SUPPORTING MATERIAL

Supplementary experimental procedures

Preparation of 10X Polymix buffer base

For preparation of the putrescine solution, 100 g of putrescine (1,4-diaminobutane) was dissolved in 600 mL of ddH_2O at 90°C, and the pH adjusted with acetic acid to 8.0 (approximately 100 mL of 100% acetic acid). After cooling to room temperature, the solution was adjusted to pH 7.6 and the volume to a final 2 L by addition of 1.134 L of ddH_2O. One 100 mL cup of activated charcoal was added and the slurry was stirred in the hood for 30 minutes before filtered through Whatman paper followed by a 0.45 µm BA85 membrane. The final solution was stored at 4°C wrapped in foil owing to the photosensitivity of putrescine.

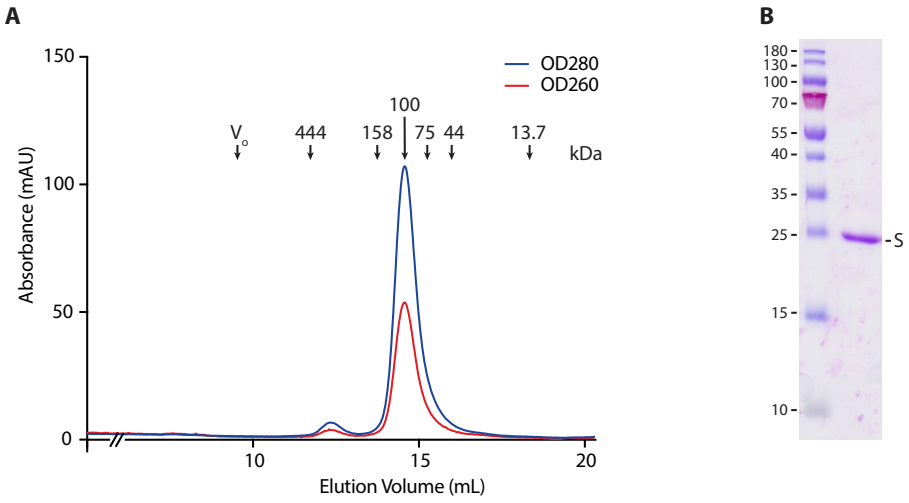
Preparation of 2 L of 10X Polymix buffer base requires 141.66 g KCl, 5.35 g NH_4Cl, 21.44 g $Mg(OAc)_2 \cdot 4H_2O$, 1.47 g $CaCl_2 \cdot 2H_2O$, 5.092 g spermidine, and 160 ml of putrecine solution prepared as described above. The salts were dissolved in app. 1500 mL ddH_2O and mixed well before adding the putrecine solution. Spermidine was dissolved in a small volume of ddH_2O and added to the mixture. The pH was adjusted to 7.5 with concentrated acetic acid or 5 M KOH, after which the volume was adjusted by adding ddH_2O to 2 L. The buffer was filtered through 0.2 µm nitrocellulose filter, before aliquoting and storage at -20°C.

Working Hepes:Polymix buffer was made using 10X Polymix buffer base, 1 M DDT and 1 M Hepes-KOH, pH 7.5 and contains 20 mM Hepes-KOH, pH 7.5, 2 mM DTT, 5 mM $MgOAc_2$, 95 mM KCl, 5 mM NH_4Cl, 0.5 mM $CaCl_2$, 8 mM putrescine, and 1 mM spermidine.

