Structural basis for (p)ppGpp synthesis by the *Staphylococcus aureus* small alarmone synthetase RelP

Melek Cemre Manav\(^1\), Jelena Beljantseva\(^2\), Martin Saxtorm Bojer\(^3\), Tanel Tenson\(^2\), Hanne Ingmer\(^3\), Vasili Hauryliuk\(^4,5\) and Ditlev Egeskov Brodersen\(^1\)

Centre for Bacterial Stress Response and Persistence, \(^1\)Department of Molecular Biology and Genetics, Gustav Wieds Vej 10c, DK-8000 Aarhus C, Denmark, \(^2\)University of Tartu, Institute of Technology, Nooruse 1, 50411 Tartu, Estonia, \(^3\)Department of Veterinary and Animal Sciences, University of Copenhagen, \(^4\)Department of Molecular Biology, Umeå University, SE-901 87 Umeå, Sweden, \(^5\)Laboratory for Molecular Infection Medicine Sweden (MIMS), Umeå University, SE-901 87 Umeå, Sweden.

Running title: *Crystal structure of S. aureus RelP*

To whom correspondence should be addressed: Ditlev E. Brodersen, Centre for Bacterial Stress Response and Persistence, \(^1\)Department of Molecular Biology and Genetics, Gustav Wieds Vej 10c, DK-8000 Aarhus C, Denmark, Telephone: +45 21669001; E-mail: deb@mbg.au.dk; ORCID ID: 0000-0002-5413-4667

**Keywords:** stress response, bacterial pathogenesis, *Staphylococcus aureus*, crystallography, enzyme mechanism, SAS enzyme, Firmicute

The stringent response is a global reprogramming of bacterial physiology that renders cells more tolerant to antibiotics and induces virulence gene expression in pathogens in response to stress. This process is driven by accumulation of the intracellular alarmone guanosine-5'-di(tri)phosphate-3'-diphosphate ([p]ppGpp), which is produced by enzymes of the RelA SpoT homologue (RSH) family. The Gram-positive Firmicute pathogen, *Staphylococcus aureus*, encodes three RSH enzymes: a multi-domain RSH (Rel) that senses amino acid starvation on the ribosome and two small alarmone synthetase (SAS) enzymes, RelQ (SAS1) and RelP (SAS2). In *Bacillus subtilis*, RelQ (SAS1) was shown to form a tetramer that is activated by pppGpp and inhibited by single stranded RNA, but the structural and functional regulation of RelP (SAS2) is unexplored. Here, we present crystal structures of *S. aureus* RelP in two major functional states, pre-catalytic (bound to GTP and the non-hydrolyzable ATP analogue, AMPCPP) and post-catalytic (bound to pppGpp). We observed that RelP also forms a tetramer, but unlike RelQ (SAS1), it is strongly inhibited by both pppGpp and ppGpp and is insensitive to inhibition by RNA. We also identified putative metal ion-binding sites at the subunit interfaces that were consistent with the observed activation of the enzyme by Zn\(^{2+}\) ions. The structures reported here reveal the details of the catalytic mechanism of SAS enzymes and provide a molecular basis for understanding differential regulation of SAS enzymes in Firmicute bacteria.

The bacterial stringent response is a wide-ranging transcriptional and metabolic reprogramming that is induced in response to a range of stress conditions such as amino acid starvation and heat shock (1,2). Activation of the stringent response causes a complete transcriptional reprogramming and is accompanied by inhibition of ribosome assembly, protein translation, and replication, and consequently induces a halt in cell division until growth conditions improve (3). In addition, the stringent response is of potential medical importance since it regulates virulence gene expression in some bacterial species and can render bacteria tolerant to antibiotic treatment (4,5).

At the molecular level, the stringent response is mediated by two alarmone nucleotides,
guanosine-5’-diphosphate-3’-diphosphate (ppGpp) and guanosine-5’-triphosphate-3’-diphosphate (pppGpp), which are 3’ hyper-phosphorylated versions of GDP and GTP, respectively (6). Synthesis and degradation of the two alarmones, collectively referred to as (p)ppGpp, are mediated by enzymes belonging to the RelA-SpoT homologue (RSH) family. This family comprises both the large multi-domain enzymes (the "long" RSH’s), RelA and SpoT, as well as two classes of single-domain RSH’s, the small alarmone synthetases (SASs) and small alarmone hydrolases (SAHs) that synthesize and hydrolyze (p)ppGpp, respectively (7). In the long RSHs, the N-terminal half contains both (p)ppGpp synthesis (SYNTH) and hydrolysis (HD) domains while the C-terminal domains are purely regulatory (7-10). The crystal structure of a bifunctional catalytic fragment of *Streptococcus equisimilis* Rel (SeRel) revealed two reciprocal conformations, one in complex with the substrate GDP (hydrolase-OFF, synthetase-ON) and another bound to both GDP and an unusual cyclic (p)ppGpp derivative, guanosine 5’-diphosphate 2’-3’ cyclic monophosphate (hydrolase-ON, synthetase-OFF) (11). The (p)ppGpp synthesis activities of the ribosome-associated Rel and RelA enzymes are induced by binding of uncharged tRNA to the A-site during amino acid starvation (12,13). Structural work has demonstrated that upon ribosome binding, the C-terminal domains are removed from the synthetase domain, thus activating the enzyme in the ribosome-bound form (8-10).

