mol Microbiol. 2003; 49(3):807–21. https://doi.org/10.1046/j.1365-2958.2003.03599.x PMID: 12864861

14. Geiger T, Kast B, Gratani FL, Horvatek P, Goerke C, Wolz C. Two small (p)ppGpp synthases in Staphylococcus aureus mediate tolerance against cell envelope stress conditions. J Bacteriol. 2014; 196(4):894–902. https://doi.org/10.1128/JB.01201-13 PMID: 24336937

15. Kuroda M, Kuroda H, Oshima T, Takeuchi F, Mori H, Hiramatsu K. Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in Staphylococcus aureus. Mol Microbiol. 2003; 49(3):807–21. https://doi.org/10.1046/j.1365-2958.2003.03599.x PMID: 12864861

16. Geiger T, Kast B, Gratani FL, Horvatek P, Goerke C, Wolz C. Two small (p)ppGpp synthases in Staphylococcus aureus mediate tolerance against cell envelope stress conditions. J Bacteriol. 2014; 196(4):894–902. https://doi.org/10.1128/JB.01201-13 PMID: 24336937

17. Steichen W, Schuhmacher JS, Altegoer F, Fage CD, Srinivasan V, Linne UC, et al. Catalytic mechanism and allosteric regulation of an oligomeric (p)ppGpp synthetase by an alarmon. Nat Struct Mol Biol. 2013; 20(6):620–9. https://doi.org/10.1038/nsmb.2450 PMID: 23686403

18. Beljantseva J, Kudrin P, Andrensen L, Shingler V, Atkinson GC, Tenson T, et al. Negative allosteric regulation of Enterococcus faecalis small alarmone synthetase RelQ by single-stranded RNA. Proc Natl Acad Sci U S A. 2015; 112(5):13348–53. https://doi.org/10.1073/pnas.1505271112 PMID: 26460002

19. Beljantseva J, Kudrin P, Andrensen L, Shingler V, Atkinson GC, Tenson T, et al. Negative allosteric regulation of Enterococcus faecalis small alarmone synthetase RelQ by single-stranded RNA. Proc Natl Acad Sci U S A. 2015; 112(5):13348–53. https://doi.org/10.1073/pnas.1505271112 PMID: 26460002

20. Beljantseva J, Kudrin P, Andrensen L, Shingler V, Atkinson GC, Tenson T, et al. Negative allosteric regulation of Enterococcus faecalis small alarmone synthetase RelQ by single-stranded RNA. Proc Natl Acad Sci U S A. 2015; 112(5):13348–53. https://doi.org/10.1073/pnas.1505271112 PMID: 26460002

21. Beljantseva J, Kudrin P, Andrensen L, Shingler V, Atkinson GC, Tenson T, et al. Negative allosteric regulation of Enterococcus faecalis small alarmone synthetase RelQ by single-stranded RNA. Proc Natl Acad Sci U S A. 2015; 112(5):13348–53. https://doi.org/10.1073/pnas.1505271112 PMID: 26460002

22. Beljantseva J, Kudrin P, Andrensen L, Shingler V, Atkinson GC, Tenson T, et al. Negative allosteric regulation of Enterococcus faecalis small alarmone synthetase RelQ by single-stranded RNA. Proc Natl Acad Sci U S A. 2015; 112(5):13348–53. https://doi.org/10.1073/pnas.1505271112 PMID: 26460002
21. Yang N, Xie S, Tang NY, Choi MY, Wang Y, Watt RM. The Ps and Qs of alarmone synthesis in Staphylococcus aureus. PLoS One. 2019; 14(10):e0213630. https://doi.org/10.1371/journal.pone.0213630 PMID: 31613897

22. Geiger T, Francois P, Liebeke M, Fraunholz M, Goerke C, Krismer B, et al. The stringent response of Staphylococcus aureus and its impact on survival after phagocytosis through the induction of intracellular PSMs expression. PLoS Pathog. 2012; 8(11):e1003016. https://doi.org/10.1371/journal.ppat.1003016 PMID: 23209405