Analytical gel filtration of SaRelP mutants

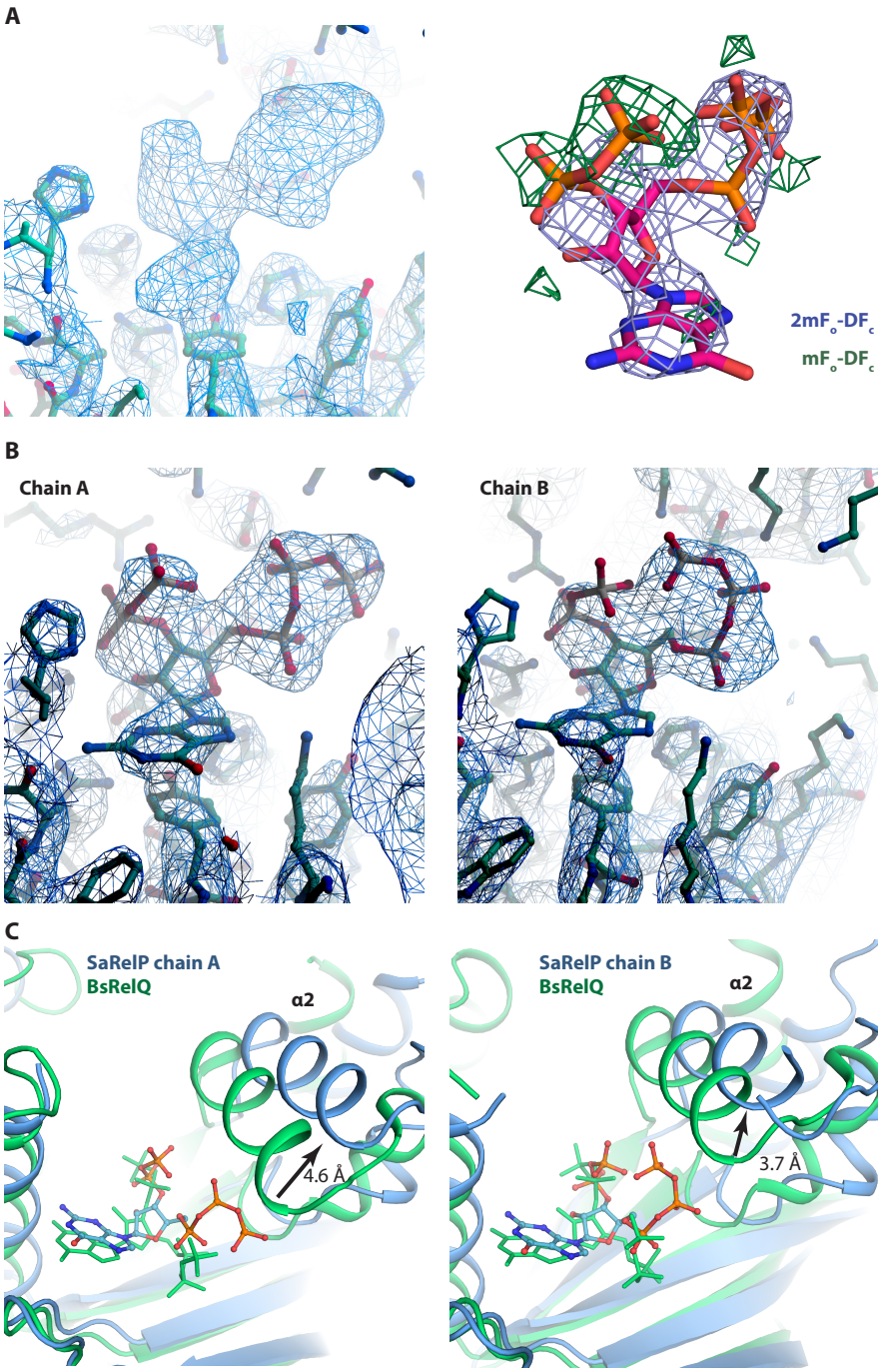
A 3 mL Superdex 200 Increase 5/150 GL (GE Healthcare) column was preequilibrated with a running buffer containing 30 mM Tris-HCl, pH 8.5, 300 mM NaCl, 5 mM $MgCl_2$, 5 mM BME, and 5 % glycerol on a Micro-ÄKTA system (GE Healthcare) at 4°C. 10 µL samples, prepared in running buffer and containing 50 µM *wt* SaRelP or one of the mutants (Y151A and H73A/H74A) were applied and resolved using standard protocols.