Small alarmone synthetase (SAS) enzymes lack both the hydrolase and C-terminal regulatory domains of the long RSHs and are thus minimalist (p)ppGtt synthetase enzymes (7). Two such enzymes, RelQ (SAS1, YjbM), and RelP (SAS2, YwaC), were first identified in the Firmicute, *Streptococcus mutans* (14). Both alkaline shock and cell wall stress (such as exposure to cell-wall active antibiotics) induce expression of the SAS enzymes, cause accumulation of (p)ppGpp and thus improve the fitness of the bacterial cells (15,16). The human pathogen, *Staphylococcus aureus*, also encodes both the RelP (SAS2) and RelQ (SAS1) enzymes (17). Clinically, *S. aureus* gives rise to diverse array of diseases ranging from mild skin infections to life threatening conditions such as endocarditis and osteomyelitis that can be difficult to treat with antibiotics (18). Similar to *S. mutans*, expression of the SAS enzymes in *S. aureus* is strongly induced by cell-wall active antibiotics such as ampicillin and vancomycin, and mutants lacking the relP and relQ genes show a significantly reduced survival rate upon exposure to these antibiotics (17). A study of *Bacillus subtilis* RelQ (BsRelQ) revealed that the protein forms a homotetramer with four active sites and structures were determined of the enzyme in complex with either ATP (one of the two substrates) or pppGpp (product) (16). This study further revealed two allosteric nucleotide binding sites at the subunit interface and provided evidence that binding of pppGpp (but not ppGpp) to these sites dramatically increases catalytic activity of the enzyme. Based on the BsRelQ structure it was also proposed that RelP (SAS2) would have a similar structure and catalytic mechanism but be differently regulated. Finally, RelQ from *Enterococcus faecalis* (EfRelQ) is negatively regulated by single-stranded RNA, likely through binding to the same allosteric site as the effect is mutually exclusive with regulation by pppGpp (19).

Here, we present crystal structures of *S. aureus* RelP (SAS2, SaRelP) in the complete pre-catalytic state (i.e. bound to both GTP and the non-hydrolysable ATP analogue, AMPCPP) as well as in the post-catalytic state (bound to pppGpp). Together, these structures complete the view of the reaction cycle of the bacterial SAS enzymes and allow us to propose a detailed reaction mechanism. We also demonstrate that the allosteric pppGpp-binding site is not present in RelP (SAS2). This is consistent with functional analysis showing that neither ppGpp nor pppGpp induce the enzymatic activity of SaRelP and instead inhibit the enzyme, likely through active site binding. In contrast to EfRelQ, SaRelP does not bind and is not inhibited by single-stranded RNA, underscoring the difference in regulatory logic between the two classes of SAS enzymes. Finally, we identify two putative divalent metal ion binding sites at the subunit interfaces and show that Zn\(^{2+}\) is a potent activator of SaRelP.

**Results**
**Structure determination and overall structure of SaRelP**

To understand why some Gram positive bacteria require two very similar SAS enzymes and delineate the molecular basis for the proposed differences in regulation, we decided to determine the crystal structure of SaRelP by x-ray crystallography. The protein has a monomer mass of app. 25 kDa but elutes with an apparent molecular mass of ~100 kDa during size exclusion chromatography, suggesting that it forms a tetramer similar to BsRelQ (Supplementary Figure S1) (16). The purified protein was concentrated and incubated with equimolar amounts of ATP and GTP prior to crystallization. Large, single crystals belonging to the centered tetragonal space group I4 \_222 and diffraction to 2.8 Å were obtained and the structure was determined by molecular replacement using the structure of BsRelQ bound to pppGpp (PDB ID 5DED) (16) as a search model (see Table 1 for crystallographic data collection and refinement statistics). There are two SaRelP molecules per crystallographic asymmetric unit (ASU), which are arranged as a dimer, and the final structure covers 194 residues out of a total of 230. The poorly conserved N-terminus (residues 1-29) and the loop region 194-201 are disordered and not visible in the electron density. The final structure has R=21.8 % (R_free=26.3%) with good overall geometry (Table 1).

The SaRelP monomer displays a Rossmann-like fold consisting of a five-stranded anti-parallel β-sheet sandwiched between six α-helices (Figure 1A). In accordance with the behavior of the protein in solution and as previously observed for BsRelQ, four SaRelP molecules come together to form a ring-shaped tetramer with a large cavity at the center (Figure 1B). In the I4 \_222 space group, this tetramer is generated across a crystallographic two-fold axis (parallel to the horizontal line in Figure 1B), so that the two dimers are perfectly identical. This is in contrast to the BsRelQ structures, which have one or two full tetramers in the crystallographic asymmetric unit and thus allow for asymmetry. Nevertheless, the overall structure of SaRelP is very similar to both BsRelQ (Figure 1C) as well as the catalytic domain of SeRel (Figure 1D).

**pppGpp is found in two different conformations inside the active site**

Inspection of the active site cavities of the two SaRelP monomers revealed clear density compatible with neither of the added ligands (ATP and GTP) but only pppGpp (Supplementary Figure S2A). This suggested that the reaction took place prior to crystallization and left the product in the active site similarly to what was observed for BsRelQ (16). The active site pocket is formed mainly by the β-strands and the loops β1/α2, α3/β2, and β3/β4 in addition to two small helices, α2 and α5 (Figure 1A). pppGpp adopts different conformations inside the two active sites (chains A and B) of the dimer (Figure 1E and Supplementary Figure S2B). Both of these conformations are different from the one observed in BsRelQ, where the two phosphate arms (5’ and 3’) display an open conformation and point in opposite directions. In contrast, in SaRelP, the phosphate arms either point in the same direction (chain A, Figure 1E, left and Supplementary Figure S2B) or towards each other (chain B, Figure 1E, right). In chain A where the 5’ γ-phosphate groups face away from the 3’ phosphate arm, α2 is shifted by approximately 4.6 Å compared to BsRelQ, apparently to adapt to the more distant position of the 5’ γ-phosphate (Supplementary Figure S2C, left). This shift is less (3.7 Å) but still significant in chain B, where the two phosphate arms face each other (Supplementary Figure S2C, right). Together, these data suggest that the product nucleotide is loosely bound within the enzyme post catalysis, possibly to promote its dissociation.