23. Corrigan RM, Bellows LE, Wood A, Grundling A. ppGpp negatively impacts ribosome assembly affecting growth and antimicrobial tolerance in Gram-positive bacteria. Proc Natl Acad Sci U S A. 2016; 113(12):E1710–9. https://doi.org/10.1073/pnas.1522179113 PMID: 26951678

24. Gao W, Chua K, Davies JK, Newton JJ, Seemann T, Harrison PF, et al. Two novel point mutations in clinical Staphylococcus aureus reduce linezolid susceptibility and switch on the stringent response to promote persistent infection. PLoS Pathog. 2010; 6(6):e1000944. https://doi.org/10.1371/journal.ppat.1000944 PMID: 20548948

25. Dordel J, Kim C, Chung M, Pardos de la Gandara M, Holden MT, Parkhill J, et al. Novel determinants of antibiotic resistance: identification of mutated loci in highly methicillin-resistant subpopulations of methicillin-resistant Staphylococcus aureus. MBio. 2014; 5(2):e01000. https://doi.org/10.1128/mBio.01000-13 PMID: 24713324

26. Matsuura M, Hiramatsu M, Singh M, Sasaki T, Hishinuma T, Yamamoto N, et al. Genetic and Transcriptomic Analyses of Ciprofloxacin-Tolerant Staphylococcus aureus Isolated by the Replica Plating Tolerance Isolation System (REPTIS). Antimicrob Agents Chemother. 2019; 63(2):e02019–18. https://doi.org/10.1128/AAC.02019-18 PMID: 30509938

27. Katayama Y, Azechi T, Miyazaki M, Takata T, Sekine M, Matsu H, et al. Prevalence of Slow-Growth Vancomycin Nonsusceptibility in Methicillin-Resistant Staphylococcus aureus. Antimicrob Agents Chemother. 2017; 61(11). https://doi.org/10.1128/AAC.00452-17 PMID: 28827421

28. Kriel A, Bittner AN, Kim SH, Liu K, Tehranchi AK, Zou WY, et al. Direct regulation of GTP homeostasis by (p)ppGpp: a critical component of viability and stress resistance. Molecular Cell. 2012; 48(2):231–41. https://doi.org/10.1016/j.molcel.2012.08.009 PMID: 22981860

29. Krasny L, Tiserova H, Jonak J, Rejman D, Sanderova H. The identity of the transcription +1 position is crucial for changes in gene expression in response to amino acid starvation in Escherichia coli. EMBO J. 2004; 23(22):4473–83. https://doi.org/10.1038/sj.emboj.7600423

30. Katayama Y, Azechi T, Miyazaki M, Takata T, Sekine M, Matsu H, et al. Prevalence of Slow-Growth Vancomycin Nonsusceptibility in Methicillin-Resistant Staphylococcus aureus. Antimicrob Agents Chemother. 2017; 61(11). https://doi.org/10.1128/AAC.00452-17 PMID: 28827421

31. Krasny L, Tiserova H, Jonak J, Rejman D, Sanderova H. 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. 2008; 69(1):42–54. https://doi.org/10.1111/j.1365-2958.2008.06256.x PMID: 18433449

32. Kastle B, Geiger T, Gratani FL, Reisinger R, Goerke C, Borisova M, et al. rRNA regulation during growth and under stringent conditions in Staphylococcus aureus. Environ Microbiol. 2015; 17(11):4394–405. https://doi.org/10.1111/1462-2920.12867 PMID: 25845735

33. Krasny L, Gourse RL. An alternative strategy for bacterial ribosome synthesis: Bacillus subtilis rRNA transcription regulation. EMBO J. 2004; 23(22):4473–83. https://doi.org/10.1038/sj.emboj.7600423 PMID: 15496697