SUPPLEMENTARY FIGURES



Supplementary Figure S1. SaRelP is a tetramer in solution

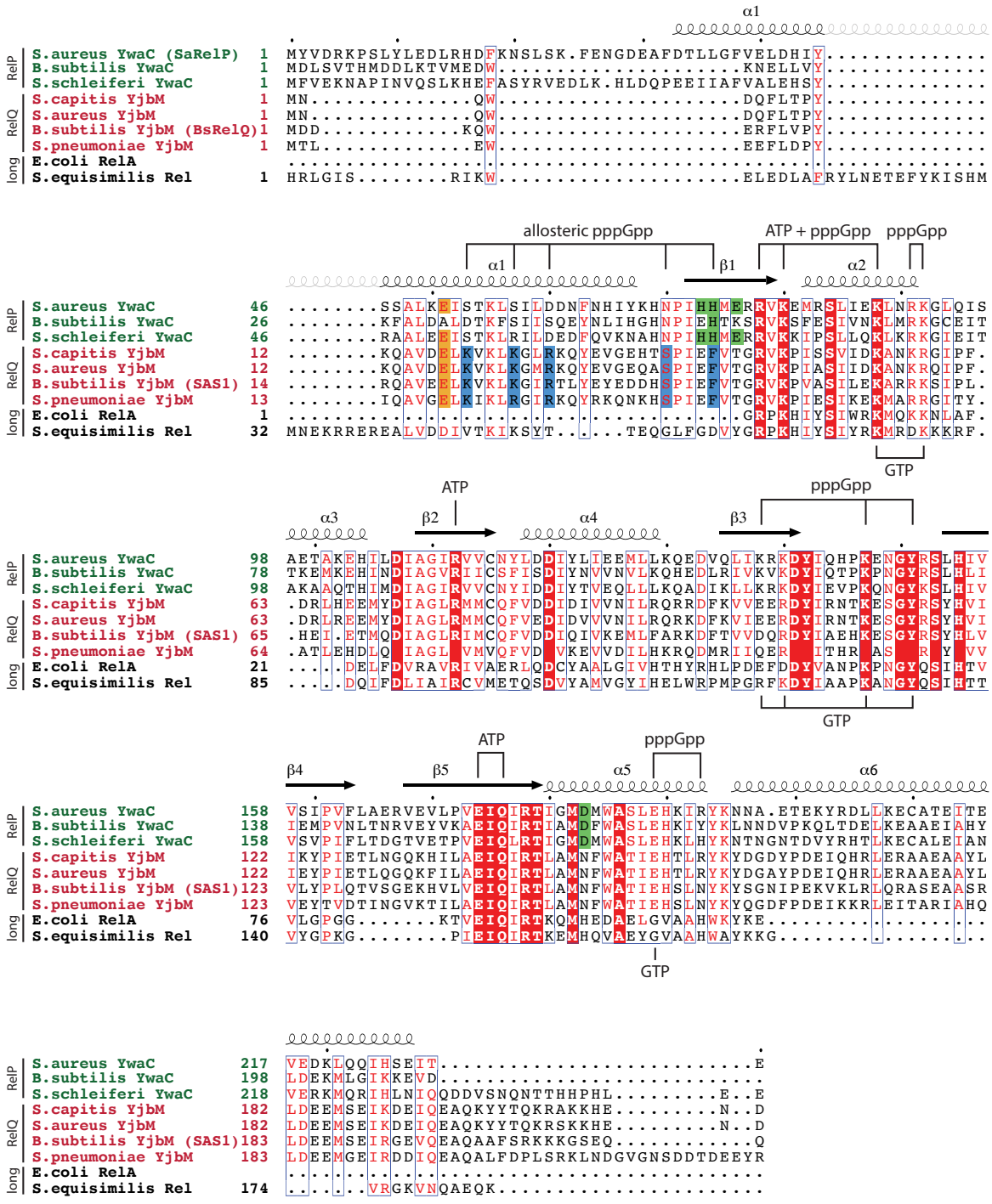
A. Size exclusion chromatography profile of SaRelP. The curves show OD_{280} (blue) and OD_{260} (red) measured in mAU as afunction of elution volume. The elution volumes of standard proteins are indicated with short arrows and the apparent molecular mass of SaRelP (100 kDa) highlighted with a longer arrow. **B.** SDS-PAGE showing the purified protein prior to crystallization. The left lane shows standard protein markers with molecular masses (in kDa) as indicated.