The interaction between pppGpp and SaRelP is stabilized by the universally conserved Tyr151, which stacks on the guanine base, while Glu189 forms a hydrogen bond with the N1 atom. The combined negative charge of the phosphates is balanced by the addition of a basic residue (Lys138) interacting with the 5’ γ-phosphate and a second basic residue (Lys80) interacting with the 3’ phosphate. Not surprisingly, several of the residues involved in the interaction between SaRelP and pppGpp (Arg78, Lys80, Lys88, Arg91, Lys92, Lys138, Lys147, and Arg193) (Figure 1E).
Structure of SaRelP in the pre-catalytic state

SAS enzymes catalyze transfer of a pyrophosphate moiety from ATP to the 3'-OH group of either GDP or GTP forming ppGpp or pppGpp, respectively. However, no structure of the complete pre-catalytic state of a SAS enzyme, i.e. bound to both GTP and ATP, has so far been determined. In order to understand the molecular details of the catalytic mechanism, we therefore determined the crystal structure of SaRelP in the pre-catalytic state by incubating the protein with GTP and the non-hydrolysable ATP analogue, AMPCPP, prior to crystallization. Complete data were collected to 2.2 Å and the structure was determined by molecular replacement using the pppGpp-bound SaRelP structure (Table 1). In these crystals, which belong to the same space group as those for the post-catalytic state, we observe clear electron density for both AMPCPP and GTP in both active sites (Figure 2A). Moreover, the overall B factors are lower and we were able to build the flexible loop region 194-201, which was disordered in the pppGpp-bound state, suggesting the enzyme became stabilized. The conformation of the nucleotides is identical in chains A and B with GTP most closely resembling the pppGpp conformation found in chain B of the post-catalytic state. The 3'-OH of GTP is positioned in close proximity (3.2Å) to the β-phosphate of AMPCPP, and thus poised for the nucleophilic attack (Figure 2C). The adenine base of AMPCPP is sandwiched through π-stacking between Arg78 and Arg112, and Glu174 further stabilizes the base through interaction with its N6 group (Figure 2A and 2C). Glu174 also coordinates two water molecules that organize a single magnesium ion, which is further coordinated by oxygen atoms from the β and γ phosphate groups of AMPCPP as well as the 3'-OH of GTP (Figure 2C). The 3'-OH of GTP does not make any direct interactions with SaRelP but interacts with Glu174 and Asp107 indirectly through the magnesium ion (Figure 2C).

The orientation of both the ribose and base of GTP is very similar to pppGpp in the post-catalytic state (Figure 2D), suggesting that no major rearrangements of these groups take place during the reaction. In contrast, the phosphate arms move significantly so that the 5' γ phosphate group of pppGpp assumes the position of the γ phosphate group of ATP prior to the reaction. Moreover, helix α2 is approximately 1.5 Å closer to the guanosine nucleotide in the pre-catalytic state compared to the post-catalytic state. The guanine base stacks on Tyr151 in the pre-catalytic state similar to what was observed for SeRel (PDB ID 1VJ7) in complex with GDP, where it corresponds to Tyr308 (Supplementary Figure S3) (11). Consistent with the central role of this residue, mutation of Tyr151 to alanine renders SaRelP completely inactive (Figure 2B) without affecting the tetrameric state of the enzyme (Supplementary Figure S4A). Recognition of GDP in SeRel also involves Lys304, Asn306, Asp264, Glu323, and His312, which are all conserved in SaRelP and correspond to Lys147, Asn149, Asp107, Glu174, and His155, respectively. Together, the pre-catalytic and post-catalytic structures presented here thus complete our picture of the reaction taking place in the SAS enzymes and allow for a detailed analysis of how these enzymes specifically transfer a pyrophosphate from ATP to the 3'-OH position of guanosine nucleotides.

SaRelP lacks the cleft allosteric pppGpp binding site

The structure of BsRelQ revealed an unexpected allosteric binding site at the subunit interface between two dimers that was shown to positively affect activity upon binding of pppGpp but not ppGpp (16). In this site, the negative charges of pppGpp is balanced by pairs of Lys21, Lys25, and Arg28 residues originating from neighboring chains on either side of the subunit cleft (Figure 3A). Phe42 stacks with the guanine base, one Arg28 residue interacts with the N2 atom while Glu41 forms a hydrogen bond with the N1 position ensuring specificity for guanosine (16). Interestingly, none of the residues that interact specifically with pppGpp at the BsRelQ allosteric site are conserved in SaRelP, (Figure 3B and Supplementary Figure S3). Lys21 and Lys25 have both been replaced by serine residues (Ser53 and Ser57, respectively), Arg28 has been replaced by Asp50 and thus has an inverted charge, while Phe42 is replaced by His73. Moreover, Ser38 that allows Arg28 to contact the guanine base in BsRelQ, is replaced by the larger Asn70 in SaRelP. Altogether, a clear pattern of differences between RelP (YwaC) and RelQ (YjbM) enzymes
in the region around $\alpha 1$ and $\beta 1$ emerges that indicate that they bind different regulatory molecules. In summary, structural and sequence data strongly indicate that SaRelP does not bind pppGpp at the cleft allosteric binding site and consequently that it may be regulated in a different way.

**SaRelP is inhibited by (p)pppGpp but not single-stranded RNA**

Since the cleft allosteric pppGpp binding site appears to be lacking in SaRelP, we next wanted to understand if the product nucleotides pppGpp and ppGpp have any effect on the activity of the enzyme. For this analysis, we employed the approach previously used for EfRelQ, where the synthesis of pppGpp is followed by thin layer chromatography after incubation of purified enzyme with $^3$H-labelled GDP and ATP as substrates (19). For SaRelP, upon addition of increasing amounts of either ppGpp (Figure 3C) or pppGpp (Figure 3D) to this reaction, we observe gradual inhibition of pppGpp synthesis activity, with a complete loss of activity occurring at 1 mM concentration of either product nucleotide. We note that pppGpp appears to be a slightly more potent inhibitor ($IC_{50}^{pppGpp} = 45\pm8 \mu M$) than ppGpp ($IC_{50}^{pppGpp} = 94\pm26 \mu M$).

For EfRelQ, allosteric activation by pppGpp (but not ppGpp) was found to be mutually exclusive with inhibition through binding of single-stranded RNA suggesting that RNA competes for binding at the cleft allosteric site (19). Using EfRelQ as a positive control, we next tested if SaRelP is also affected by single-stranded RNA. However, unlike EfRelQ, SaRelP is neither inhibited by model mRNA (Figure 3E) nor forms a protein-RNA complex as judged by electrophoretic mobility shift assay (EMSA) (Figure 3F).

