

RESEARCH ARTICLE

WILEY

Dual role of a (p)ppGpp- and (p)ppApp-degrading enzyme in biofilm formation and interbacterial antagonism

Wieland Steinchen¹ | Shehryar Ahmad^{2,3} | Martina Valentini ^{4,5} | Kira Eilers⁵ |
Mohamad Majkani¹ | Florian Altegoer¹ | Marcus Lechner¹ | Alain Filloux ⁵ |
John C. Whitney ^{2,3,6} | Gert Bange ¹

¹Center for Synthetic Microbiology (SYNMIKRO) & Faculty of Chemistry, Philipps-University Marburg, Marburg, Germany

²Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON, Canada

³Michael G. DeGroote Institute for Infectious Disease Research, McMaster University, Hamilton, ON, Canada

⁴Department of Microbiology and Molecular Medicine, Faculty of Medicine, University of Geneva, Geneva, Switzerland

⁵MRC Centre for Molecular Bacteriology and Infection, Department of Life Sciences, Imperial College London, London, UK

⁶David Braley Centre for Antibiotic Discovery, McMaster University, Hamilton, Ontario, Canada

Correspondence

Wieland Steinchen and Gert Bange, Center for Synthetic Microbiology (SYNMIKRO) & Faculty of Chemistry, Philipps-University Marburg, Germany.
wieland.steinchen@synmikro.uni-marburg.de (W. S.) and gert.bange@synmikro.uni-marburg.de (G. B.)

Funding information

Canadian Institutes of Health Research, Grant/Award Number: PJT-156129; Deutsche Forschungsgemeinschaft, Grant/Award Number: SPP1879

Abstract

The guanosine nucleotide-based second messengers ppGpp and pppGpp (collectively: (p)ppGpp) enable adaptation of microorganisms to environmental changes and stress conditions. In contrast, the closely related adenosine nucleotides (p)ppApp are involved in type VI secretion system (T6SS)-mediated killing during bacterial competition. Long RelA-SpoT Homolog (RSH) enzymes regulate synthesis and degradation of (p)ppGpp (and potentially also (p)ppApp) through their synthetase and hydrolase domains, respectively. Small alarmone hydrolases (SAH) that consist of only a hydrolase domain are found in a variety of bacterial species, including the opportunistic human pathogen *Pseudomonas aeruginosa*. Here, we present the structure and mechanism of *P. aeruginosa* SAH showing that the enzyme promiscuously hydrolyses (p)ppGpp and (p)ppApp in a strictly manganese-dependent manner. While being dispensable for *P. aeruginosa* growth or swimming, swarming, and twitching motilities, its enzymatic activity is required for biofilm formation. Moreover, (p)ppApp-degradation by SAH provides protection against the T6SS (p)ppApp synthetase effector Tas1, suggesting that SAH enzymes can also serve as defense proteins during interbacterial competition.

KEYWORDS

(p)ppApp, (p)ppGpp, biofilm formation, interbacterial competition, *Pseudomonas aeruginosa*, small alarmone hydrolase

1 | INTRODUCTION

Microorganisms have to continuously adapt to environmental changes and stress conditions. The stringent response is conserved among bacteria and relies on the nucleotide-based second

messengers ppGpp and pppGpp (collectively referred to as (p)ppGpp). Both second messengers regulate a wide variety of cellular processes including amino acid/nucleotide/lipid metabolism, replication, transcription, translation, and the biogenesis of ribosomes (Haurlyuk et al., 2015; Steinchen & Bange, 2016). As such, (p)ppGpp

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2021 The Authors. *Molecular Microbiology* published by John Wiley & Sons Ltd.

has a pleiotropic effect on bacterial physiology and consequently, also impacts motility, biofilm formation, virulence of pathogens, and bacterial dormancy (Dalebroux & Swanson, 2012; Hauryliuk et al., 2015; Liu et al., 2015; Potrykus & Cashel, 2008).

Central to the metabolism of (p)ppGpp are members of the RelA-SpoT Homolog (RSH) protein family (Figure 1a). RSH enzymes are characterized by the presence of (p)ppGpp synthetase or hydrolase domains and can be categorized into "long" and "short" RSH proteins.

The (p)ppGpp synthetase domain catalyzes the production of ppGpp and pppGpp through the transfer of pyrophosphate from ATP to the ribose 3'-OH of GDP or GTP, respectively (Sy & Lipmann, 1973). *Pseudomonas aeruginosa* possesses the "long" RSH proteins SpoT and RelA. Both are multidomain enzymes harboring (p)ppGpp synthetase and hydrolase domains as well as additional domains located within their C-terminal portion that are critical for the regulation of the opposing enzymatic activities (Figure 1a). SpoT activity is

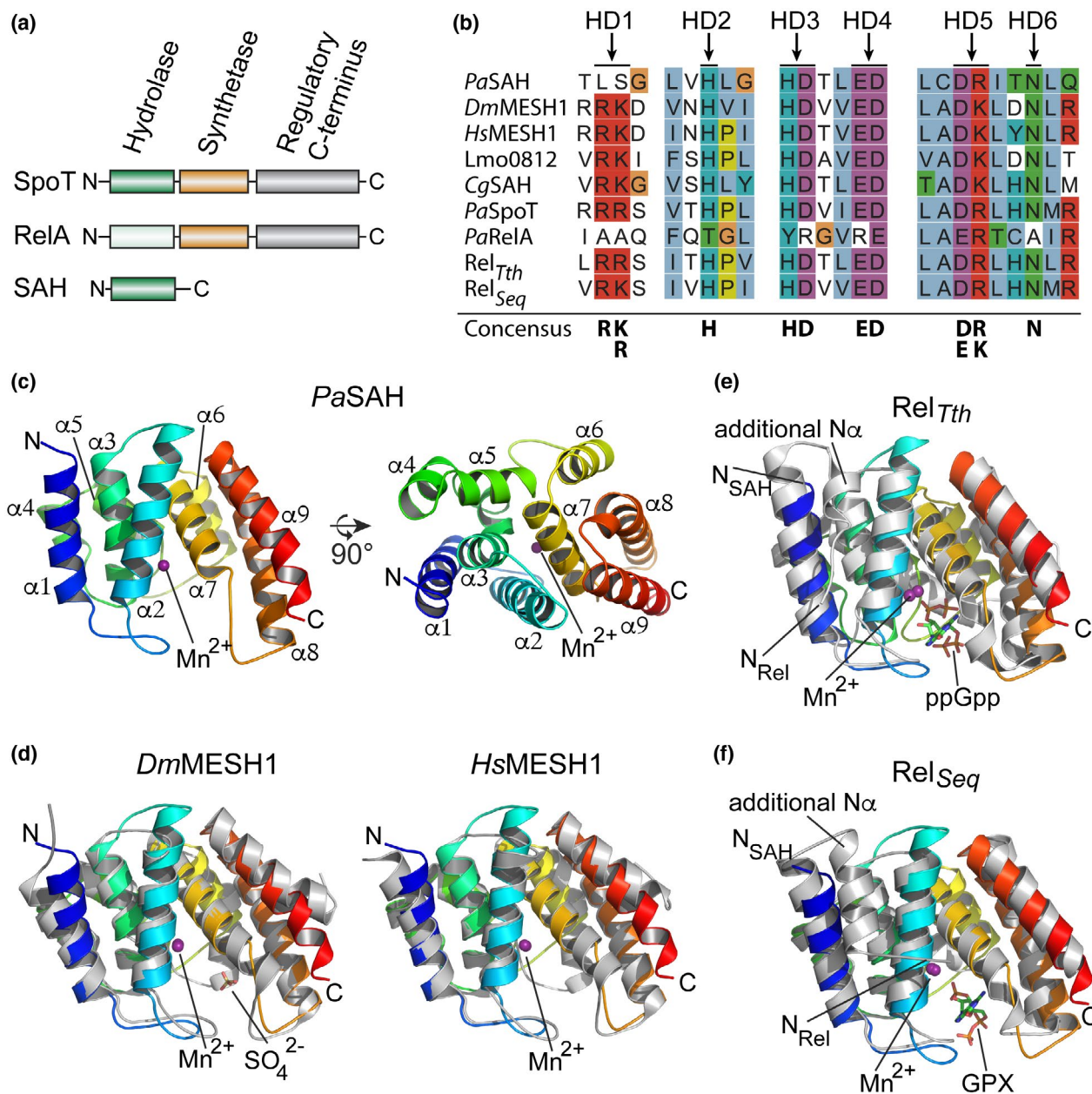


FIGURE 1 Crystal structure of PaSAH. (a) Domain architecture of RSH proteins in *P. aeruginosa*. (b) Hydrolase domain (HD) motifs 16 present in *P. aeruginosa* (Pa) SAH, *D. melanogaster* (Dm) and *H. sapiens* (Hs) MESH1, the putative SAH enzyme Lmo0812 from *L. monocytogenes*, *C. glutamicum* (Cg) SAH, PaSpoT, and PaRelA and the bifunctional Rel enzymes from *T. thermophilus* (Rel_{Tth}) and *S. equisimilis* (Rel_{Seq}). (c) The crystal structure of PaSAH is shown in cartoon representation and colored in rainbow from its N- to C-terminus. The purple sphere represents a manganese ion. (d-f) Superimposition of the crystal structures of PaSAH colored in rainbow with the grey-colored structures of (d) the metazoan DmMESH1 and HsMESH1 enzymes, (e) the hydrolase domain (amino acids 1–200, PDB: 6S2T) of Rel_{Tth} bound to ppGpp, and (f) the hydrolase domain (amino acids 1–200 of monomer B, PDB: 1VJ7) of Rel_{Seq} bound to guanosine 5'-diphosphate 2':3'-cyclic monophosphate (GPX). The purple spheres represent manganese ions. The sulfate ion (SO₄²⁻), ppGpp and GPX are shown as sticks

regulated by fatty acid starvation conditions and carbon downshifts, while RelA-dependent (p)ppGpp synthesis is triggered during amino acid starvation through binding to ribosomes blocked by uncharged tRNAs at the A-site (reviewed in: (Hauryliuk et al., 2015; Ronneau & Hallez, 2019)). Degradation of (p)ppGpp occurs through hydrolase domains catalyzing the removal of the 3'-diphosphate moiety of (p)ppGpp to generate GDP or GTP. The N-terminal portions of SpoT and Rel harbor active (p)ppGpp hydrolase domains; however, the equivalent domain in *P. aeruginosa* RelA is inactive due to the lack of amino acid residues essential for catalytic activity (Figure 1b). Stand-alone (p)ppGpp synthetase and hydrolase domain-containing proteins (SAS and SAH for small alarmone synthetase and small alarmone hydrolase, respectively) constitute the class of "short" RSH proteins. Bacterial SAS proteins synthesize ppGpp and pppGpp independently of the ribosome and have previously been characterized structurally and biochemically (Beljantseva et al., 2017; Gaca et al., 2015; Manav et al., 2018; Steichen et al., 2015, 2018).

Structural knowledge about (p)ppGpp hydrolase domains in bacteria is limited to those contained in the Rel/RelA proteins from *Thermus thermophilus* (Tamman et al., 2020), *Bacillus subtilis* (Pausch et al., 2020), *Mycobacterium tuberculosis* (Singal et al., 2017), *Escherichia coli* (Arenz et al., 2016; Brown et al., 2016; Loveland et al., 2016) and *Streptococcus equisimilis* (Hogg et al., 2004). However, the crystal structures of two metazoan SAH proteins (MESH1) from *Drosophila melanogaster* and *Homo sapiens* showed that they are capable of hydrolyzing ppGpp in vitro in a strictly manganese-dependent manner (Sun et al., 2010). Deletion of the MESH1-encoding gene in *D. melanogaster* resulted in retarded body growth and impaired starvation resistance implying critical regulatory functions during development (Sun et al., 2010). Besides possessing (p)ppGpp hydrolytic activity, *H. sapiens* MESH1 is an efficient NADPH phosphatase that facilitates ferroptosis (Ding et al., 2020).