34. Pohl K, Francois P, Stenz L, Schlink F, Geiger T, Herbert S, et al. CodY in Staphylococcus aureus: a regulatory link between metabolism and virulence gene expression. J Bacteriol. 2009; 191(9):2953–63. https://doi.org/10.1128/JB.01395-06 PMID: 17020360

35. Majerczyk CD, Dunman PM, Luong TT, Lee CY, Sadykov MR, Somerville GA, et al. Direct targets of CodY in Staphylococcus aureus. J Bacteriol. 2010; 192(11):2861–77. https://doi.org/10.1128/JB.01000-20 PMID: 20363936

36. Eymann C, Homuth G, Scharf C, Hecker M. Bacillus subtilis functional genomics: global characterization of the stringent response by proteome and transcriptome analysis. J Bacteriol. 2002; 184(9):2500–20. https://doi.org/10.1128/JB.184.9.2500-2520.2002 PMID: 11948165

37. Kazmierczak KM, Wayne KJ, Rechtsteiner A, Winkler ME. Roles of rel(Snp) in stringent response, global regulation and virulence of serotype 2 Streptococcus pneumoniae D39. Mol Microbiol. 2009; 72(3):590–611. https://doi.org/10.1111/j.1365-2958.2009.06669.x PMID: 19426208

38. Gaca AO, Abranches J, Kajfasz JK, Lemos JA. Global transcriptional analysis of the stringent response in Enterococcus faecalis. Microbiology. 2012; 158(Pt 8):1994–2004. https://doi.org/10.1099/micro.0.06023-0 PMID: 22653948

39. Nascimento MM, Lemos JA, Abranches J, Lin VK, Burne RA. Role of RelA of Streptococcus mutans in global control of gene expression. J Bacteriol. 2008; 190(1):28–36. https://doi.org/10.1128/JB.01395-07 PMID: 17951382

40. Sanchez-Vazquez P, Dewey CN, Kitten N, Ross W, Gourse RL. Genome-wide effects on Escherichia coli transcription from ppGpp binding to its two sites on RNA polymerase. Proc Natl Acad Sci U S A. 2019; 116(17):8310–9. https://doi.org/10.1073/pnas.1819682116 PMID: 30971496

41. Hughes J, Mellows G. Interaction of pseudomonic acid A with Escherichia coli 5S RNA synthetase. Biochem J. 1980; 191(1):209–19. https://doi.org/10.1042/bj1910209 PMID: 6258580
40. Augagneur Y, King AN, Germain-Amiot N, Sass M, Fitzgerald JW, Sahukhal GS, et al. Analysis of the CodY RNAse reveals RsaD as a stress-responsive riboregulator of overflow metabolism in Staphylococcus aureus. Mol Microbiol. 2020; 113(2):309–25. https://doi.org/10.1111/mmi.14418 PMID: 31696578

41. Queck SY, Jameson-Lee M, Villaruz AE, Bach TH, Khan BA, Sturdevant DE, et al. RNAIII-independent target gene control by the agr quorum-sensing system: insight into the evolution of virulence regulation in Staphylococcus aureus. Mol Cell. 2008; 32(1):150–8.

42. Gaupp R, Ledala N, Somerville GA. Staphylococcal response to oxidative stress. Front Cell Infect Microbiol. 2012; 2:33. https://doi.org/10.3389/fcimb.2012.00033 PMID: 22919625

43. Novichkov PS, Kazakov AE, Ravcheev DA, Leyn SA, Kovaleva GY, Sutormin RA, et al. RegPrecise 3.0—a resource for genome-scale exploration of transcriptional regulation in bacteria. BMC Genomics. 2013; 14:745. https://doi.org/10.1186/1471-2164-14-745 PMID: 24175918

44. Ballal A, Manna AC. Control of thioredoxin reductase gene (trxB) transcription by SarA in Staphylococcus aureus. MBio. 2018; 9(6). https://doi.org/10.1128/mBio.01851-18 PMID: 30459192