**SaRelP contains an iron-binding site at the dimer interface**

Inspection of the $2mF_o - DF_c$ density at the SaRelP dimer interface revealed a significant, spherical blob between the side chains of two equivalent Glu51 residues from adjacent molecules, i.e. at the non-crystallographic two-fold symmetry axis between monomers (Supplementary Figure S5A). To understand if this peak represents a metal ion binding site, we performed broad wavelength range fluorescence scans on crystals of SaRelP in the pppGpp-bound form, which indicated that both Zn and Fe were present (data not shown). To check whether one of these metals could correspond to the site identified between the Glu51 side chains, complete anomalous data sets were collected both above and below the K-edge absorption edges for Zn and Fe and used for calculation of anomalous difference maps (Table 1 and Supplementary Figure S5B). For the data collected above the Fe K-edge at 7.200 keV, a significant anomalous peak (3.2$\sigma$) was observed between the two Glu51 side chains, whereas there was no signal in the anomalous map below the absorption edge (6.900 keV) nor in the map calculated from data collected above the Zn K-edge at 9.800 keV.

Based on these data and the presence of a peak in the $2DF_o - rF_c$ refined maps of both SaRelP structures (pre-catalytic and post-catalytic), we conclude that iron ($Fe^{2+}$ or $Fe^{3+}$) binds at the dimerisation interface. Since no iron was present in the buffers used for purification and crystallization, this ion must have been present in the protein during expression and carried along during purification. The ion displays a clear octahedral coordination geometry involving carboxylate oxygen atoms from the two Glu51 side chains and four surrounding water molecules (Figure 4A). In this context, we note that octahedral configuration is the preferred coordination for iron, while the zinc ion ($Zn^{2+}$) prefers tetragonal coordination and is very rarely octahedral (20). Glu51 is highly conserved in the SAS enzyme family, but is, intriguingly, replaced by alanine in BsRelP (YwaC) suggesting that the iron-binding site is not present in this protein (Supplementary Figure S3). To check for a possible regulatory effect on enzyme activity, we repeated the activity assay in the presence of increasing concentrations of $Fe^{2+}$ (Figure 4B), which revealed no strong effect. Since $Fe^{2+}$ and $Fe^{3+}$ are readily interchangeable in aqueous solutions, $Mn^{2+}$ is often used as a surrogate (21). We therefore also tested the effect of $Mn^{2+}$ on the catalytic activity of SaRelP and consistently found no strong effect (Supplementary Figure S4B). We therefore conclude that SaRelP binds $Fe^{2+}$ or $Fe^{3+}$ at the dimer interface and that this might represent a structural ion binding site.
**SaRelP contains a putative Zn$^{2+}$-binding site**

Binding of pppGpp at the cleft allosteric site in BsRelP requires Arg28, Glu41 and Phe42, which interact with the guanine N1/N2 atoms and stack with the base, respectively (Figure 3A) (16). In SaRelP, these residues have been replaced by two histidines (His73 and His74) suggestive of another putative metal (possibly zinc) binding site. The two histidine residues are located across from the equivalent residues in the other half of the tetramer and thus form a four-histidine site (Figure 4C). Intriguingly, this unique combination of residues appears to be conserved among certain RelP/SAS2 enzymes, such as Staphylococcus shleiferi RelP (Supplementary Figure S3), while B. subtilis RelP (YwaC), contains one residue (Glu) from the BsRelQ motif (EF) and a histidine from the SaRelP motif (HH). To understand if Zn$^{2+}$ could bind to this site, crystals of SaRelP in the pppGpp-bound state were soaked in 5 mM ZnCl$_2$ before data collection. However, no difference electron density peaks were observed following refinement (data not shown) and it is therefore presently uncertain if this site is able to bind a metal ion. Surprisingly, activity tests carried out in the presence of increasing amount of Zn$^{2+}$ demonstrated that the ion acts as an activator at lower concentrations (peaking at ~5 µM), and as an inhibitor at higher concentrations (Figure 4D). Ni$^{2+}$, used here as a specificity control, had only the inhibitory effect, nearly completely abolishing the enzymatic activity at 40 µM (Figure 4D). To test if this effect is mediated by the HH motif, we finally mutated the two histidine residues (His73 and His74) to alanine. Surprisingly, this mutation completely abolished the activity of the enzyme (Figure 2B) but did not affect the tetrameric state (Supplementary Figure S4A). Thus, despite our inability to demonstrate structurally that zinc binds at the His site, our data suggest that the site is indeed functionally important, and also that divalent metal ions can affect the enzyme activity. In summary, RelP/SAS2 (as opposed to RelQ/SAS1) enzymes appear to be regulated by metal ions and future investigations should therefore focus on revealing the mechanism by which this occurs.

**Discussion**

In this paper, we present crystal structures of *S. aureus* RelP/SAS2 in two key functional states, pre-catalytic and post-catalytic. Together, these structures provide a clear picture of the catalytic mechanism of the single-domain SAS enzymes (Figure 5). The complete pre-catalytic state presented here demonstrates that Glu174 plays a key role in organizing two water molecules that form part of the interaction sphere of a Mg$^{2+}$ ion in the active site (Figure 2C and Figure 5). This residue corresponds to Glu139 in BsRelQ, which was proposed to function as a general base and deprotonate the 3' OH of GTP directly during the reaction (16). This appears not to be the case in SaRelP, since the Mg$^{2+}$ ion is the only group that directly contacts the 3' OH group of GTP in the pre-catalytic state. It is therefore rather the electrophilic Mg$^{2+}$ ion that promotes deprotonation of the 3' OH group and subsequently the nucleophilic attack of the resulting alkoxide (R-O') on the β phosphate of ATP (Figure 5, left, curved arrow). The pentavalent transition state is stabilized by a large number of positively charged residues in the vicinity, one of which likely also serves as general acids to protonate the phosphate leaving group on AMP. Interestingly, the conformation of the 5' and 3' phosphate arms in the product nucleotide (pppGpp) deviate from the position of the corresponding groups in the pre-catalytic state suggesting that some conformational change takes place during the reaction (Figure 5).