No structural data about bacterial SAH proteins are available so far and their function is just starting to be addressed. A physiological role has recently been put forward for a SAH protein from *Cellulomonas marina* (ATfaRel, (Jimmy et al., 2020)) as a type IV antitoxin (Harms et al., 2018). ATfaRel counteracts the toxic SAS (toxSAS) protein FaRel, a (p)ppGpp and ppApp synthetase encoded in the same operon as ATfaRel, through degradation of the otherwise noxiously accumulating (p)ppGpp and ppApp nucleotides. The antitoxin activity of ATfaRel, and similarly that of HsMESH1 and the SAH enzymes PVP-SE1 (Santos et al., 2011) and SSU5 (Kim et al., 2012) present in *Salmonella* phages, is not restricted to toxSAS proteins encoded in the same operon. Instead, they are capable of detoxifying the activity of toxSAS proteins from other species upon co-expression in *E. coli* thus suggesting a broader role of SAH enzymes in toxin-antitoxin modules (Jimmy et al., 2020). Nullification of FaRel toxSAS activity by the long RSH SpoT from *E. coli* (Jimmy et al., 2020) contrasting to the inability of *P. aeruginosa* SpoT to abrogate toxicity of a closely related (p)ppApp synthetase toxin, Tas1 (Ahmad et al., 2019), hints to species-specific differences in hydrolase activity and/or different potency of (p)ppGpp and (p)ppApp nucleotides to inhibit microbial growth. Another RSH-related toxin-antitoxin

module consisting of the (p)ppGpp synthetase gp29 and its antitoxin gp30 has been identified in the mycobacterial Cluster N bacteriophage Phrann. However, the mode of gp30 detoxification of gp29 activity is unclear (Dedrick et al., 2017).

In the present study, we further investigate the functions of bacterial SAH proteins by characterizing a putative SAH enzyme from *P. aeruginosa* (PaSAH). Structural analysis of PaSAH unambiguously identifies the protein as having significant structural homology to the metazoan MESH1 enzymes and the hydrolase domain present in bacterial long RSH proteins. We show that the manganese-dependent PaSAH enzyme efficiently degrades (p)ppGpp, but even better the related (p)ppApp nucleotides, an emerging class of bacterial second messengers (Bruhn-Olszewska et al., 2018; Sobala et al., 2019) and weaponry of type VI secretion systems (T6SSs, (Ahmad et al., 2019)). Our in vivo analysis shows that the deletion of the PaSAH-encoding gene does neither affect the growth kinetics of *P. aeruginosa* nor its swimming, swarming, and twitching motilities but is detrimental for its capability to form biofilms. Most interestingly, the (p)ppApp degrading activity of PaSAH alleviates the toxicity evoked by the (p)ppApp synthetase of the T6SS effector protein Tas1 employed by *P. aeruginosa* to kill bacterial competitors. Therefore, PaSAH not only serves as a critical element during the (p)ppGpp-dependent regulation of biofilm formation, but also enables immunity against (p)ppApp-based killing mechanism during inter-bacterial competition.

2 | RESULTS

2.1 | Crystal structure of PaSAH

To clarify structure, function, and mechanism of bacterial small alarmone hydrolases, we chose the predicted SAH present in the Gram-negative pathogen *P. aeruginosa*, PA0431 (named: PaSAH from here on), which is classified as a PbcSpo2 subfamily RSH enzyme (Atkinson et al., 2011; Jimmy et al., 2020). Comparison of the amino acid sequences of hydrolase domain-containing proteins shows that PaSAH harbors five of the six catalytic motifs (HD motifs 1–6 (Steichen & Bange, 2016)) required for manganese ion coordination, substrate binding, and hydrolysis (Figures 1b and S1). However, PaSAH lacks the otherwise highly conserved HD1 motif, which is critically involved in coordination of the substrates' guanine base in structures of Rel proteins from *T. thermophilus* (Rel_{Tth} (Tamman et al., 2020)) and *S. equisimilis* (Rel_{seq} (Hogg et al., 2004)) and its essentiality for hydrolysis underscored by mutational analysis (Hogg et al., 2004; Sun et al., 2010; Tamman et al., 2020). This raises the question of whether or not PaSAH would be an active (p)ppGpp hydrolase.

To elucidate this, first, we determined the crystal structure of PaSAH to a resolution of 1.85 Å (Table 1). The structure was solved by molecular replacement employing the hydrolase domain of Rel_{seq} (PDB: 1VJ7 (Hogg et al., 2004)), and refined to R_{work}/R_{free} values of 19/22%. The structure shows a monomer of PaSAH consisting of a bundle of nine anti-parallel α -helices that form a deep cavity at one

TABLE 1 Crystallographic data collection and refinement statistics

	<i>PaSAH</i>
Data collection	
Space group	C2
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	85.33, 85.38, 122.17
α , β , γ (°)	90, 102.059, 90
Wavelength (Å)	1.072
Resolution (Å)	45.78–1.85 (1.92–1.85)
<i>R</i> _{merge}	0.147 (0.622)
<i>I</i> / σ <i>I</i>	8.09 (1.92)
Completeness (%)	99.13 (99.08)
Redundancy	3.7 (3.8)
CC1/2	0.99 (0.69)
Refinement	
Resolution (Å)	48.75–1.85
No. reflections	72,548 (7,192)
<i>R</i> _{work} / <i>R</i> _{free}	0.19/0.22
No. atoms	6,532
Protein	5,632
Ligand/ion	4
Water	896
B-factors	
Protein	25.69
Ligand/ion	28.81
Water	35.01
R.m.s. deviations	
Bond lengths (Å)	0.004
Bond angles (°)	0.90

Note: Values in parentheses are for highest-resolution shell.

side (Figure 1c). Although we did not deliberately add manganese to *PaSAH* prior to crystallization, refinement of the electron density present within the cavity indicates an approximately 50% occupation with a Mn²⁺ ion (Figure 1c). The structure of *PaSAH* shares significant homology to the structures of the *D. melanogaster* (*DmMESH1*, PDB: 3NQW) and *H. sapiens* (*HmMESH1*, PDB: 3NR1) MESH1 enzymes (Figure 1d (Sun et al., 2010)). Search of the protein data bank for homologous structures further revealed the protein Lmo0812 from *Listeria monocytogenes* (Figure S2, PDB-ID: 4YF1) as structurally highly similar, and thus, as a putative SAH enzyme. The structure of *PaSAH* also superimposes well with the hydrolase domains from structures of Rel_{Th} (Figure 1e, PDB: 6S2T (Tamman et al., 2020)) and Rel_{Seq} (Figure 1f, PDB: 1VJ7 (Hogg et al., 2004)). One difference in

topology between the structures of *PaSAH* and Rel relates to the presence of an additional N-terminal helix in the latter (Figure 1e,f), which leans against the two subsequent helices and protrudes the N-termini of Rel_{Th} and Rel_{Seq} to the opposite direction as found in *PaSAH*. Taken together, our analysis structurally establishes *PaSAH* as protein of the RSH family harboring a (p)ppGpp hydrolase domain.

2.2 | *PaSAH* is a manganese-dependent (p)ppGpp hydrolase

Our structure of *PaSAH* showed that a manganese ion is coordinated within its central cavity by two histidines (H40, H70) and two aspartates (D71, D131) (Figure 2a), an arrangement that is also observed in the crystal structures of the MESH1, Lmo0812, and Rel enzymes (Figure S3). We thus, second, probed the enzymatic activity of *PaSAH* biochemically using ppGpp as a substrate and MnCl₂ as cofactor. Hydrolysis of the 3'-pyrophosphate moiety of the ppGpp substrate is evidenced by the appearance of a GDP product (Figure 2b). Variation of two metal-coordinating residues (H70, D71) into alanines completely abolished the ability of *PaSAH* to degrade ppGpp suggesting that the enzyme activity depends on the presence of a metal ion cofactor (Figure 2b). As magnesium and manganese ion cofactors may in some proteins, for example, DNA and RNA polymerases, be interchangeable but result in different enzymatic properties (Esyunina et al., 2016; Vashishtha & Konigsberg, 2016), we assayed the hydrolysis of (p)ppGpp in presence of increasing concentrations of either manganese or magnesium (both provided as chloride salts) observing efficient degradation only in presence of manganese (Figure 2c). An enzyme kinetic analysis of (p)ppGpp hydrolysis in presence of 2.5 mM MnCl₂ showed that *PaSAH* degraded ppGpp and pppGpp with similar *K_m* and *V_{max}* values (Figure 2d). These experiments evidence *PaSAH* as a manganese-dependent (p)ppGpp hydrolase.

2.3 | *PaSAH* employs a catalytic mechanism similar to long RSH enzymes

Despite intensive trials, we could not obtain a crystal structure of *PaSAH* bound to (p)ppGpp. We, therefore, employed the structures of the hydrolase domains of Rel_{Th} bound to ppGpp (Figure 3a, PDB: 6S2T (Tamman et al., 2020)) and Rel_{Seq} (Figure 3b, PDB: 1VJ7 (Hogg et al., 2004)), which has the unusual nucleoside guanosine 5'-diphosphate 2':3'-cyclic monophosphate (GPX) residing within its active site, to approximate the substrate binding site in *PaSAH* (Figure 3c,d). Common between both Rel structures and *PaSAH* is the position of the ppGpp/GPX molecules relative to the manganese ion cofactor and the glutamate and aspartate residues constituting the HD4 motif (E80/D81 in Rel_{Th}, E81/D82 in Rel_{Seq}, and E71/D72 in *PaSAH*), which was proposed by Hilgenfeld and coworkers to facilitate the nucleophilic attack on the 3'-phosphate moieties (Hogg et al., 2004). Slightly different configurations of the