Supplementary Figure S2. Binding of pppGpp in the SaRelP active site

A. Left, 2*m*Fo-*D*Fc OMIT map (contoured at 1.5 σ) covering the SaRelP active site (chain A) and calculated before inclusion of ligands in the model; Right, 2*m*Fo-*D*Fc map (blue) covering the active site ligand after refinement with GTP and contoured at 2.0 σ. The *m*Fo-*D*Fc difference density (green, 2.5 σ) clearly indicates the missing phosphate groups. **B.** 2*m*Fo-*D*Fc density (1.5 σ) showing the two different conformations of pppGpp found in chains A (left) and B (right) of

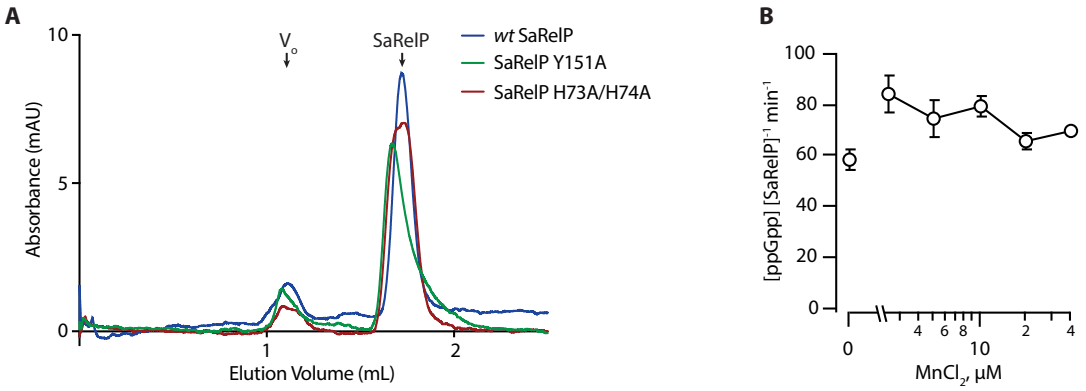
the structure. **C.** Comparison of the locations of helix α2 and pppGpp in the two RelP molecules (chain A, left and chain B, right, both green) in the crystal compared to BsRelQ (green) (21). The shift of the helix is indicated with arrows and the distance in Å.