While the reaction mechanisms of RelP and RelQ enzymes are very likely the same, our data also highlight the functional and structural differences between these homologous enzymes in Firmicute bacteria: We demonstrate that SaRelP, unlike BsRelQ, is not stimulated by pppGpp binding, in fact, we observe that the activity drops in the presence of both ppGpp and pppGpp. This, together with the observation that the allosteric pppGpp binding site is absent from SaRelP, suggests that the effect we observe is orthosteric, i.e. arising from simple competitive product binding at the active site. In this scenario, one would predict that the relative level of inhibition would increase as the substrate (GDP) concentration is lowered due to competition for the same binding site. However, we do not observe this effect, which suggests that GDP and (p)ppGpp do not compete for the same binding sites directly (data not shown). It is, however, possible, that the effect is related to the tetrameric state of the
enzyme and thus, cross-talk between active sites. For example, binding of a product nucleotide to one active site could negatively affect the other active sites in the tetramer without directly competing with binding of GDP.

If not regulated by (p)ppGpp, the question remains: What, if anything, regulates RelP/SAS2 in Firmicute bacteria, and why are there two homologous SAS enzymes? We present evidence for the existence of an iron-binding site located between monomers of a dimer and identify a second metal binding site at the dimer-dimer interface. Surprisingly, we were not able to observe any significant effect on the catalytic rate upon addition of Fe\(^{3+}\), while Zn\(^{2+}\), which could not be located at the second binding site, did have an effect. Moreover, we note that the in vitro activity of SaRelP in the presence of Zn\(^{2+}\) shows a biphasic response curve: up to 4 \(\mu\)M the ion acts as an activator while at higher concentrations, inhibition occurs (Figure 4D). The location of two histidine residues adjacent to two equivalent histidines from the other half of the tetramer is unusual and highly suggestive of a zinc binding site. But we have not been able to identify zinc at this site, not even when the crystals were soaked in millimolar concentrations of Zn\(^{2+}\). Nevertheless, we speculate that the three unique combinations of residues (HH, EH, and EF) observed among homologous SAS sequences at this position (Supplementary Figure S3, green boxes) represent different specificities for ligands yet to be uncovered. The four histidine residues located very close to each other in SaRelP thus represent a different regulatory site than the one that has been observed in BsRelQ, which has Glu-Phe at this position. Although we have not been able to identify what binds to this site in SaRelP, we note the presence of a significant and continuous blob of electron density at the interface between dimers in the structure of the pre-catalytic state that is not compatible with a bound nucleotide (Supplementary Figure S5C). This density is found adjacent to the putative zinc site, close to Arg77 and the backbone of residues 73-77 and was only observed for the structure of the pre-catalytic state determined in the presence of AMPCPP and GTP.

Altogether, our results suggest that Firmicute bacteria contain two separate SAS enzymes because they need to respond to more environmental signals than can be handled by a single allosteric site. Most likely, RelP/SAS2 arose by gene duplication and diversification eventually led to two enzymes catalyzing the same reaction but responding to different signals. Although the details of this diverse regulation are not yet clear, RelP/SAS2 appears to respond to metal ions to a greater extent than RelQ/SAS1, which likely responds only to nucleotides. In this context, it is interesting to note that the presence of zinc ions in the medium has been observed to induce stringent response and (p)ppGpp production in B. subtilis and that YwaC (SAS2/RelP) is one of several proteins that are upregulated as a response (22). A working hypothesis could therefore be that RelP evolved to respond to oxidative stress induced by chelation of low amounts of metal ions present in the environment. This could also explain why we only see activation of SaRelP at low Zn\(^{2+}\) concentrations. At higher concentrations, it is possible that the presence of zinc negatively affects the enzyme in a non-physiological way. Future research in the field should now be focused on understanding where metal ions interact with RelP/SAS2 and how this affects activity. Secondly, it remains possible that the enzyme is regulated by additional (larger) compounds, as indicated by our unexplained density at the tetramer interface. Careful purification and MS analysis of protein produced under stress conditions in S. aureus might allow identification of such metabolite(s).

**Experimental procedures**

**Cloning of SaRelP**

The complete S. aureus relP ORF (NWMN_2405) was amplified by PCR (Phusion Hot Start II DNA Polymerase, ThermoFisher Scientific) using S. aureus strain Newman chromosomal DNA as template and the primer pair: 5'\'-GATACATCTAGATTTAAGAAGG AGATATACCATGTATGTAGATCGAAAACC ATC-3'; 5'CGCGGTACCTAGATGATGGT GATGGTATGCACCCTGTATTTCAAGAT GAAATTG-3', which contain an upstream ribosomal binding site (rbs) and include the coding sequence for a C-terminal hexa-histidine tag. The PCR fragment was cloned into pET28a using the XbaI/BamHI restriction sites and verified by sequencing. The Y151A and H73A/H74A mutants
were constructed using the Agilent QuikChange Multi Lightning Kit, protocol according to the instructions by the manufacturer.