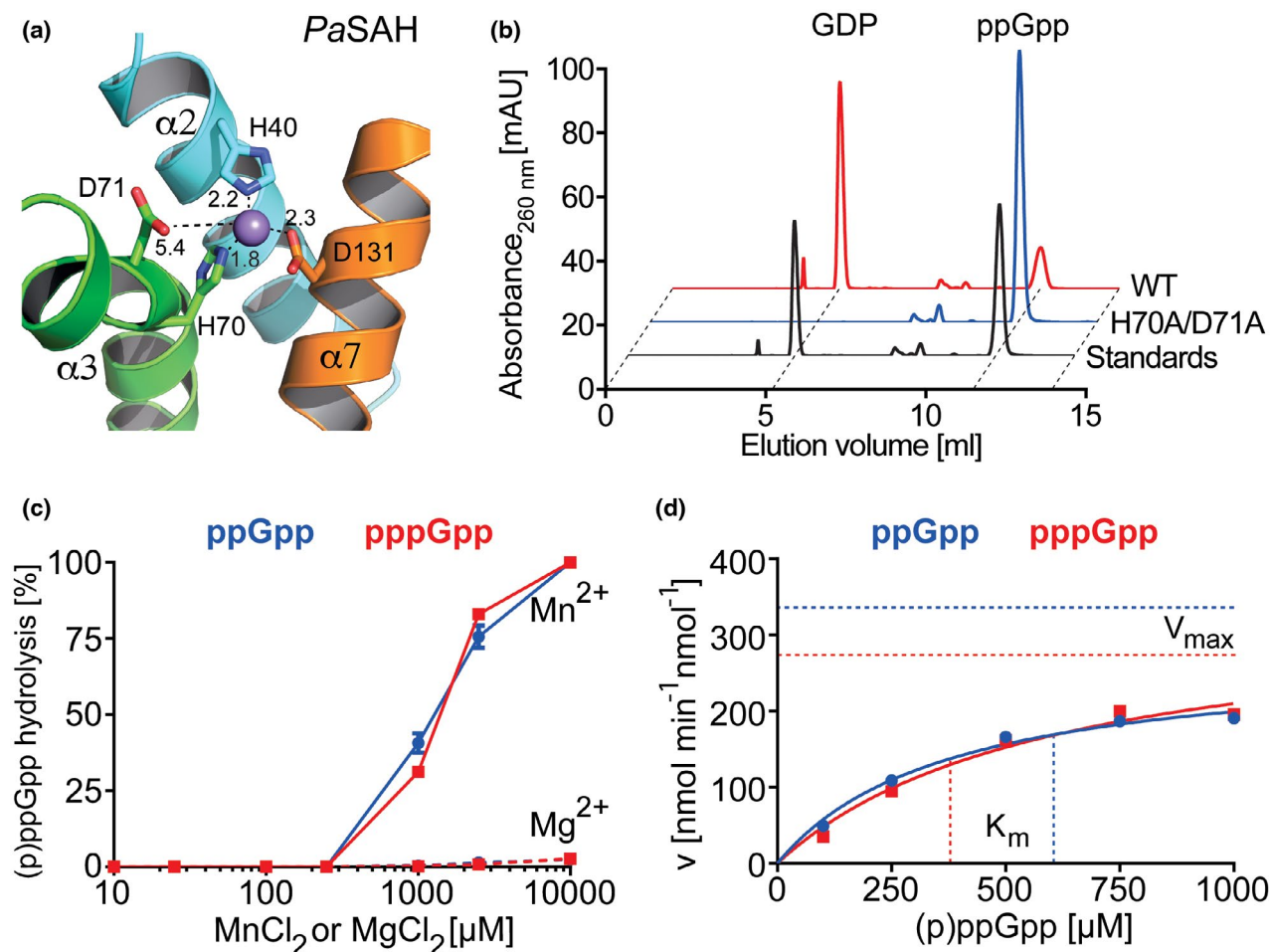


FIGURE 2 *PaSAH* is a manganese-dependent (p)ppGpp hydrolase. (a) Coordination of the manganese ion (purple sphere) within the active site of *PaSAH* is indicated by dashed lines; nearby numbers denote the distance in Å. (b) UV chromatogram of an enzymatic reaction containing ppGpp and native *PaSAH* (WT, red trace) or a variant deficient in manganese coordination (H70A/D71A, blue trace). A mixture of GDP and ppGpp (black trace) served as standard. (c) (p)ppGpp hydrolysis of 1 mM ppGpp (blue circles) and pppGpp (red squares) was assayed in presence of 0.5 μ M *PaSAH* and increasing concentrations of MnCl_2 (solid lines) or MgCl_2 (dashed lines). The amount of hydrolysis is given in %. Error bars indicate the SD of $n = 3$ replicates. (d). Velocity (v)/substrate (S) characteristic of ppGpp (blue circles) and pppGpp (red squares) hydrolysis by *PaSAH* in presence of 2.5 mM MnCl_2 . Data were fitted according to the Michaelis–Menten equation. Vertical and horizontal dashed lines indicate the Michaelis–Menten constants (K_m) and maximal velocities ($V_{\max}$), respectively. K_m (in μ M) and $V_{\max}$ (in $\text{nmol}/\text{min nmol}^{-1}$) were: ppGpp (K_m : 378 ± 84 and $V_{\max}$: 275 ± 24) and pppGpp (K_m : 604 ± 236 and $V_{\max}$: 336 ± 65)

α - and β -phosphates are observed for ppGpp (Figure 3a,c) and GPX (Figure 3b,d), the latter further lacking the ϵ -phosphate present in ppGpp. The phosphates' negative charges of both molecules are in Rel and *PaSAH* counterbalanced by a set of nonconserved positively charged arginine and lysine residues (Figure 3a–d). Further shared is the position of a tyrosine residues in Rel_{Tth} (Y49) and *PaSAH* (Y37) that coordinates the ribose moiety through an H-bond with the 2'-OH group of ppGpp (Figure 3a,c).

Comparison of residues that might contribute to coordination of the guanine moiety of ppGpp/GPX to *PaSAH* reveals strong disparities to Rel_{Tth} and Rel_{Seq}. Primarily, *PaSAH* diverges in its HD1 motif-containing loop that harbors L30 and S31 (Figure 3c,d) but in Rel houses an arginine (R43 and R44 in Rel_{Tth} and Rel_{Seq}, respectively), which together with another arginine/lysine residue sandwiches the guanine base (Figure 3a,b). This arginine is in Rel positioned by H-bonds with the side chains of T153/T151 and N150/N148 (Rel_{Tth}/

Rel_{Seq}). The clamping of the guanine is further enforced by M157 and L155 in Rel_{Tth} and Rel_{Seq}, respectively, the position of which is roughly occupied by W143 in *PaSAH* (Figure 3c, d). The side chains of L30, S31, and W143 appear to be rather remote from the guanine base in the models of *PaSAH* with bound ppGpp/GPX, however, conformational changes upon substrate binding are likely to take place as observed for Rel_{Tth} (Figure S4). In Rel, specificity for the guanine base is established by H-bonding interactions between the N-1 of the base and the side chain of T153/T151 (Rel_{Tth}/Rel_{Seq}), the C-2 amino group and the main chain carbonyl of N150/N148 and between the C-6 carbonyl group and the main chain amides of R44/K45 and S45/S46 (Figure 3a,b). In *PaSAH*, the peptide backbone similarly contacts the C-2 amino and C-6 carbonyl groups of the guanine, however, interactions with N-1 are lacking because helix $\alpha 7$ of *PaSAH* is approximately one turn shorter than its counterpart $\alpha 8$ in Rel. Hereby, a stretch of prolines P138–140 (termed Triple P in

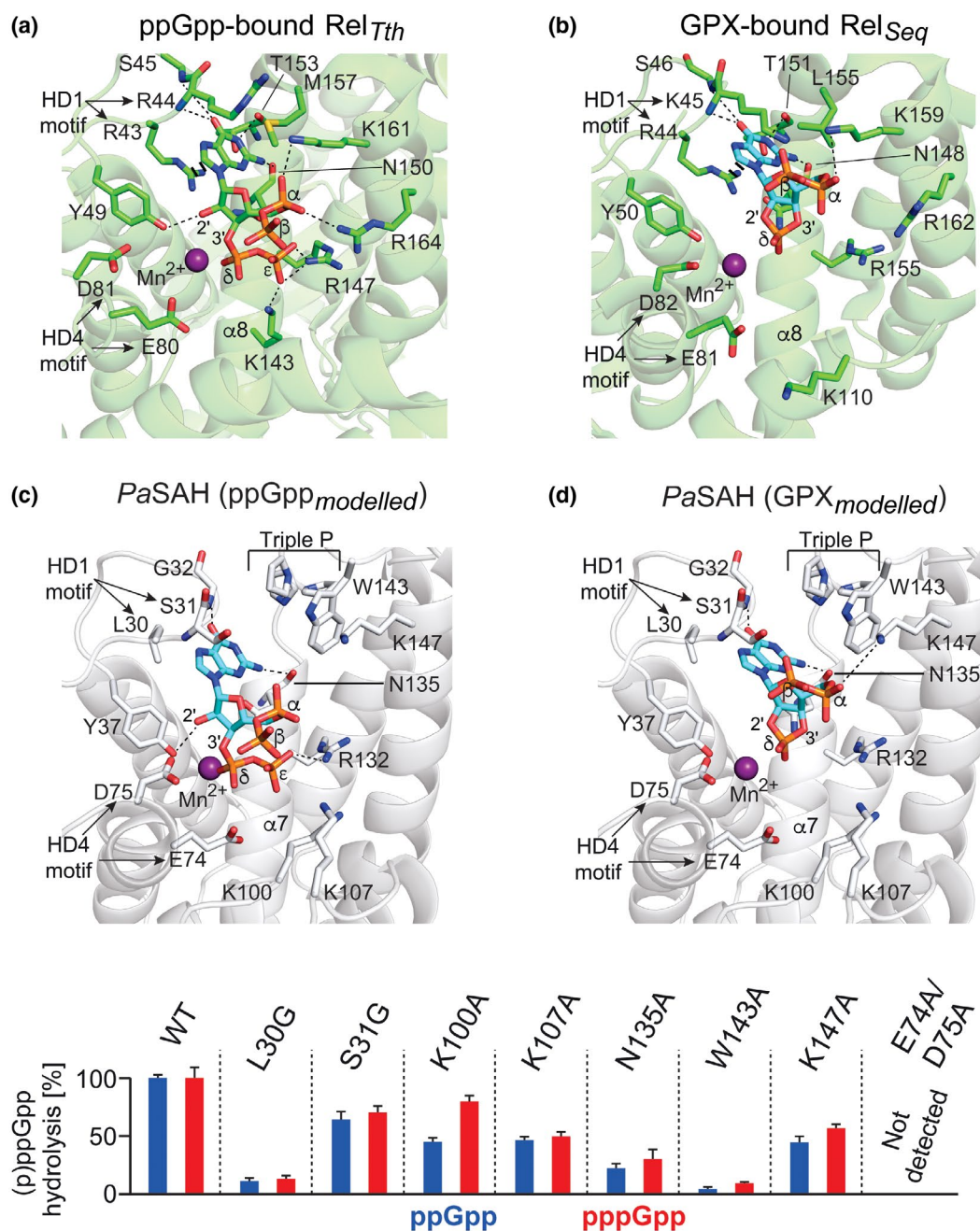


FIGURE 3 Model of substrate binding to PaSAH. (a,b) Crystal structures of (a) ppGpp-bound Rel_{Tth} (PDB: 6S2T) and (b) GPX-bound Rel_{Seq} (PDB: 1VJ7), which were used to derive the position of substrates in the active site of PaSAH. (c,d). Crystal structures of PaSAH with the location of (c) ppGpp or (d) GPX modeled based on the superimpositions with Rel_{Tth} and Rel_{Seq}. In panels (a-d), interactions within 4 Å distance are indicated by dashed lines. (e) Hydrolysis of 1 mM ppGpp (blue) and pppGpp (red) in presence of 0.5 μM native PaSAH (WT) or its variants. Hydrolysis by native PaSAH was set to 100%. Data represent mean ± SD of *n* = 3 replicates

Figure 3c,d) is present instead of threonine, which increases the distance between N-1 and the peptide backbone. It appeared to us that those differences might potentially correlate with less specificity for guanosine as the nucleobase of the substrate.

To probe the participation of active site-lining residues, we mutated them to either glycine or alanine and determined the ability of the variants to hydrolyze (p)ppGpp (Figure 3e). Variation of three lysine residues (K100, K107, K147) to alanine reduced (p)ppGpp hydrolysis approximately twofold. The different amplitude in reduction

of ppGpp and pppGpp hydrolysis observed for the K100A variant hints at a participation of K100 in the coordination of the γ-phosphate during substrate binding and/or product release. Strongly impaired in catalytic activity were variants of N135, L30, and W143 residues suggesting that they would be able and relevant to correctly position the nucleobase in PaSAH (Figure 3c,d). Surprisingly though, hydrolytic activity was still evident for the PaSAH L30G and N135A variants (Figure 3e) whereas exchange of the corresponding residues in Rel_{Tth} (N150A and R43A (Tamman et al., 2020)) and Rel_{Seq} (R44Q

(Hogg et al., 2004)) disrupted their enzymatic activity entirely. The abolishment of (p)ppGpp hydrolysis by PaSAH upon variation of E74 and D75 to alanine validates the essentiality of these residues for catalysis in agreement with earlier observations (Hogg et al., 2004; Sun et al., 2010; Tamman et al., 2020).

Taken together, these results suggest that the catalytic mechanism of (p)ppGpp degradation by the bacterial PaSAH enzyme is very similar to the hydrolase domain found in the long RSH proteins Rel_{Tth} and Rel_{Seq} albeit some differences mainly in the coordination of the nucleobase do exist.

2.4 | PaSAH does not affect planktonic growth but contributes to the ability of *P. aeruginosa* to form biofilms

Many bacterial species, including *P. aeruginosa*, often exist in one of two distinct growth states: a free-living, highly motile state or as part of a sessile, biofilm-forming community (Costerton et al., 1981). These two states have been linked to either acute inflammatory

reaction or refractory chronic infections, respectively (Valentini et al., 2018). (p)ppGpp-dependent regulation of biofilm formation has been documented for *Vibrio cholerae* (He et al., 2012), *Pseudomonas putida* (Diaz-Salazar et al., 2017; Liu et al., 2017), and *P. aeruginosa* (de la Fuente-Nunez et al., 2014; Xu et al., 2016). The deletion of both (p)ppGpp synthetases, RelA and SpoT, reduced the ability of *P. aeruginosa* to form biofilms (de la Fuente-Nunez et al., 2014), exhibit swarming behavior, and express its host cell-targeting type III secretion system (Xu et al., 2016). These findings prompted us to test whether PaSAH is involved in the regulation of biofilm formation or motility by *P. aeruginosa*.