Supplementary Figure S3. Sequence alignment of bacterial (p)ppGpp synthetases

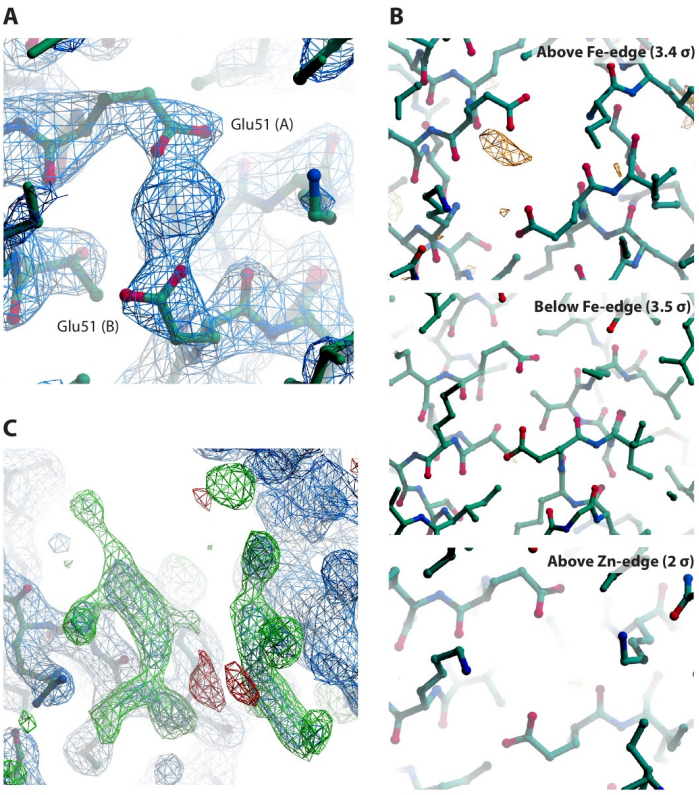
The sequence of SaRelP (*S. aureus* YwaC) aligned with representative RelP (YwaC, green) and RelQ (YjbM, red) sequences as well as the corresponding synthetase domains from two "long" RSHs, *E. coli* RelA and *S. equisimilis* Rel (black). The observed secondary structure of SaRelP is shown with arrows (β-strands) and curly lines (α-helices) above the amino acid sequences, highly conserved residues are shown on a red background, residues found to be interacting with pppGpp

at the allosteric binding in BsRelQ (SAS1, *B. subtilis* YjbM) are shown on a blue background, Glu51 that interacts with Fe^{3+} in SaRelP is shown on an orange background, and the residues involved in the putative Zn^{2+} binding site on a green background, where conserved. Residues specifically interacting with pppGpp, ATP, and GTP at the active site as well as pppGpp at the allosteric site are indicated with brackets. GenBank accession numbers are as follows: *S. aureus* YwaC (this study, WP_063651169.1), *B. subtilis* YwaC (WP_019716176.1), *S. capitis* YwaC (WP_070664381.1), *S. schleiferi* YwaC (WP_050345179.1), *S. aureus* YjbM (WP_064129082.1), *B. subtilis* YjbM (SAS1, WP_003224620.1), *S. pneumoniae* YjbM (WP_000171678), *E. coli* RelA (WP_074156896), and *S. equisimilis* Rel (WP_043030187.1). The alignment was prepared using ESPript (http://espript.ibcp.fr).



Supplementary Figure S4. Conformational stability of site-specific SaRelP mutants and the effect of Mn^{2+} on catalysis

A. Analytical gel filtration of *wt* SaRelP (blue) superimposed on separate runs of the Y151A (green) and H73A/H74A (red) mutants. The curves show OD_{280} measured in mAU as function of elution volume. The void volume and standard elution volumes of RelP proteins are indicated with short arrows. **B.** Enzymatic activity of SaRelP (measured as production of ppGpp from GDP and ATP per enzyme per minute) as a function of increasing $MnCl_2$ concentrations. Assays were performed using 250 nM SaRelP, 200 µM ^{3}H GDP, and 1 mM ATP.



Supplementary Figure S5. Analysis of metal and ligand binding to SaRelP

A. 2*m*Fo-*D*Fc OMIT map covering the Fe^{3+} binding site and contoured at 3.0 σ with the two Glu51 residues indicated. **B.** Top, anomalous difference map calculated using data measured above the Fe K-edge at 7.200 keV contoured at 3.4 σ; middle, anomalous difference map calculated from data measured below the Fe K-edge at 6.900 keV contoured at 3.5 σ; bottom, anomalous difference map calculated using data measured above the Zn K-edge at 9.800 keV contoured at 2 σ. **C.** A continuous blob of difference electron density found at the tetramer interface. The $2mF_o$-DF_c map (blue) is contoured at 2.5 σ while the mF_o-DF_c map (green) is contoured at 3.5 σ.