**Protein expression and purification**

_E. coli_ BL21(DE3) competent cells carrying the pET28a-SArelP-His expression plasmid were grown overnight in lysogeny broth (LB) medium supplemented with 50 μg/mL kanamycin at 37°C, then used to inoculate 4 L LB medium supplemented with kanamycin and grown at 37°C until the OD_{600} reached ~0.6-0.7. The culture was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and protein expression allowed to proceed for 3 hours at 30°C in a shaking incubator. Cells were harvested (~7.000 x g for 15 min), resuspended in a lysis buffer containing 50 mM Tris-HCl, pH 8.5, 500 mM NaCl, 5 mM MgCl₂, 10 mM imidazole, 3 mM beta-mercaptoethanol (BME), and 100 mM PMSF, and disrupted by sonication. Cell debris was pelleted by a fast centrifugation (~23.000 x g for 45 min) and the supernatant applied to a 1 mL HisTrap HP Ni-affinity column (GE Healthcare) equilibrated with the lysis buffer. After washing with 5-10 column volumes (CV), the protein was eluted with a 5-step gradient using 30 mM, 50 mM, 75 mM, 150 mM, and 300 mM imidazole over 10 CV. At this stage, protein for biochemistry was concentrated and exchanged into 30 mM Tris, pH 8, 300 mM NaCl, 5 mM MgCl₂, 5 mM BME, and 5 % glycerol and used directly. Protein for crystallization was further purified on a 1 mL Source15Q (GE Healthcare) anion exchange column equilibrated in 50 mM Tris-HCl, pH 8.5, 100 mM NaCl, and 5 mM BME and eluted using a linear gradient into 1 M NaCl. The eluate was concentrated by spin filtration (VivaSpin 30.000 MWCO, Sartorius) and applied to a Superdex 200 increase 10/300 size exclusion column (GE Healthcare) equilibrated in 30 mM Tris-HCl, pH 8.5, 300 mM NaCl, 5 mM MgCl₂, and 5 mM BME. Peak fractions were pooled and concentrated to 10-15 mg/mL and the final protein purity was assessed by SDS-PAGE. Protein concentrations were measured spectrophotometrically using OD_{280}.

**Crystallization**

All crystallization experiments were carried out using the sitting drop vapor diffusion method, where the protein sample was mixed 1:1 with reservoir buffer in 96-well Swissci trays (Molecular Dimensions) using a Mosquito® liquid handler (TTP Labtech). For the pppGpp-bound structure, purified SaRelP (10 mg/mL) was incubated with 1 mM ATP and 1 mM GTP at 30°C for 5 min, then kept on ice. Initial crystal hits were obtained after 2 days and optimized to a final condition containing 30-40% (v/v) pentaerythril propoxylate (5/4 PO/OH), 0.2 M sodium thiocyanate, and 0.1 M Hepes-NaOH, pH 7.0 at 19°C. For the AMPCPP·GTP-bound structure, SaRelP (11 mg/mL) was incubated with 1 mM AMPCPP and 1 mM GTP at 30°C for 5 min, then kept on ice. Crystals were obtained at 19°C in a buffer containing 25% (w/v) PEG 3350 and 0.1 M Tris-HCl, pH 8.5.

**Data collection and structure determination**

Crystals of SaRelP on the pppGpp-bound form grown in 32% (v/v) pentaerythril propoxylate (5/4 PO/OH), 0.2 M sodium thiocyanate, and 0.1 M Hepes-NaOH, pH 7.0 were cryo-protected in the mother liquor by addition of 20% glycerol while crystals on the AMPCPP·GTP-bound form were cryo-protected by addition of 30% glycerol. Complete native diffraction data were collected at 100 K at the European Synchrotron Radiation Facility (ESRF) beamline ID29, Grenoble, France (pppGpp-bound form) and PETRA III, beamline P13, Hamburg, Germany (AMPcpp·GTP-bound form and anomalous data). All data were processed using the xia2 automated processing system (23) using the XDS pipeline (24). Data quality was assessed using Phenix.xtriage (25). The structures were determined by molecular replacement in Phenix, Phaser (26) using the BsRelQ structure (PDB ID 5DED) as search model (16). The structures were built in Coot (27) and iteratively refined in Phenix.refine (25) with manual rebuilding. Figures were created using PyMOL (28). Anomalous data were collected from a crystal of SaRelP on the pppGpp-bound form above the Zn K absorption edge at 1.26 Å (9.800 keV), and both below (1.79Å, 6.900 keV) and above (1.71Å, 7.200 keV) the Fe K-edge. The structure was refined briefly against each of these data sets before anomalous difference maps were calculated in Phenix.

**Enzymatic assays**
Experiments were performed at 37°C in Hepes:Polymix buffer with 5 mM Mg\textsuperscript{2+} (29,30) supplemented with 1 mM BME (see Supplementary experimental procedures for details). Reaction mixtures containing 250 nM SaRelP or EfRelQ and 200 µM \textsuperscript{3}H GDP were pre-incubated for 2 min at 37°C in the presence of increasing concentrations of different effectors; nucleotides, metal ions (Ni\textsubscript{2}SO\textsubscript{4}, ZnCl\textsubscript{2}, or FeCl\textsubscript{3}), or model mRNA coding for a Met-Phe (MF) dipeptide, 5'-GGCAAGGGUAAAAUGUUCAA-3' (19).

Reactions were initiated by addition of 1 mM ATP after which 5 µl aliquots were taken throughout the time course of the reaction. Aliquots were quenched with 4 µl 70% formic acid supplemented with a cold nucleotide standard (10 mM GDP + 10 mM GTP) and analyzed by UV-shadowing after separation on PEI-TLC plates (Macherey-Nagel). TLC analysis was performed as described by Mechold and colleagues (31) with modifications: Nucleotides were resolved in a 0.5 M KH\textsubscript{2}PO\textsubscript{4}, pH 3.5 buffer, and plates were dried and cut into sections as guided by UV-shadowing. \textsuperscript{3}H radioactivity was quantified by scintillation counting using Optisafe-3 (Fisher) scintillation cocktail. Conversion of substrate to product was quantified as described previously (32).

**Electrophoretic mobility shift assay (EMSA)**

Reactions were carried out in 8 µl total in Hepes:Polymix buffer as described above. Prior to assembling the reaction mixtures, stock RNA was incubated for 2 min at 65°C in order to melt potential secondary structure. Reaction mixtures were assembled by adding increasing concentrations of SaRelP or EfRelQ to the RNA (0.19 µM final concentration), followed by addition of 4 U/µl RiboLock RNase Inhibitor (Thermo Scientific). After incubation for 10 min at 37°C, 5 µl loading dye, 40% (w/v) sucrose supplemented with bromphenol blue, was added per 8 µl (i.e. per 1.5 pmol of mRNA) and the samples resolved on 12-15% Tris:borate:EDTA (TBE) gels run at 4°C (120-140 V) for 1.5-2 hours. Gels were stained with SYBR Gold nucleic acid stain (Life Technologies) for 20 minutes prior to visualization using a Typhoon Trio variable mode imager (Amersham Biosciences).