First, we compared the growth kinetics of wild-type *P. aeruginosa* PAO1 with that of a strain lacking PaSAH (Δ SAH). Neither in rich medium (nutrient broth) nor in chemically defined M9 supplemented with 20% (w/v) glucose did we observe differences in growth between the wild-type and Δ SAH strain (Figure 4a). Next, we analyzed the impact of PaSAH on swimming, swarming, and twitching motility (Figures 4b and S5a–c). Again, no differences between the wild-type PAO1 and the Δ SAH strain for any of these types of motility were observed. We then studied the impact of PaSAH on the

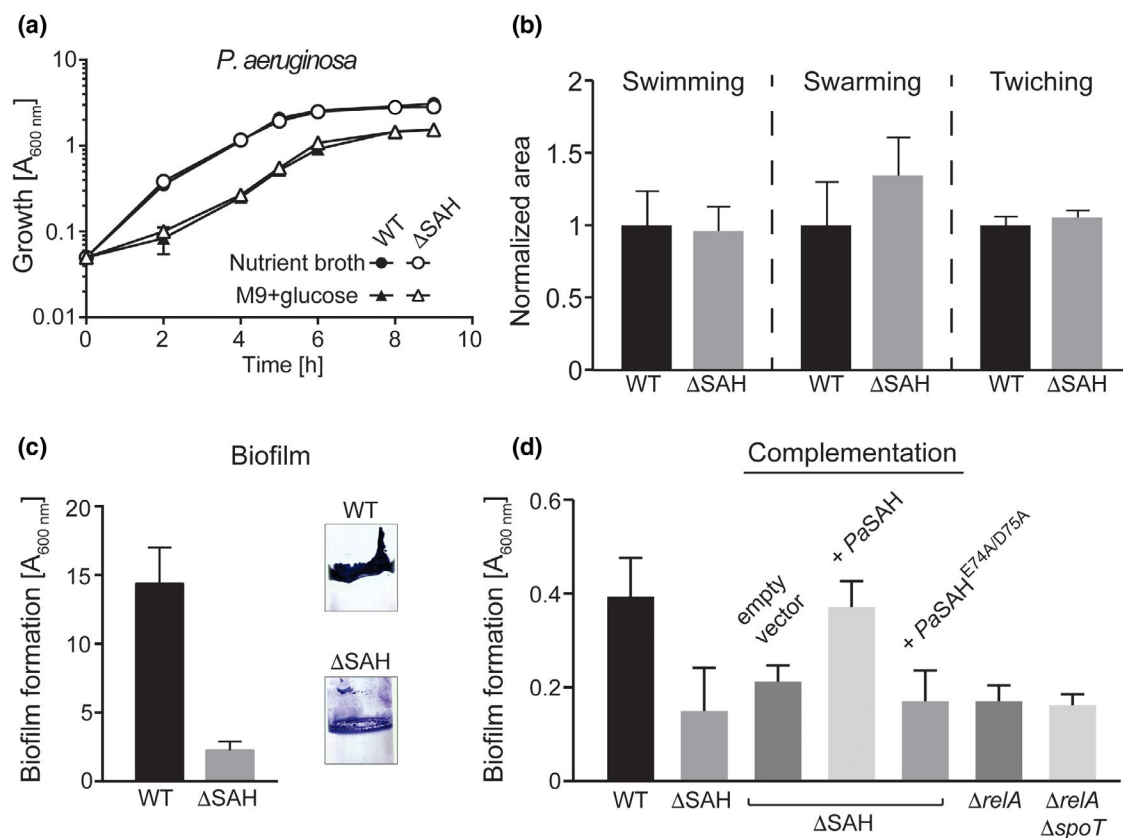


FIGURE 4 PaSAH does not affect planktonic growth but biofilm formation of *P. aeruginosa*. (a) Growth of *P. aeruginosa* PAO1 wild-type (WT, filled symbols) and a PaSAH-deficient strain (Δ SAH, empty symbols) in rich (nutrient broth, circles) and chemically defined medium (M9 supplemented with 20% glucose, triangles). (b) Swimming, swarming and twitching behavior of *P. aeruginosa* PAO1 wild-type and Δ SAH strains. Error bars indicate the SD of $n = 3$ biological replicates each consisting of $n = 3$ technical replicates. Data were normalized to the wild-type strains. (c) Biofilm formation of *P. aeruginosa* PAO1 wild-type and Δ SAH strains was monitored in glass tubes after 16 hr of stasis. Data represent mean $\pm$ SD of $n = 3$ independent samples. The insets depict representative images of the staining with crystal violet solution. (d) Biofilm formation of *P. aeruginosa* PAO1 strains was assayed in 24-well polystyrene microtiter plates after 16 hr of stasis. Complementation experiments were implemented using the mini-CTX system. Data represent mean $\pm$ SD of $n = 3$ independent samples

biofilm-forming properties of *P. aeruginosa*. To do so, the wild-type and the Δ SAH strains of PAO1 were inoculated in nutrient broth and grown at 37°C for 16 hr of stasis, after which the formed biofilms were stained with crystal violet and the amount of dye bound by the biofilm matrix quantified photometrically. In the Δ SAH strain, the amount of biofilm was significantly reduced, implying a functional role of *PaSAH* in biofilm formation (Figure 4c).

We asked whether the lack of the enzymatic, that is, (p)ppGpp-hydrolyzing activity of *PaSAH*, was underlying this inhibition of biofilm formation. To address this question, we introduced a gene encoding either native *PaSAH* or a variant, in which the catalytically essential HD4 motif (Figure 1b) was mutated (*PaSAH*^{E74A/D75A}), into the genome of the biofilm-defective PAO1 Δ SAH strain. When assaying the biofilm forming capability of those strains, the Δ SAH strain complemented with native *PaSAH* displayed a full recovery in its biofilm forming capacity (Figure 4d). However, introduction of *PaSAH*^{E74A/D75A} into Δ SAH resulted in a strain still impaired in biofilm formation (Figure 4d). Western blots using extracts from the complemented strains demonstrate that both native *PaSAH* and *PaSAH*^{E74A/D75A} were present at similar levels (Figure S5d). As a control for the implication of (p)ppGpp, and to reconcile observations from earlier studies (de la Fuente-Nunez et al., 2014; Xu et al., 2016), we constructed PAO1 strains lacking the synthetase RelA (Δ relA) or both RelA and SpoT synthetases (Δ relA Δ spoT) generating a ppGpp⁰ strain. Both of these PAO1 strains were similarly impaired in biofilm formation as the Δ SAH strain (Figure 4d).

These experiments suggest that the (p)ppGpp-degrading activity of *PaSAH* is involved in effective biofilm formation by *P. aeruginosa* PAO1.

2.5 | *PaSAH* confers resistance against killing by the Tas1 toxin

Our structural analysis of substrate binding to *PaSAH* revealed a less tight coordination of the guanine base of ppGpp than observed in the structures of the long RSH enzymes Rel_{Th} and Rel_{seq}, which might enable *PaSAH* to accept other substrates. Several recent studies indicate that some bacteria possess enzymes, for example, toxSAS, capable of (p)ppApp synthesis through pyrophosphorylation of ADP or ATP at the 3'-OH group (Ahmad et al., 2019; Bruhn-Olszewska et al., 2018; Sobala et al., 2019; Jimmy et al., 2020). These (p)ppApp nucleotides differ from (p)ppGpp only by the presence of an adenine nucleobase instead of guanine. The enzyme HsMESH1 was demonstrated to possess ppApp-degrading activity (Jimmy et al., 2020; Potrykus et al., 2020). In a structure of HsMESH1 bound to NADPH (PDB: 5VXA (Ding et al., 2020)), the dinucleotides' adenine moiety resided in the same position as the guanine of (p)ppGpp in our model of substrate-bound *PaSAH*, suggesting that *PaSAH* might similarly exhibit substrate promiscuity (Figure S6a,b). Consequently, we tested the activity of *PaSAH* toward (p)ppApp and found that both ppApp and pppApp are degraded with approximately 5- to 10-fold higher maximal velocity than their ppGpp and pppGpp counterparts (Figures 5a and S6c).

In the *P. aeruginosa* strain PA14, (p)ppApp synthesis activity has been demonstrated for the T6SS effector protein Tas1, which upon injection into target bacterial cells, rapidly synthesizes (p)ppApp, but not (p)ppGpp (Ahmad et al., 2019). The Tas1-catalyzed accumulation of (p)ppApp results in a concomitant depletion of ATP and dysregulation of essential metabolic pathways, ultimately causing cell death. Self-protection of PA14 against the Tas1 toxin is achieved via its cognate immunity protein Tis1 that, upon binding Tas1, physically blocks its active site (Ahmad et al., 2019). Since *PaSAH* displays high hydrolytic activity for (p)ppApp, we hypothesized that it might provide enzymatic immunity against Tas1 through degradation of Tas1-produced (p)ppApp.

To assess this, we measured the growth of *E. coli* cells expressing the Tas1 toxin in presence of Tis1, *PaSAH* or *PaSAH*^{E74A/D75A}. Co-expression with *PaSAH* resulted in a marked increase of *E. coli* growth compared to Tas1 alone, though, this effect was intermediate to Tas1 and Tas1/Tis1-expressing cells (Figure 5b). By contrast, *PaSAH*^{E74A/D75A} was unable to restore *E. coli* growth indicating that *PaSAH* is detoxifying Tas1 enzymatically by (p)ppApp degradation. We then quantified the impact of *PaSAH* on cell viability of *E. coli* expressing Tas1 in the presence of Tis1 or *PaSAH* by determining the number of colony-forming units before and 1 hr after Tas1 toxin induction. Similar to our growth rate experiments, *PaSAH* conferred moderate protection, reducing the Tas1-dependent loss in cell viability by approximately 1,000-fold compared to cells expressing the toxin in isolation. This protective effect is ~10-fold lower than the Tis1-expressing cells, which did not show any reduction in viability compared to a control strain (Figure 5c).

Having established a protective effect of *PaSAH* against Tas1 in a heterologous system, we probed whether it may also protect against Tas1 during interbacterial competition. The *P. aeruginosa* strains PA14 and PAO1 both harbor an SAH homolog (PA5338 and PA0431, respectively), however, PA14 has a significant Tas1-dependent growth advantage over PAO1 because the latter strain lacks the Tas1/Tis1 effector-immunity pair (Ahmad et al., 2019). Co-cultivation of a T6SS-active strain of PA14 with a *PaSAH*-deficient strain of PAO1 (Δ SAH) resulted in a 12-fold increase in competitive index relative to a competition involving the PAO1 parent strain, suggesting that the Δ SAH mutant strain is more susceptible to T6SS-delivered Tas1 (Figure 5d). Plasmid-borne expression of *PaSAH*, but not *PaSAH*^{E74A/D75A}, in the Δ SAH recipient strain abolished the Tas1-dependent fitness advantage of PA14 over PAO1. Competition of a PA14 donor strain harboring a Tas1-inactivating mutation (E382A) with PAO1 recipients did not reveal any *PaSAH*-dependent fitness advantages demonstrating that *PaSAH* specifically provides protection against Tas1 and not other T6SS effectors (Figure 5d). We also noted that changing the ratio of PAO1 recipient to PA14 donor cells in favor of the donor, while overall resulting in less viability of PAO1, reduced the protective effect granted by *PaSAH* (Figure S7). This likely occurs because an increasing number of PA14 cells coincides with more frequent Tas1-injection events upon which the number of *PaSAH* copies in PAO1 cells becomes insufficient to overcome the (p)ppApp-induced toxicity.