45. Heinrichs JH, Bayer MG, Cheung AL. Characterization of the sar locus and its interaction with agr in Staphylococcus aureus. J Bacteriol. 1996; 178(2):418–23. https://doi.org/10.1128/JB.178.2.418-423.1996 PMID: 8550461

46. Zielinska AK, Beenken KE, Joo HS, Mrak LN, Griffin LM, Luong TT, et al. Defining the strain-dependent impact of the Staphylococcal accessory regulator (sarA) on the alpha-toxin phenotype of Staphylococcus aureus. J Bacteriol. 2011; 193(12):2948–58. https://doi.org/10.1128/JB.01517-10 PMID: 21478342

47. Sun F, Ding Y, Ji Q, Liang Z, Deng X, Wong CC, et al. Protein cysteine phosphorylation of SarA/MgrA mediates bacterial virulence and antibiotic resistance. Proc Natl Acad Sci U S A 2012; 109(38):15461–6. https://doi.org/10.1073/pnas.1205952109 PMID: 22927394

48. Ballal A, Mannan AC. Control of thioredoxin reductase gene (trxB) transcription by SarA in Staphylococcus aureus. J Bacteriol. 2010; 192(1):336–45. https://doi.org/10.1128/JB.01202-09 PMID: 19854896

49. Grosser MR, Weiss A, Shaw LN, Richardson AR. Regulatory Requirements for Staphylococcus aureus Nitric Oxide Resistance. J Bacteriol. 2016; 198(15):2043–55. https://doi.org/10.1128/JB.00229-16 PMID: 27185828

50. George SE, Hrubesch J, Breuing I, Vetter N, Korn N, Hennemann K, et al. Oxidative stress drives the selection of quorum sensing mutants in the Staphylococcus aureus population. Proc Natl Acad Sci U S A 2019; 116(38):667–73. https://doi.org/10.1073/pnas.1902752116 PMID: 31488708

51. Reiss S, Pane-Farré J, Fuchs S, Mehlan H, Bernhardt J, Hennig A, Michalik S, Surmann K, et al. AureoWiki: The repository of Staphylococcus aureus the family transcriptional regulators mediates bacterial virulence and antibiotic resistance. Proc Natl Acad Sci U S A 2012; 109(38):15461–6. https://doi.org/10.1073/pnas.1205952109 PMID: 22927394

52. Li L, Bayer AS, Cheung A, Lu L, Abdelhady W, Donegan NP, et al. The stringent response contributes to persistent methicillin-resistant Staphylococcus aureus endovascular infection through the purine biosynthetic pathway. J Infect Dis. 2020. https://doi.org/10.1093/infdis/jiaa202 PMID: 32333768

53. Cheung GY, Joo HS, Chatterjee SS, Otto M. Phenol-soluble modules—critical determinants of staphylococcal virulence. FEMS Microbiol Rev. 2014; 38(4):698–719. https://doi.org/10.1111/1574-6976.12057 PMID: 24372362

54. Peschel A, Otto M. Phenol-soluble modules and staphylococcal infection. Nat Rev Microbiol. 2013; 11(10):667–73. https://doi.org/10.1038/nrmicro3110 PMID: 24018382

55. Schlatterer K, Beck C, Hanzelmann D, Lebig M, Fehrenbacher B, Schaller M, et al. The Mechanism behind Bacterial Lipoprotein Release: Phenol-Soluble Modules Mediate Toll-Like Receptor 2 Activation via Extracellular Vesicle Release from Staphylococcus aureus. MBio. 2018; 9(6). https://doi.org/10.1128/mBio.01851-18 PMID: 30459192

56. Wang X, Thompson CD, Weidenmaier C, Lee JC. Release of Staphylococcus aureus extracellular vesicles and their application as a vaccine platform. Nat Commun. 2018; 9(1):1379. https://doi.org/10.1038/s41467-018-03647-z PMID: 29643357