**Acknowledgements** - We are thankful to Rasmus Koch Flygaard and beamline staff at the ESRF and EMBL PETRA beamlines ID29 and P13 for help during data collection. This work was supported by the Danish National Research Foundation (grant DNRF120 to D.E.B.), the Estonian Research Council (grant IUT2-22 to T.T.), the European Regional Development Fund through the Centre of Excellence for Molecular Cell Technology (V.H. and T.T.), the Swedish Research Council (Vetenskapsrådet, grant 2013-4680 to V.H.), and the Ragnar Söderberg foundation (V.H.). Atomic coordinates and crystallographic structure factors have been deposited in the Protein Data Bank with ID codes 6EX0 (pppGpp-bound form) and 6EWZ (AMPCPP-GTP-bound form).

**Conflicts of interest** - The authors declare that they have no conflicts of interest with the contents of this article.

**Author contributions** - Ditlev E. Brodersen and Hanne Ingmer conceived the project, Ditlev E. Brodersen, Hanne Ingmer, Tanel Tenson and Vasili Hauryliuk designed the experiments, Martin S. Bojer cloned the initial protein construct, M. Cemre Manav purified the protein, determined the structures, and carried out the structural analysis, Jelena Beljantseva carried out the enzymatic and binding assays. M. Cemre Manav, Jelena Beljantseva, Martin S. Bojer, Hanne Ingmer, Tanel Tenson, Vasili Hauryliuk, and Ditlev E. Brodersen wrote the paper.

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10

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### Table 1. Crystallographic data collection and refinement statistics

|                     | SaRelP – AMPCPP:GTP | SaRelP – pppGpp | Below Fe edge | Above Fe edge | Above Zn edge |
|---------------------|----------------------|-----------------|--------------|--------------|--------------|
| **Data Collection** |                      |                 |              |              |              |
| Wavelength (Å)      | 0.979                | 0.979           | 1.79 (6.900 keV) | 1.71 (7.200 keV) | 1.26 (9.800 keV) |
| Resolution range    | 63.1 – 2.24          | 53.7 – 2.78     | 69.0 – 3.07   | 54.3 – 3.09   | 54.4 – 2.98   |
| (2.32 – 2.24) *     | (2.88 – 2.78)        | (3.18 – 3.07)   | (3.20 – 3.09) | (3.09 – 2.98) |              |
| Space group         | I4,22                | I4,22           | I4,22         | I4,22         | I4,22        |
| Unit cell dimensions| a, b, c, (Å)         | 125.1, 125.1, 219.1 | 124.3, 124.3, 213.2 | 126.4, 126.4, 217.0 | 126.4, 126.4, 217.3 |
|                     | a, b, c, (Å)         | 90, 90, 90      | 90, 90, 90    | 90, 90, 90    | 90, 90, 90    |
| Total reflections   | 556,165 (53,841)     | 133,328 (10,390) | 122,971 (11,057) | 126,224 (13,018) | 119,309 (11887) |
| Unique reflections  | 42,049 (4098)        | 21,284 (2055)   | 16,809 (1621) | 16,498 (1622) | 18,140 (1738) |
| Multiplicity        | 13.2 (13.1)          | 6.3 (5.1)       | 7.3 (7.0)     | 7.7 (8.0)     | 6.6 (6.5)     |
| Completeness (%)    | 99.9 (99.9)          | 99.3 (96.9)     | 99.9 (99.2)   | 99.8 (99.8)   | 99.9 (99.2)   |
| Rmerge (%)          | 5.5 (181)            | 7.3 (105)       | 10.1 (135)    | 5.9 (113)     | 13.5 (172)    |
| I/σ(I)              | 24.8 (1.4)           | 12.6 (1.4)      | 8.6 (1.2)     | 21.5 (1.7)    | 8.0 (1.3)     |
| CC1/2               | 1.00 (0.66)          | 1.00 (0.53)     | 0.99 (0.51)   | 0.99 (0.62)   | 0.99 (0.53)   |
| **Refinement**      |                      |                 |              |              |              |
| Average B-factor (Å²) | 83.0                | 97.5            | -            | -            | -            |
| No. of reflections  | 42,042 (4098)        | 21,297 (2055)   | -            | -            | -            |
| No. of reflections (free) | 2100 (227)     | 1049 (90)       | -            | -            | -            |
| R-work (%)          | 19.3 (29.8)          | 22.4 (34.5)     | -            | -            | -            |
| R-free (%)          | 23.2 (35.7)          | 26.4 (43.6)     | -            | -            | -            |
| No. of atoms        |                      |                 |              |              |              |
| protein             | 3386                 | 3246            | -            | -            | -            |
| solvent             | 99                   | 25              | -            | -            | -            |
| ligand              | 129                  | 81              | -            | -            | -            |
| RMSD bonds (Å)      | 0.015                | 0.010           | -            | -            | -            |
| RMSD angles (°)     | 1.17                 | 1.12            | -            | -            | -            |
| Ramachandran statistics |                     |                 |              |              |              |
| Favored (%)         | 96.2                 | 96.4            | -            | -            | -            |
| Allowed (%)         | 3.3                  | 2.6             | -            | -            | -            |
| Outliers (%)        | 0.5                  | 1.0             | -            | -            | -            |

*Numbers in parentheses refer to the outermost resolution shell
Figure 1. Overall structure of SaRelP in the post-catalytic state. A. Crystal structure of the ppGpp-bound SaRelP monomer shown in cartoon with N and C termini and secondary structure elements indicated and ppGpp in ball and stick. The disordered loop 194-201 is shown with a dashed line. B. Overview of the SaRelP homotetramer with the four chains in various shades of blue. The horizontal line indicates the location of the two-fold crystallographic axis that generates the tetramer. C. Structural alignment of SaRelP (blue) and BsRelQ (PDB ID 5DED, green) (16). The ppGpp molecule bound at the active site in both structures is shown in ball and stick in matching colors. D. Structural alignment of SaRelP (blue) with the catalytic domain of ScRel (PDB ID 1VJ7, brown) (11). The GDP molecule in the ScRel active site is shown in ball and stick. The hydrolase domain of ScRel is highlighted with a darker shade of brown. E. Details of the interactions between ppGpp and SaRelP in chains A and B of the
Crystal structures of S. aureus RelP

structure. The pppGpp molecule is shown in ball and stick with relevant interacting residues and secondary structure elements are labelled.
Crystal structures of S. aureus RelP