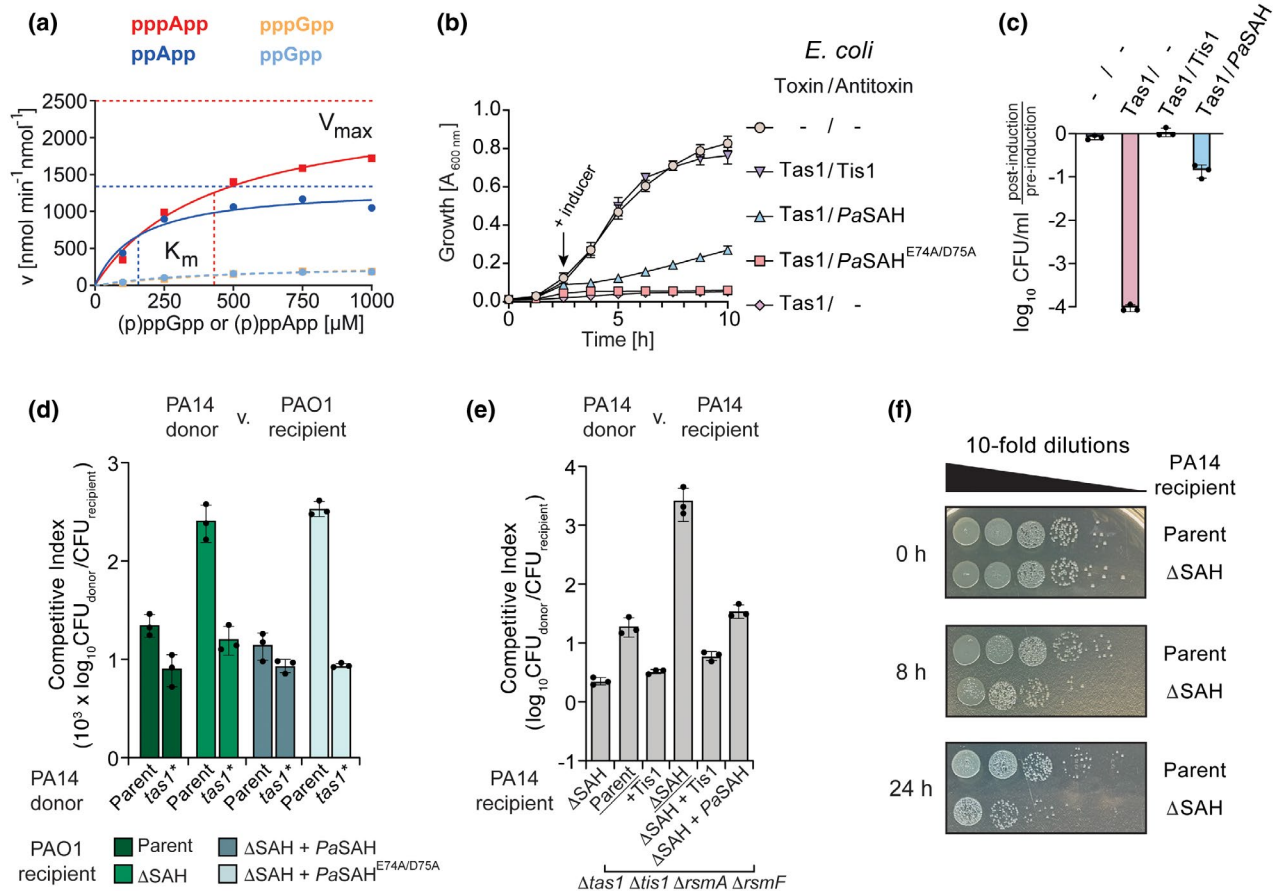


FIGURE 5 PaSAH degrades (p)ppApp and reduces the toxicity of the (p)ppApp synthetase Tas1. (a) Velocity (v)/substrate (S) characteristic of ppApp (blue circles) and pppApp (red squares) hydrolysis by PaSAH in presence of 2.5 mM MnCl_2 . Data were fitted according to the Michaelis–Menten equation. Vertical and horizontal dashed lines indicate the Michaelis–Menten constants (K_m) and maximal velocities ($V_{\max}$), respectively. K_m (in μM) and $V_{\max}$ (in $\text{nmol/min nmol}^{-1}$) were: ppApp (K_m : 155 ± 58 and $V_{\max}$: $1,336 \pm 132$) and pppApp (K_m : 429 ± 112 and $V_{\max}$: $2,507 \pm 274$). The velocities for hydrolysis of ppGpp (pale blue circles) and pppGpp (orange squares) are shown for direct comparison (see Figure 2d). (b) Growth of *E. coli* cells harboring plasmids that encode for the indicated inducible proteins in LB medium. The arrow indicates the addition of inducer after 2.5 hr. Data represent mean $\pm$ SD of $n = 3$ replicates. (c) Ratio of colony forming units (CFU)/ml postinduction and preinduction of the *E. coli* strains shown in panel (b) determined on LB agar without inducer. Data represent mean $\pm$ SD of $n = 3$ replicates. (d) Growth competition assay between *P. aeruginosa* PA14 donor and PAO1 recipient strains. PA14 harboring an inactive Tas1 (E382A variant, denoted tas1*) was used as a control. The parental backgrounds are ΔretS and $\Delta\text{rsmA} \Delta\text{rsmF}$ for PAO1 and PA14, respectively; both of these mutations stimulate H1-T6SS activity (Goodman et al., 2004; Marden et al., 2013). The PAO1 parent had a significant T6SS-independent growth advantage over PA14, and therefore, all ratios for the competitive index were multiplied by 10^3 to better visualize the changes in PA14 CFU. Data represent mean $\pm$ SD of $n = 3$ replicates. (e) Growth competition assay between *P. aeruginosa* PA14 donor and recipient strains. The donor strain genotype is $\Delta\text{rsmA} \Delta\text{rsmF}$. All recipient strains, except the ΔSAH strain, were generated from a $\Delta\text{tas1} \Delta\text{tis1} \Delta\text{rsmA} \Delta\text{rsmF}$ parental strain. The competitive index denotes the $\log_{10}$ of the donor/recipient CFU ratio after co-cultivation of both strains for 24 hr. Data represent mean $\pm$ SD of $n = 3$ replicates. (f) Viability of *P. aeruginosa* PA14 recipient strains after co-cultivation with a PA14 donor at the indicated times, determined on LB agar without inducer. The PA14 donor genotype is $\Delta\text{rsmA} \Delta\text{rsmF}$. The recipient strains (underlined in panel (e)) were generated from a $\Delta\text{tas1} \Delta\text{tis1} \Delta\text{rsmA} \Delta\text{rsmF}$ parental strain. Data represent mean $\pm$ SD of $n = 3$ replicates

In order to directly compare the protective effects of Tis1, the cognate immunity protein to Tas1, and PaSAH, we conducted intraspecific competition assays using *P. aeruginosa* PA14 donor and recipient strains. The recipient strain deleted for the Tas1/Tis1 effector-immunity pair displayed a reduction in its competitive index after co-cultivation with the donor strain compared to a $\Delta\text{tas1} \Delta\text{tis1}$ recipient with plasmid-borne Tis1 expression (Figure 5e) consistent with earlier observations (Ahmad et al., 2019). The viability of this Tis1-containing strain was very similar to a recipient only lacking PaSAH (ΔSAH), likely

because the Tis1 present in the ΔSAH strain can still compensate for the Tas1 delivered by the donor. However, deletion of PaSAH in the $\Delta\text{tas1} \Delta\text{tis1}$ mutant background resulted in a 150-fold increase in the donor competitive index compared to the $\Delta\text{tas1} \Delta\text{tis1}$ parent (Figure 5e) and resulted in a massive loss in cell viability of the recipient strains (Figure 5f). Expression of individual Tis1 or PaSAH from plasmids improved the competitive fitness of the $\Delta\text{SAH} \Delta\text{tas1} \Delta\text{tis1}$ recipient strain, but neither provided protection to the same extent as the expression of Tis1 in the recipient lacking *tas1* and *tis1*.

Collectively, these results suggest that *PaSAH* reduces the toxicity of T6SS-delivered Tas1 in *P. aeruginosa* strains lacking Tis1 by enzymatically degrading (p)ppApp.

3 | DISCUSSION

3.1 | SAH proteins typify the minimal (p)ppGpp hydrolase domain

Small alarmone hydrolases (SAHs) belong to an enzyme class within the RelA-SpoT Homolog (RSH) protein family with limited structural characterization. As of now, the structures of two metazoan (Sun et al., 2010) and two bacterial SAHs are available (Midwest Center for Structural Genomics and this study). Comparison of the crystal structures of *PaSAH* with those of MESH1 (Sun et al., 2010) and the hydrolase domains of the Rel_{seq} (Hogg et al., 2004) and Rel_{th} (Tamman et al., 2020) evidences *PaSAH* as a minimal (p)ppGpp hydrolase domain that largely contains the motifs required for (p)ppGpp degradation (Atkinson et al., 2011; Steinchen & Bange, 2016).

We noted that SAH proteins lack an α -helix exclusively present at the N-terminus of long RSH proteins' hydrolase domains (Figures 1 and S1), which is rather remote from its catalytic cavity (Figure S8a). In the crystal structure of a truncated form of *B. subtilis* Rel, this helix is in close proximity to an interaction interface established between the two molecules of the putative homodimer (PDB: 6YXA (Pausch et al., 2020)). Hereby, the α -helical domain contained in the regulatory C-terminus of one monomer of Rel (Figure 1a) inserts into a cleft formed in between the hydrolase and synthetase domains of the second monomer (Figure S8b), a process thought to be of importance for intramolecular regulation of Rel's reciprocal (p)ppGpp degrading and synthesizing activities (Pausch et al., 2020). One might speculate that the N-terminal α -helix could partake in this regulation, although no strong interactions are established with the α -helical domain of Rel in the reported structure and further conformational rearrangements might have to occur (Figure S8c). The α -helix might also be relevant for the interactions of the other RSH, SpoT, with numerous proteins including the acyl carrier protein (Battesti & Bouveret, 2006, 2009), Rsd (Lee et al., 2018), components of nitrogen-related phosphotransferase systems (Ronneau et al., 2016, 2019) and the ribosomal biogenesis GTPase CgtA/ObgE (Jiang et al., 2007; Raskin et al., 2007; Wout et al., 2004). For most of these, the regulatory C-terminus of SpoT has been implicated as the major interaction site. A mechanism similar to the intramolecular regulation of Rel involving relocation of C-terminal domains to the hydrolase/synthetase entity is conceivable (Ronneau et al., 2019), which should either induce a conformational change within the hydrolase domain or sterically block the catalytic cavity resulting in altered enzymatic activity. Further investigation will be needed to determine if the first α -helix is involved in this particular mode of regulation.

3.2 | Catalytic mechanism of the *PaSAH* enzyme

PaSAH, MESH1 as well as Rel_{seq} and Rel_{th} harbor the amino acid residues critical for coordination of a divalent metal ion cofactor (i.e., HD motifs 2, 3, and the aspartate of HD5, Figure S1) in similar structural arrangements. Likewise, *PaSAH* exhibited a strong preference for Mn²⁺ as a cofactor for (p)ppGpp hydrolysis over Mg²⁺ in vitro, the same preference documented biochemically for *C. glutamicum* SAH (Ruwe et al., 2018), DmMESH1 and HsMESH1 (Sun et al., 2010) and the bifunctional Rel enzymes from *S. equisimilis* (Mechold et al., 2002), *Mycobacterium tuberculosis* (Avarbock et al., 2000), *B. subtilis* (Takada et al., 2020), *T. thermophilus* (Van Nerom et al., 2019), and *Staphylococcus aureus* (Yang et al., 2019). The concentration of Mn²⁺ in vivo is estimated to be in the low micromolar range (Foster et al., 2014) and thus, 1,000-fold less than the concentration necessary to promote hydrolytic activity in our in vitro assays (1–10 mM). Similar high concentrations of Mn²⁺ were required for the in vitro activity of other (p)ppGpp hydrolases (see above) despite the dissociation constant of Rel_{th}/Mn²⁺ was quantified with 420 nM (Van Nerom et al., 2019). It seems possible that the activity of *PaSAH* and other (p)ppGpp hydrolases observed in vitro would drastically overestimate their in vivo efficiency. Conversely, the high requirement for Mn²⁺ in vitro might also be reasoned by, for example, the ability of divalent cations to interact with nucleotides. In this regard, we observed a marked drop in velocity of hydrolysis by *PaSAH* at high concentrations of substrates (p)ppGpp and (p)ppApp in our enzyme kinetic experiments (Figure S9). Ruwe et al. observed a similar behavior for the *C. glutamicum* SAH and Rel proteins in presence of (p)ppGpp and attributed it to substrate inhibition by an unclear mechanism (Ruwe et al., 2018). We noted, however, that the degree of this presumed substrate inhibition correlated with the concentration of manganese ions present in the enzymatic reactions (Figure S9). Also, substrate inhibition was not apparent for *C. glutamicum* SAH when magnesium ions were employed as a cofactor (Ruwe et al., 2018). As a similar phenomenon had been observed for Rel from *M. tuberculosis* (Avarbock et al., 2000), we suspect that the drop in hydrolysis by *PaSAH* is simply caused by complex formation of the (p)ppGpp/(p)ppApp substrates with manganese ions (the dissociation constant of Mn/ATP may be used as proxy and is roughly 10 μ M (O'Sullivan & Cohn, 1966)) thereby decreasing the effective concentration of both the cofactor and the substrates. It remains to be determined whether the metal-controlled environment of the bacterial cytoplasm would nevertheless ensure optimal metalation of (p)ppGpp/(p)ppApp hydrolases in the living cell.