57. Bojer MS, Lindemose S, Vestergaard M, Ingmer H. Quorum Sensing-Regulated Phenol-Soluble Modules Limit Persister Cell Populations in Staphylococcus aureus. Front Microbiol. 2018; 9:255. https://doi.org/10.3389/fmicb.2018.00255 PMID: 29515541

58. Xu T, Wang XY, Cui P, Zhang YM, Zhang WH, Zhang Y. The Agr Quorum Sensing System Represses Persister Formation through Regulation of Phenol Soluble Modules in Staphylococcus aureus. Front Microbiol. 2017; 8:2189. https://doi.org/10.3389/fmicb.2017.02189 PMID: 29163457

59. Zapf RL, Wiemels RE, Keogh RA, Holzschu DL, Howell KM, Trzeziak E, et al. The Small RNA Teg41 Regulates Expression of the Alpha Phenol-Soluble Modules and Is Required for Virulence in Staphylococcus aureus. MBio. 2019; 10(1). https://doi.org/10.1128/mBio.02484-18 PMID: 30723124
60. Jiang Q, Jin Z, Sun B. MgrA Negatively Regulates Biofilm Formation and Detachment by Repressing the Expression of psm Operons in Staphylococcus aureus. Appl Environ Microbiol. 2018; 84(16). https://doi.org/10.1128/AEM.01008-18 PMID: 2984758

61. Liu B, Sun B. Rsp promotes the transcription of virulence factors in an agr-independent manner in Staphylococcus aureus. Emerg Microbes Infect. 2020;1–42.

62. Fang B, Liu B, Sun B. Transcriptional regulation of virulence factors Hla and phenol-soluble modulins α by AraC-type regulator Rbf in Staphylococcus aureus. Int J Med Microbiol. 2020; 310(5):151436. https://doi.org/10.1016/j.ijmm.2020.151436 PMID: 32654771

63. Sojka L, Kouba T, Barvík I, Sanderová H, Maderová Z, Jonák J, et al. Rapid changes in gene expression: DNA determinants of promoter regulation by the concentration of the transcription initiating NTP in Bacillus subtilis. Nucleic Acids Res. 2011; 39(11):4598–611. https://doi.org/10.1093/nar/gkr032 PMID: 21303765

64. Fritsch VN, Loi VV, Busche T, Tung QN, Lill R, Horvátková M, English AM, Nguyen D. Superoxide dismutase activity confers (pp)pGpp-mediated antibiotic tolerance to stationary-phase Pseudomonas aeruginosa. Proc Natl Acad Sci U S A. 2018; 115(39):9797–802. https://doi.org/10.1073/pnas.1804525115 PMID: 30201715

65. Kim HY, Go J, Lee KM, Oh YT, Yoon SS. Guanosine tetra- and pentaphosphate increase antibiotic tolerance by reducing reactive oxygen species production in Vibrio cholerae. J Biol Chem. 2018; 293(15):5679–94. https://doi.org/10.1074/jbc.RA117.00383 PMID: 29475943

66. Hanna N, Ouahrami-Bettache S, Drake KL, Adams LG, Kohler S, Gilmore MS, et al. The (pp)pGpp synthetase RelA contributes to stress adaptation and virulence in Enterococcus faecalis V583. Microbiology. 2009; 155(Pt 10):3226–37. https://doi.org/10.1099/mic.0.02614-0 PMID: 19606070

67. Holley C, Gangaiah D, Li W, Fortney KR, Janowicz DM, Ellinger S, et al. A (pp)pGpp-null mutant of Hae-mophilus ducreyi is partially attenuated in humans due to multiple conflicting phenotypes. Infect Immun. 2014; 82(8):3492–502. https://doi.org/10.1128/IAI.01994-14 PMID: 24914217