Figure 2. Structures of SaRelP in the pre- and post-catalytic states. A. Binding of AMPCPP and GTP in the active site of SaRelP in the pre-catalytic state. The nucleotides are shown in ball and stick with interacting residues as labeled sticks. The OMIT difference map (calculated before inclusion of nucleotides in the model), is shown contoured at 2.0 σ. B. The fraction of GDP converted to ppGpp by wt SaRelP and the Y151A and H73A/H74A mutants at various time points. Error bars represent standard deviations of three independent measurements. C. Overview of the pre-catalytic state of SaRelP bound to GTP and AMPCPP. Interacting residues are shown in labelled sticks along with the magnesium ion (green sphere) and relevant water molecules (red spheres). GTP is shown in a purple, AMPCPP in green, and the two phosphate groups of AMPCPP (Pβ and Pγ) that get transferred during the reaction are colored yellow. Stacking interactions are shown with grey areas. D. Overview of the post-catalytic state of SaRelP (chain B) bound to pppGpp. Residues interacting with pppGpp are shown with labeled sticks and the phosphate groups that were transferred (3′-Pα and 3′-Pβ) are colored yellow.
**Figure 3. SaRelP is negatively regulated by (p)ppGpp-binding to the active site.** **A.** Overview of the allosteric pppGpp binding site in BsRelQ (PDB entry 5DED) with relevant interacting side chains labelled (21). **B.** The same site in SaRelP with equivalent residues (as per the alignment) labelled. The positively charged Lys21, Lys25 and Arg28 have been replaced by Ser53, Ser57, and Asp60, respectively, thus changing the electrostatic behavior of the binding site. His73 and His74, which are unique to SaRelP, are shown in blue. **C.** Activity of SaRelP (measured as production of ppGpp from GDP and ATP per enzyme per minute) in the presence of increasing amounts of ppGpp. The calculated IC$_{50}$ value is shown. **D.** Same titration as in C but using pppGpp. Error bars represent standard deviations of the turnover estimates determined by linear regression. Each experiment was performed at least three times. **E.** Effect of model single-stranded mRNA (MF) on the enzymatic activity of EfRelQ (empty circles) and SaRelP (filled circles) measured as production of ppGpp from GDP and ATP per enzyme per minute. Enzymatic assays were performed with 250 nM (62.5 nM tetramer) enzyme, 200 µM $^3$H GDP, and 1 mM ATP. Error bars represent standard deviations of the turnover estimates determined by linear regression; each experiment was performed at least three times. **F.** Electrophoretic mobility shift (EMSA)
analysis of mRNA (MF) complex formation using increasing concentrations of EfRelQ and SaRelP. EMSA assays were performed using 0.19 µM mRNA (MF) and increasing concentrations of SaRelP or EfRelQ as indicated on the figure.
Figure 4. Metal ion ligand binding sites in SaRelP. A. A top view of the SaRelP dimer shown with Fe\(^{3+}\) bound at the subunit-subunit interface of the dimer and coordinated by the two Glu51 residues originating from neighboring chains. The inset displays a close-up view with the octahedral coordination of iron indicated. Fe\(^{3+}\) is shown as a brown sphere, relevant water molecules with red spheres, and the glutamate residues with green sticks. B. Enzymatic activity of SaRelP (measured as production of ppGpp from GDP and ATP per enzyme per minute) as a function of increasing FeCl\(_3\) concentrations. Assays were performed using 250 nM SaRelP, 200 µM \(^3\)H GDP, and 1 mM ATP. C. A putative second metal binding site is found at the dimer-dimer interface of the tetramer. The inset shows a close-up view of the region with residues forming the site shown in green sticks. D. Enzymatic activity of SaRelP as a function of increasing Ni\(_2\)SO\(_4\) and ZnCl\(_2\) concentrations. Error bars represent standard deviations of the turnover estimates determined by linear regression. Each experiment was performed at least three times.
**Figure 5. Mechanism of (p)ppGpp synthesis by SAS enzymes.** Detailed catalytic mechanism for (p)ppGpp synthesis by SAS enzymes based on the structures of the pre-catalytic (left) and post-catalytic (right) state structures of SaRelP. In the pre-catalytic state (left), a Mg\(^{2+}\) ion is held firmly in place by tight octahedral coordination facilitated by the β and λ phosphate oxygen atoms of ATP, two water molecules (which again are constrained by both carboxylate oxygen atoms of Glu 174), one carboxylate oxygen from Asp107, and the 3' OH group of GTP. The electrophilic metal ion promotes deprotonation of the 3' OH group on GTP and subsequently a nucleophilic attack (large, curved arrow) of the resulting alkoxide ion onto the β phosphate group of ATP causing hydrolysis of the phosphate ester between the α and β phosphate groups (small, curved arrow). The developing negative charge of the pentavalent transition state is stabilized by the combined positive charge of two arginine residues and six lysine residues in the neighborhood (not all shown). In the post-catalytic state (right), the 5' β and λ phosphate groups of pppGpp flip over and the λ phosphate group assumes the position of the λ phosphate group of ATP in the pre-catalytic state, stabilized through hydrogen bonding with Asp 107. Likewise, the new 3' α and β phosphate groups shift position to generate the U-shaped conformation of pppGpp found in chain A of the post-catalytic state.
Structural basis for (p)ppGpp synthesis by the Staphylococcus aureus small alarmone synthetase RelP
Melek Cemre Manav, Jelena Beljantseva, Martin Saxtorph Bojer, Tanel Tenson, Hanne Ingmer, Vasili Hauryliuk and Ditlev Egeskov Brodersen

J. Biol. Chem. published online January 11, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA117.001374

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