As the structure of *PaSAH* represents the apo-state of the enzyme, we approximated the position of the substrates in *PaSAH* by superimposition with the structures of ppGpp-bound Rel_{th} (Tamman et al., 2020) and GPX-bound Rel_{seq} (Hogg et al., 2004). Hence, rearrangements of amino acid side chains upon binding of substrate have to be considered. Despite this caveat, the mechanism of substrate binding and catalysis by *PaSAH* appears to be similar to that of the Rel_{th} and Rel_{seq} hydrolase domains although

some alterations are apparent. We infer from the model of substrate-bound PaSAH and our mutational analysis of active site-lining residues (Figure 3) that the nucleobase of a (p)ppGpp or (p)ppApp substrate would be sandwiched by the side chains of L30 and W143, the latter probably capable of establishing π - π interactions with the nucleobase and the former roughly occupying the position of an arginine residue commonly found in the HD1 motif of (p)ppGpp hydrolases (Figure 1b). It is unlikely that the (p)ppApp degrading activity of PaSAH would solely be attributable to the divergence in HD1 because HsMESH1, containing the canonical arginine, can hydrolyze ppApp (Jimmy et al., 2020; Potrykus et al., 2020). Surprisingly, mutational studies validated the HD1 arginine as indispensable for Rel_{Th}, Rel_{Seq}, and HsMESH1 hydrolase activity (Hogg et al., 2004; Sun et al., 2010; Tamman et al., 2020). Accommodation of the ribose moiety is practically identical between PaSAH and Rel_{Th}/Rel_{Seq} facilitated by conserved tyrosine and asparagine residues (Figure 3). This defines the direction of the phosphates present at the 5'-end toward nonconserved positively charged residues while the 3'-pyrophosphate moiety protrudes toward the manganese cofactor. In this position, priming of a water molecule for subsequent nucleophilic attack as postulated by Hilgenfeld and coworkers (Hogg et al., 2004) would be enabled by the aspartate/glutamate pair found in HD motif four strictly invariant in all hydrolase domains of the RSH family (Aravind & Koonin, 1998; Atkinson et al., 2011). To what extent, if at all, the discrepancy in HD1 of PaSAH and the shortened helix $\alpha 7$ (Figure 3c,d) might give rise to differences in its substrate spectrum is unclear.

3.3 | Functional role of SAH enzymes within the (p)ppGpp framework

We observed a reduction in the biofilm forming ability of *P. aeruginosa* upon deletion of PaSAH, which could only be restored by complementation with the native but not an enzymatically inactive variant (PaSAH^{E74A/D75A}) of the protein. This suggests that the (p)ppGpp hydrolytic activity of PaSAH, instead of interactions between PaSAH and other proteins or factors in the cell, is specifically responsible for the observed defect in biofilm formation by *P. aeruginosa* upon deletion of PaSAH. Besides the presence of PaSAH, regulation of biofilm formation also relies on the long RSH enzymes RelA and SpoT (de la Fuente-Nunez et al., 2014; Xu et al., 2016). Conversely, deletion of the monofunctional (p)ppGpp synthetase RelA or of both RelA and SpoT synthetases, which generates a strain without any ppGpp (ppGpp⁰), had the same effect on biofilm formation of *P. aeruginosa* as a lack of PaSAH. One plausible explanation for this could be that PaSAH acts on biofilm formation outside of the (p)ppGpp metabolic network (RelA, SpoT), however, its enzymatic activity would still be required to fulfill this unknown function. In this regard, it is conceivable that PaSAH could, in addition to (p)ppGpp and (p)ppApp, also hydrolyze-related compounds, for example, NADPH as shown for HsMESH1 (Ding et al., 2020). Nevertheless, our data could suggest that deviations of the intracellular (p)ppGpp to an

elevated (Δ SAH) or reduced (Δ relA or Δ relA Δ spoT) niveau would be detrimental for biofilm formation. The importance of (p)ppGpp for bacterial physiology is best studied in *B. subtilis* ppGpp⁰ strains, which are auxotrophic for several amino acids due to the lack of regulation by the alarmones (Bittner et al., 2014; Kriel et al., 2012, 2014). To what degree (p)ppGpp would exert its opposing activities either directly, through altered RNA polymerase (RNAP) activity (reviewed in: (Dalebroux & Swanson, 2012; Gourse et al., 2018)) or by recruiting further proteins/factors via other second messengers like c-di-GMP or c-di-AMP is unclear (Jenal et al., 2017; Liu et al., 2017; Molina-Henares et al., 2017; Rao et al., 2010). The complexity of (p)ppGpp signaling could also explain why PaSAH-deficiency did neither influence the motility properties of *P. aeruginosa* nor alter its growth kinetics. Future studies on (p)ppGpp, taking into account the presence of PaSAH, might shed further light on SAH function.

3.4 | SAH enzymes as an enzymatic defense against (p)ppApp intoxication

A number of potential functional roles of (p)ppApp in bacteria have emerged. Older reports linked (p)ppApp to the initiation of sporulation in *B. subtilis* (Rhaese & Groscurth, 1976; Rhaese et al., 1977). It was also shown that (p)ppApp can bind near the *E. coli* RNAP active site and enhance transcription from the *rrnB* P1 promoter as opposed to (p)ppGpp, which allosterically inhibits RNAP activity (Bruhn-Olszewska et al., 2018). Furthermore, ppApp and ppGpp bind in highly similar fashion to PurF from *E. coli* and inhibit its activity with similar potency (Ahmad et al., 2019). Whether, if at all, (p)ppApp might exert a cellular regulatory role similar to (p)ppGpp is currently unknown.

In the current view, the occurrence of (p)ppApp is linked to toxic effects on the microbial cell (Ahmad et al., 2019; Jimmy et al., 2020). The T6SS effector protein Tas1 is a (p)ppApp synthetase whose toxicity is either mediated by (p)ppApp and/or through ATP depletion in the intoxicated cell (Ahmad et al., 2019). Tis1, the cognate immunity protein for Tas1, provides complete protection against Tas1 toxicity because it sterically blocks the toxin active site. Enzymatic abrogation of ppApp-mediated toxicity was demonstrated for the SAH enzymes present in the *Salmonella* phages PVP-SE1 and SSU5, *C. marina* ATfaRel and HsMESH1 (Jimmy et al., 2020). The expression of PaSAH provides a similar form of protection against the Tas1 toxin by acting as an enzymatic, nonsteric form of immunity. The lower levels of protection conferred by PaSAH relative to Tis1 are likely caused by the rate of (p)ppApp degradation by PaSAH being approximately 70-fold lower than the rate of (p)ppApp synthesis by Tas1 in vitro. This difference in rate is probably compounded by the fact that the synthesis of one molecule pppApp consumes two ATP molecules but its hydrolysis only replenishes one equivalent of ATP. Likely due to these differences in enzymatic efficiency, PaSAH can alleviate the bactericidal effect of Tas1 but not fully restore growth of *P. aeruginosa*. Likewise, the bifunctional (p)ppGpp hydrolase/synthetase SpoT present in *P. aeruginosa* exhibits only weak (p)

ppApp-degrading activity in vitro and upon overexpression is unable to reduce the toxic effects of (p)ppApp-producing Tas1 in vivo (Ahmad et al., 2019). In contrast, SpoT from *E. coli* could sufficiently restore growth when co-expressed with the otherwise growth-inhibiting toxSAS FaRel (Jimmy et al., 2020). Potential causes for this discrepancy include divergent (p)ppApp hydrolytic activities of the *E. coli* and *P. aeruginosa* SpoT enzymes, a lower (p)ppApp synthetic activity of FaRel compared to Tas1 or a different toxicity elicited by the ppApp and pppApp molecules synthesized by FaRel and Tas1, respectively.

The dual role of PaSAH in defense against foreign T6SS effectors and biofilm formation of *P. aeruginosa* exemplified in our study further expands the functional relevance of SAH enzymes for bacteria.

4 | MATERIALS AND METHODS

4.1 | Cloning and mutagenesis

All primers used in this study are listed in Table 2. For production of PaSAH in *E. coli*, PaSAH (PA0431) was amplified from *P. aeruginosa* PAO1 genomic DNA by polymerase chain reaction (PCR) using Phusion High-Fidelity DNA polymerase (NEB) according to the manufacturer's manual. The resulting PCR fragment was introduced into pET24d (Novagen) and encoded an N-terminal His₆-tag in frame with the DNA sequence of PaSAH. Variants of PaSAH were generated by overlapping PCR (Table 2).

For deletion of PaSAH from the *P. aeruginosa* PAO1 genome, a 500-bp fragment containing the upstream region and the first four codons of PaSAH and a 500-bp fragment containing the PaSAH stop codon were amplified by PCR using the primer pairs pPA0431.1/2 and pPA0431.3/4, respectively. The products were fused by overlapping PCR and cloned into the suicide vector pME3087 (Voisard et al., 1994) yielding plasmid pME3087ΔPaSAH. This plasmid, carried by *E. coli* DH5α, was then introduced into *P. aeruginosa* PAO1 by triparental mating, using the *E. coli* HB101 (pRK2013) helper strain (Sambrook et al., 1989) to generate a ΔSAH strain. Strains were selected as described (Ye et al., 1995) and successful deletion of PaSAH verified by PCR. For complementation of the PAO1 ΔSAH strain, PaSAH preceded by an optimized ribosome binding site (RBS) and encoding a product with an N-terminal His₆-tag was amplified by PCR. Site-directed mutagenesis yielding inactive PaSAH^{E74A/D75A} for complementation studies was introduced by PCR. Both variants were cloned into mini-CTX-plac (Hoang et al., 2000) and conjugated into the genome of *P. aeruginosa* PAO1 ΔSAH under control of the lac promoter at the CTX attP site by triparental mating.

In-frame deletions of *relA* (PA0934) and *spoT* (PA5338) and genetic modifications of *P. aeruginosa* PAO1 and PA14 for growth competition assays were completed similarly with some modifications. In-frame deletion constructs were cloned into pEXG2 suicide vectors using primers listed in Table 2 (primer names: PA0934_KO, PA5338_KO, PA0431_KO, and PA14_05600_KO). The vector was transferred into *P. aeruginosa* PAO1 and PA14 by conjugation using

E. coli SM10. Selection for allelic exchange was carried out as previously described (Hmelo et al., 2015). For complementation of strains in competition assays, the constructs for PaSAH for both PAO1 and PA14 or PaSAH^{E74A/D75A} for PAO1 were cloned into a pPSV39-CV vector (Castang et al., 2008) using primers PA0431_F_HcpRBS_SacI_1/PA14_05600_F_HcpRBS_SacI_1 and PA0431_R_XbaI_4/PA14_05600_R_XbaI_4 for PaSAH and additionally, PA0431_E74A_D75A_overlap_2 and PA0431_E74A_D75A_overlap_3 for PaSAH^{E74A/D75A}. Note that the reverse primer (PA0431_R_XbaI_4/PA14_05600_R_XbaI_4) does not contain a stop codon to express the VSV-G tag present in pPSV39-CV. The pPSV39-CV complementation vectors containing PaSAH or PaSAH^{E74A/D75A} were transformed into *E. coli* SM10 and subsequently introduced into *P. aeruginosa* PAO1 or PA14 ΔSAH strains via conjugative transfer.

4.2 | Protein production and purification

Native and varied PaSAH proteins were overproduced in *E. coli* BL21 (DE3) (NEB) carrying the corresponding plasmids. Cells were grown in lysogeny broth (LB) medium supplemented with 50 μg/ml kanamycin and 12.5 g/L D(+)-lactose-monohydrate for 20 hr at 30°C and harvested by centrifugation (3,500g, 20 min, 4°C). The cells were resuspended in lysis buffer (20 mM of HEPES-Na pH 7.5, 20 mM KCl, 250 mM NaCl, and 40 mM imidazole) and lysed by French Press (SLM Aminco) at 1,000 psi pressure. After removal of cell debris by centrifugation (47,850g, 20 min, 4°C), the supernatant was loaded on a 1-ml HisTrap column (GE Healthcare) equilibrated with 10 column volumes (CV) lysis buffer. After washing with 10 CV of lysis buffer, PaSAH was eluted with 5 CV elution buffer (lysis buffer containing 500 mM imidazole). The protein was concentrated (Amicon Ultracel-10K (Millipore)) and applied to size-exclusion chromatography (SEC) on a HiLoad 26/600 Superdex 200 pg column (GE Healthcare) equilibrated with SEC buffer (20 mM of HEPES-Na, pH 7.5, 20 mM KCl, and 200 mM NaCl). Fractions containing PaSAH were pooled, concentrated (Amicon Ultracel-10K (Millipore)), deep-frozen in liquid nitrogen and stored at -80°C. Protein concentration was determined by a spectrophotometer (NanoDrop Lite, Thermo Scientific).