68. Wang J, Tian Y, Zhou Z, Zhang L, Zhang W, Lin M, et al. Identification and Functional Analysis of RelA/SpoT Homolog (RSH) Genes in Deinococcus radiodurans. J Microbiol Biotechnol. 2016; 26(12):2106–15. https://doi.org/10.4014/jmb.1601.01017 PMID: 27558436

69. Abranches J, Martinez AR, Kajfasz JK, Miller JH, Wang JD, Abranches J, et al. The molecular alarmone (pp)pGpp mediates stress responses, vancomycin tolerance, and virulence in Enterococcus faecalis. J Bacteriol. 2009; 191(7):2246–56. https://doi.org/10.1128/JB.01726-08 PMID: 19168608

70. Surewaard BG, de Haas CJ, Vervoort F, Rigby KM, DeLeo FR, Otto M, et al. Staphylococcal alpha-pheno- nol soluble modulins contribute to neutrophil lysis after phagocytosis. Cell Microbiol. 2013; 15(8):1427–37. https://doi.org/10.1111/cmi.12130 PMID: 23470014

71. Yang J, Anderson BW, Turliev A, Turliev H, Stevenson DM, Amador-Noguez D, et al. The nucleotide pGpp acts as a third alarmone in Bacillus, with functions distinct from those of (pp)pGpp. Nat Commun. 2020; 11(1):5388. https://doi.org/10.1038/s41467-020-19166-1 PMID: 33097692

72. Fernández-Coll, Chashel m,. Possible roles for basal levels of (pp)pGpp: growth efficiency vs surviving stress. Front Microbiol. in press. https://doi.org/10.3389/fmicb.2020.529278 PMID: 33162969

73. Gaca AO, Kajfasz JK, Miller JH, Liu K, Wang JD, Abranches J, et al. Basal levels of (pp)pGpp in Entero-occus faecalis: the magic beyond the stringent response. MBio. 2013; 4(5):e00646–13. https://doi.org/10.1128/mBio.00646-13 PMID: 24069631

74. Bae T, Schnurrwind O. Allelic replacement in Staphylococcus aureus with inducible counter-selection. Plasmid. 2006; 55(1):58–63. https://doi.org/10.1016/j.plasmid.2005.05.005 PMID: 16051359

75. Fey PD, Endres JL, Yajjala VK, Widhalm TJ, Boissy RJ, Bose JL, et al. A genetic resource for rapid and comprehensive phenotype screening of nonessential Staphylococcus aureus genes. MBio. 2013; 4(1):e00337–12. https://doi.org/10.1128/mBio.00337-12 PMID: 23404388

76. Goerke C, Campaña S, Bayer MG, Doring G, Botzenhart K, Wolz C. Direct quantitative transcript analy- sis of the agr regulon of Staphylococcus aureus during human infection in comparison to the expression profile in vitro. Infect Immun. 2000; 68(3):1304–11. https://doi.org/10.1128/iai.68.3.1304-1311.2000 PMID: 10678942
79. Juengert JR, Borisova M, Mayer C, Wolz C, Brigham CJ, Sinskey AJ, et al. Absence of ppGpp Leads to Increased Mobilization of Intermediately Accumulated Poly(3-Hydroxybutyrate) in *Ralstonia eutropha* H16. Appl Environ Microbiol. 2017; 83(13).

80. Meyer H, Liebeke M, Lalk M. A protocol for the investigation of the intracellular *Staphylococcus aureus* metabolome. Anal Biochem. 2010; 401(2):250–9. https://doi.org/10.1016/j.ab.2010.03.003 PMID: 20211591

81. Mader U, Nicolas P, Depke M, Pane-Farre J, Debarbouille M, van der Kooi-Pol MM, et al. *Staphylococcus aureus* Transcriptome Architecture: From Laboratory to Infection-Mimicking Conditions. PLoS Genet. 2016; 12(4):e1005962. https://doi.org/10.1371/journal.pgen.1005962 PMID: 27035918