4.3 | Crystallization and structure determination

For crystallization trials, PaSAH was purified in SEC buffer supplemented with 20 mM MgCl₂. Crystallization was conducted at 20°C by sitting drop vapor diffusion method in SWISSCI MRC 2-well plates (Jena Bioscience) with a reservoir volume of 50 μl. 0.5 μl of PaSAH concentrated to 1 mM was mixed with 0.5 μl of a solution containing 2.0 M sodium formate and 0.1 M sodium acetate pH 4.6. After three days, 0.5 μl of a cryo-protecting solution containing mother liquor supplemented with 20% (v/v) glycerol was added to the drop, crystals harvested and flash-frozen in liquid nitrogen. Diffraction data were collected at the European Synchrotron Radiation Facility (ESRF) Grenoble, France, at beamline ID29 under laminar nitrogen

TABLE 2 Oligonucleotides

Primer	Sequence	Purpose	
<i>PaSAH</i> -6H-Nco-F	TTAACCATGGGCCATCACCATCACCAT CACAGCGCTCCCACGATGTCC	N-terminal <i>His₆</i> - <i>PaSAH</i>	In vitro assays
<i>PaSAH</i> -Xho-R	TTAACTCGAGTCAGTAGTACTGGCGGTAG		
<i>PaSAH</i> -H70A/D71A-F	GATCGCGTCTGGCCGCCACCCTGGAAG	N-terminal <i>His₆</i> - <i>PaSAH</i> ^{H70A/}	
<i>PaSAH</i> -H70A/D71A-R	CTTCCAGGGTGGCGGCCAGGACCGCGATC	D71A	
<i>PaSAH</i> -E74A/D75A-F	GCACGACACCCTGGCAGCCACGGCGAC	N-terminal <i>His₆</i> - <i>PaSAH</i> ^{E74A/D75A}	
<i>PaSAH</i> -E74A/D75A-R	GTCGCCGTGGCTGCCAGGGTGTCTGTC		
<i>PaSAH</i> -L30G-F	CAGACCGGCAGCGGCTCG	N-terminal <i>His₆</i> - <i>PaSAH</i> ^{L30G}	
<i>PaSAH</i> -L30G-R	CGAGCCGCTGCCGGTCTG		
<i>PaSAH</i> -S31G-F	CAGACCCTCGGCGGCTCG	N-terminal <i>His₆</i> - <i>PaSAH</i> ^{S31G}	
<i>PaSAH</i> -S31G-R	CGAGCCGCCGAGGGTCTG		
<i>PaSAH</i> -K100A-F	CCTGAGCGCGCGCTGG	N-terminal <i>His₆</i> - <i>PaSAH</i> ^{K100A}	
<i>PaSAH</i> -K100A-R	CCACGCGCGCGCTCAGG		
<i>PaSAH</i> -K107A-F	GCCCCGCGCGTTCCCTCG	N-terminal <i>His₆</i> - <i>PaSAH</i> ^{K107A}	
<i>PaSAH</i> -K107A-R	CGAGGGAACGCGCGGGGC		
<i>PaSAH</i> -N135A-F	GCATCACCGCCTGCAGCC	N-terminal <i>His₆</i> - <i>PaSAH</i> ^{N135A}	
<i>PaSAH</i> -N135A-R	GGCTGCAGGGCGGTGATGC		
<i>PaSAH</i> -W143A-F	CGCAGACCGCAACCAGG	N-terminal <i>His₆</i> - <i>PaSAH</i> ^{W143A}	
<i>PaSAH</i> -W143A-R	CCTGGTTCGCGGTCTGCG		
<i>PaSAH</i> -K147A-F	CCAGGACGCGATCGCGAAC	N-terminal <i>His₆</i> - <i>PaSAH</i> ^{K147A}	
<i>PaSAH</i> -K147A-R	GTTGCGGATCGCGTCTG		
pPA0431.1	GGAATTCGGTCTGCAACATCGGCC	Deletion of <i>PaSAH</i> (PA0431) in <i>P.</i> <i>aeruginosa</i> PAO1	Behavioural assays
pPA0431.2	TCAGTAGTAGGGAGCGCTCATGCGAAC		
pPA0431.3	ATGAGCGCTCCCTACTACTGAAGTACCCGG		
pPA0431.4	CCCAAGCTTGGGTGGACCTCCGGATAG		
PA0934_KO_1_HindIII	TGTTAAGCTAAAGCTTCGGGCTGGGCAATTCTC	Deletion of <i>relA</i> (PA0934) in <i>P.</i> <i>aeruginosa</i> PAO1	
PA0934_KO_2_overlap	AACTCGAGCCGAAGCATGCTGAAGT ACCATCTTGCTACCCTTT		
PA0934_KO_3_overlap	TTCAGCATGCTTGCGGCTCGAGTTCT TGAGGCGAGCGGAAA		
PA0934_KO_4_XbaI	TGTTAAGCTATCTAGAACAACCTGCTGCGGCTCCT		
PA5338_KO_1_HindIII	TGTTAAGCTAAAGCTTCCTCAAGCGATCTTCCG	Deletion of <i>spoT</i> (PA5338) in <i>P.</i> <i>aeruginosa</i> PAO1	
PA5338_KO_2_overlap	AACTCGAGCCGAAGCATGCTGAAATGCCCGCAAGGGTTCA		
PA5338_KO_3_overlap	TTCAGCATGCTTGCGGCTCGAGTTCGAGCTGACCCGCTTTT		
PA5338_KO_4_XbaI	TGTTAAGCTATCTAGACGGCGAGGAAAATCACGC		
<i>PaSAH</i> -comp-BamHI-F	GTACGGGATCCTAACAGGAGGAATTAACCATGGGCCATCACC ATCACCATCACAGCGCTCCCACGATGTCCACG	Complementation of <i>PaSAH</i> and <i>PaSAH</i> ^{E74A/D75A} in <i>P.</i> <i>aeruginosa</i> PAO1	
<i>PaSAH</i> -comp-E74A/D75A-F	GTCCTGCACGACACCCTGGCAGCCACGGCGACCTCACC		
<i>PaSAH</i> -comp-E74A/D75A-R	GGTGAGGTGCGCGTGGCTGCCAGGGTGTCTGTCAGGAC		
<i>PaSAH</i> -comp-SacI-R	CAGGGTGGCCGCCAGGACCGCGATCTGCAGGGTT		
PA0431_KO_1_SacI	TGTTAAGCTAGAGCTCGTGGTCTGCAACATCGGCCAC	Deletion of <i>PaSAH</i> (PA0431) in <i>P.</i> <i>aeruginosa</i> PAO1	Viability and competition assays
PA0431_KO_2_overlap	TTCAGCATGCTTGCGGCTCGAGTTCGTGGGAGCGCTCATGCGAA		
PA0431_KO_3_overlap	AACTCGAGCCGAAGCATGCTGAACGCCAGTACTAC TGAAGTACCCG		
PA0431_KO_4_XbaI	TCAATCAGTATCTAGAGGGTGGACCTCCGGATAGGC		

(Continues)

TABLE 2 (Continued)

Primer	Sequence	Purpose
PA0431_F_HcpRBS_SacI_1	TGTTAAGCTAGAGCTCACGGGAGGAAAGATGAGCGCTC CCACGATGTCC	Complementation of <i>PaSAH</i> and <i>PaSAH</i> ^{E74A/D75A} in <i>E.</i> <i>coli</i> and <i>P. aeruginosa</i> PAO1
PA0431_E74A_D75A_ overlap_2	GAGGTCGCCGTTGCGGCCAGGGTGTCTGTCAGGACCG	
PA0431_E74A_D75A_ overlap_3	GCACGACACCCTGGCCGCAACGGCGACCTACCCGAGG	
PA0431_R_XbaI_4	TCAATCAGTATCTAGAGTAGTACTGGCGGTAGTGCTCG	Deletion of <i>PaSAH</i> (PA14_05600) in <i>P.</i> <i>aeruginosa</i> PA14
PA14_05600_KO_1_SacI	TCAATCAGTAGAGCTCAACATCGGCCACTTCGACAACGAG	
PA14_05600_KO_2_overlap	TTCAGCATGCTTGCGGCTCGAGTTGGGAGCGCTCATGCGAACG	
PA14_05600_KO_3_overlap	AACTCGAGCCGCAAGCATGCTGAATACTACTG AAGTACCCGGCGCTAGT	
PA14_05600_KO_4_XbaI	TCAATCAGTATCTAGATGGGGGTGGACTTCCGGATAG	Complementation of <i>PaSAH</i> (PA14_05600) in <i>P.</i> <i>aeruginosa</i> PA14
PA14_05600_F_HcpRBS_ SacI_1	TCAATCAGTAGAGCTCACGGGAGGAAAGAT GAGCGCTCCCACGATGTCC	
PA14_05600_R_XbaI_4	TCAATCAGTATCTAGAGTAGTACTGGCGGTAGTGCTCG	

flow at 100K (Oxford Cryostream 700 Series) with a Pilatus 6M detector at 1.85 Å. Data were processed with XDS (Kabsch, 2010) and the structure determined with Phenix-implemented Phaser (Adams et al., 2010) using the hydrolase domain of Rel_{Seq} (PDB: 1VJ7 chain B, amino acids 1–200, (Hogg et al., 2004)) as a search model for molecular replacement. The structure was manually built in Coot (Emsley & Cowtan, 2004) and refined with Phenix refine (Adams et al., 2010). Figures were prepared with PyMOL (www.pymol.org).

4.4 | Preparation of (p)ppGpp and (p)ppApp

Alarmones ppGpp and pppGpp were produced essentially as described previously (Pausch et al., 2018; Steinchen et al., 2015). In brief, 5 μM *B. subtilis* RelQ produced and purified as described (Steinchen et al., 2015) were incubated in a buffer containing 10 mM HEPES-Na, pH 7.5, 10 mM KCl, 10 mM MgCl₂, and 100 mM NaCl together with 10 mM ATP and 10 mM of GDP or GTP at 37°C. After 3 hr an equal volume of chloroform was added to stop the reaction followed by mixing and centrifugation (17,300g, 5 min, 4°C). The aqueous phase was subjected to anion-exchange chromatography using a Resource Q 6-ml column (GE Healthcare) at a flow rate of 6 ml/min and the nucleotides eluted with a gradient of LiCl. (p)ppGpp-containing fractions were pooled and lithium chloride powder added to a final concentration of 1 M. After addition of four volumes ethanol, incubation at –20°C for 20 min and centrifugation (5,000g, 20 min, 4°C), the resulting pellet was washed with ethanol, dried, and stored at –20°C. Nucleotides ppApp and pppApp were produced by incubating 10 μM *S. aureus* RelP produced and purified as described (Steinchen et al., 2018) in a buffer containing 10 mM HEPES-Na, pH 7.5, 10 mM KCl, 10 mM MgCl₂, and 100 mM NaCl together with 10 mM ATP and 10 mM ADP (for ppApp) or only 10 mM ATP (for ppApp) at 37°C for 24 hr. The reactions were stopped with chloroform and the (p)ppApp purified as described above for (p)ppGpp.

4.5 | Determination of (p)ppGpp hydrolase activity

The enzymatic activity of *PaSAH* or its variants was assayed by incubating 0.5 μM *PaSAH* together with 1 mM substrate (ppGpp or pppGpp) in SEC buffer supplemented with 1 mM DTT at 37°C. In experiments probing the effect of manganese and magnesium ions on *PaSAH* activity, MnCl₂ or MgCl₂ were added at a concentration of 0.01/0.025/0.1/0.25/1/2.5/10 mM to the buffer. In all other non-kinetic experiments, MnCl₂ was used in a concentration of 2.5 mM. After 10 min, the reactions were stopped by adding two volume parts chloroform, thoroughly mixed and heated at 95°C for 15 s each and immediately flash-frozen in liquid nitrogen. Immediately after thawing, the samples were centrifuged (17,300g, 30 min, 4°C) and the aqueous phase used for HPLC analysis on an Agilent 1,100 Series system (Agilent Technologies) equipped with a C18 column (EC 250/4.6 Nucleodur HTec 3 μM; Macherey-Nagel). Analytes were isocratically eluted with a buffer containing 50 mM KH₂PO₄, 50 mM K₂HPO₄, 10 mM tetrapentylammonium bromide and 15% (v/v) acetonitrile and detected at 260 nm wavelength in agreement with standards.

4.6 | Kinetic analysis of (p)ppGpp and (p)ppApp hydrolase activity

For kinetic analyses, 0.1 μM *PaSAH* was incubated in presence of 0.1/0.25/0.5/0.75/1/2.5 mM substrate (ppGpp or pppGpp) in SEC buffer supplemented with 1 mM DTT and 1 or 2.5 mM MnCl₂ at 37°C. Samples were withdrawn after 2.5/5/7.5/10 min, quenched, and quantified by HPLC as described above. Data were analyzed with GraphPad Prism version 6.04 for Windows, (GraphPad Software, San Diego, California, USA). The velocity of (p)ppGpp hydrolysis was obtained by linear regression of the amount of the products GDP or

GTP after different incubation times and the enzyme kinetic parameters (K_m and $V_{max} \pm SD$) obtained from the fit of the v/S characteristic according to the equation $v = V_{max} S / (K_m + S)$. Kinetic analyses of ppApp and pppApp hydrolysis by PaSAH was done as described for (p)ppGpp but owing to the higher velocity contained only 0.01 μM PaSAH. The velocities of (p)ppApp hydrolysis were obtained by linear regression of the amount of the products ADP or ATP after different incubation times and the enzyme kinetic parameters (K_m and $V_{max} \pm SD$) obtained from the fit of the v/S characteristic according to the equation $v = V_{max} S / (K_m + S)$.

4.7 | Analysis of *P. aeruginosa* swimming/swarming/twitching behavior and biofilm formation

Growth kinetics of *P. aeruginosa* PAO1 were performed in Nutrient Yeast Broth (NYB) (Stanisich & Holloway, 1972) or in M9 medium (Sambrook et al., 1989) supplemented 20% (w/v) glucose in 100 ml Erlenmeyer flasks filled with 20 ml of medium, with shaking at 180 rpm and at 37°C.

Swimming assays were carried out on agar plates containing 10 g/L tryptone, 5 g/L NaCl, and 3 g/L agar. Standardized overnight culture (0.5 μl with an A_{600nm} of 1.0) was injected into the surface of the agar and plates were incubated at 30°C overnight.

Swarm assays were performed in plates consisting of 0.5% (w/v) Difco bacto agar with 8 g/L Difco nutrient broth and 5 g/L glucose. Cells were inoculated onto swarm plates with standardized overnight cultures (5 μl with an A_{600nm} of 1.0) and incubated overnight at 37°C.

Twitch assays were carried out on 1% (w/v) LB agar plates and bacteria were inoculated by picking a colony using a sterile tip and stabbing to the bottom of the plates, which were incubated at 37°C overnight. The next day, the agar was peeled off the plate, and cells were stained with crystal violet for visualization. Pictures were taken from each motility plate and the ImageJ software (NIH) was used to determine the area of the plate surface covered by the bacteria. At least three independent experiments were performed for each experiment and every experiment consisted of three replicates.

Biofilm formation was performed in NYB using glass tubes or 24-well polystyrene microtiter plates, as indicated in the figures. Biofilms assays were inoculated with standardized overnight cultures (A_{600nm} of 0.1), incubated at 37°C under static conditions for 16 hr, and stained with 0.1% (w/v) crystal violet solution. The dye bound, which is proportional to the biofilm produced, was solubilized with 96% (v/v) ethanol and the absorption at 600 nm wavelength quantified with a Synergy H1 plate reader (Biotek). Data represent mean $\pm SD$ of $n = 3$ independent samples.

4.8 | Western blot analysis

SDS-PAGE and western blotting were performed as described previously (Hachani et al., 2014). Briefly, proteins were resolved 12% gels

using the Mini-PROTEAN system (Bio-Rad) by electrophoresis and transferred to nitrocellulose membrane (GE Healthcare) by blotting. Membranes were blocked with 5% (w/v) milk powder (Sigma Aldrich) dissolved in TBST buffer (20 mM Tris-Cl, 150 mM NaCl, 0.1% (v/v) Tween-20, pH 8.0) prior to incubation with the primary monoclonal anti-His₆ antibody (Sigma Aldrich) at a 1:100 dilution. Membranes were washed with TBST before incubation with HRP-conjugated secondary antibodies (Sigma Aldrich) at a dilution of 1:5,000. Blots were developed with the Luminata Forte Western HRP substrate (Millipore) according to the manufacturer's manual and detected with a LAS-3000 Luminescent Image Analyzer (Fujifilm Life Science).

4.9 | *E. coli* growth curves and Tas1 toxicity experiments

E. coli XL-1 blue was co-transformed with pSCrhaB2-CV or pSCrhaB2-CV expressing *P. aeruginosa* Tas1 and the pPSV39-CV vector or pPSV39-CV expressing *P. aeruginosa* Tis1, PaSAH or PaSAH^{E74A/D75A} (this study and (Ahmad et al., 2019)).

For growth curves, overnight cultures grown in LB medium (37°C, 200 rpm) were diluted 1:100 in 200 μl of fresh LB medium containing selection in a 96-well plate (see above). The plate was placed into a Synergy 4 (BioTek) plate reader set at 37°C with medium shaking and the A_{600nm} of the cultures were measured every 30 min for the duration of the assay. Inducers were added after 2.5 hr once the strain containing empty vectors reached mid-log optical density (A_{600nm} of 0.2).

For CFU enumeration experiments, overnight cultures grown in LB medium (37°C, 200 rpm) were used to inoculate 50 ml LB and allowed to grow to an A_{600nm} of 0.4. Samples were withdrawn from these cultures, diluted to 10^{-6} using 10-fold serial dilutions, and plated on noninducer containing agar to enumerate the CFUs before induction. Inducer was added to each culture followed by further incubation for 1 hr, after which samples for counting of postinduction CFUs were withdrawn, again diluted and plated on noninducer containing LB agar plates. CFUs were counted following 24 hr incubation on LB agar plates at 37°C.

4.10 | *P. aeruginosa* growth competition assays

For inter-strain competitions, *P. aeruginosa* PA14 donor and PAO1 recipient strains were grown in 2 ml LB medium overnight. One milliliter of each stationary-phase culture was pelleted at 7,600g for 3 min and washed twice with 1 ml of fresh LB medium. The resuspended cells were diluted to an A_{600nm} of 0.4 (approximately 3×10^5 colony-forming units (CFUs)) and mixed at a 4:3 (v/v) (PAO1:PA14) ratio. For intraspecific competitions with *P. aeruginosa* PA14 donor and recipient strains, stationary-phase cultures were directly mixed in a 1:1 (v/v) ratio except for the viability assays shown in Figure 5f, where PA14 donor and recipient strains (approximately 3×10^5 CFU) were mixed in a 10:1 (donor:recipient) ratio. PA14 donor strains contain a chromosomal *lacZ*

reporter and tetracycline-resistance cassette to permit blue-white screening on X-gal and selection on LB with tetracycline. Mixed cultures were diluted and plated on 40 µg/ml X-gal to enumerate exact starting ratio of donor and recipient. A 10 µl of each mixture were spotted on a 0.45 µm nitrocellulose membrane overlaid on a 3% (w/v) LB agar plate and incubated face up at 37°C for 9 hr (inter-strain competition) or 8 hr and 24 hr (intraspecific competition). Competition spots were harvested by resuspending each in 200 µl of LB followed by serial dilution and plating on LB with 40 µg/ml X-gal or 75 µg/ml tetracycline. Final CFUs of donor and recipient strains were normalized to initial CFUs for both strains. Images of plates were taken 24 hr after plating.

ACKNOWLEDGMENTS

We thank Anja Paulus for assistance in the early stage of the project. We thank the European Synchrotron Radiation Facility (ESRF), Grenoble, France for support during data collection. We thank Vera Bettenworth for comments on the manuscript. S.A. was supported by an Ontario Graduate Scholarship. G.B. thanks the SPP1879 priority program of the Deutsche Forschungsgemeinschaft (DFG) for financial support. J.C.W. acknowledges funding from the Canadian Institutes of Health Research (PJT-156129) and the David Braley Centre for Antibiotic Discovery.

CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

W.S., S.A., A.F., J.C.W., and G.B. designed research. W.S., S.A., M.V., K.E., and M.M. performed research. W.S., S.A., M.V., K.E., F.A., and M.L. analyzed data. W.S., S.A., A.F., J.C.W., and G.B. prepared the manuscript with input from the other authors. All authors commented on the manuscript.

DATA AVAILABILITY STATEMENT

Atomic coordinates and structure factors *P. aeruginosa* SAH were deposited in the Protein Data Bank (PDB) under accession code 6YVC.

ORCID

Martina Valentini  <https://orcid.org/0000-0002-1948-8339>

Alain Filloux  <https://orcid.org/0000-0003-1307-0289>

John C. Whitney  <https://orcid.org/0000-0002-4517-8836>

Gert Bange  <https://orcid.org/0000-0002-7826-0932>

REFERENCES

- Adams, P.D., Afonine, P.V., Bunkoczi, G., Chen, V.B., Davis, I.W., Echols, N., et al. (2010) PHENIX: A comprehensive Python-based system for macromolecular structure solution. *Acta crystallographica Section D, Biological Crystallography*, 66, 213–221.
- Ahmad, S., Wang, B., Walker, M.D., Tran, H.R., Stogios, P.J., Savchenko, A., et al. (2019) An interbacterial toxin inhibits target cell growth by synthesizing (p)ppApp. *Nature*, 575, 674–678. <https://doi.org/10.1038/s41586-019-1735-9>
- Aravind, L. & Koonin, E.V. (1998) The HD domain defines a new superfamily of metal-dependent phosphohydrolases. *Trends in Biochemical Sciences*, 23, 469–472. [https://doi.org/10.1016/S0968-0004\(98\)01293-6](https://doi.org/10.1016/S0968-0004(98)01293-6)
- Arenz, S., Abdelshahid, M., Sohmen, D., Payoe, R., Starosta, A.L., Berninghausen, O., et al. (2016) The stringent factor RelA adopts an open conformation on the ribosome to stimulate ppGpp synthesis. *Nucleic Acids Research*, 44, 6471–6481. <https://doi.org/10.1093/nar/gkw470>
- Atkinson, G.C., Tenson, T. & Haurlyuk, V. (2011) The RelA/SpoT homolog (RSH) superfamily: Distribution and functional evolution of ppGpp synthetases and hydrolases across the tree of life. *PLoS One*, 6, e23479. <https://doi.org/10.1371/journal.pone.0023479>
- Avarbock, D., Avarbock, A. & Rubin, H. (2000) Differential regulation of opposing RelMtb activities by the aminoacylation state of a tRNA. ribosome.mRNA.RelMtb complex. *Biochemistry*, 39, 11640–11648.
- Battesti, A. & Bouveret, E. (2006) Acyl carrier protein/SpoT interaction, the switch linking SpoT-dependent stress response to fatty acid metabolism. *Molecular Microbiology*, 62, 1048–1063. <https://doi.org/10.1111/j.1365-2958.2006.05442.x>
- Battesti, A. & Bouveret, E. (2009) Bacteria possessing two RelA/SpoT-like proteins have evolved a specific stringent response involving the acyl carrier protein-SpoT interaction. *Journal of Bacteriology*, 191, 616–624. <https://doi.org/10.1128/JB.01195-08>
- Beljantseva, J., Kudrin, P., Andresen, L., Shingler, V., Atkinson, G.C., Tenson, T., et al. (2017) Negative allosteric regulation of Enterococcus faecalis small alarmone synthetase RelQ by single-stranded RNA. *Proceedings of the National Academy of Sciences of the United States of America*, 114, 3726–3731.
- Bittner, A.N., Kriel, A. & Wang, J.D. (2014) Lowering GTP level increases survival of amino acid starvation but slows growth rate for *Bacillus subtilis* cells lacking (p)ppGpp. *Journal of Bacteriology*, 196, 2067–2076. <https://doi.org/10.1128/JB.01471-14>
- Brown, A., Fernandez, I.S., Gordiyenko, Y. & Ramakrishnan, V. (2016) Ribosome-dependent activation of stringent control. *Nature*, 534, 277–280. <https://doi.org/10.1038/nature17675>
- Bruhn-Olszewska, B., Molodtsov, V., Sobala, M., Dylewski, M., Murakami, K.S., Cashel, M., et al. (2018) Structure-function comparisons of (p)ppApp vs (p)ppGpp for *Escherichia coli* RNA polymerase binding sites and for rrnB P1 promoter regulatory responses in vitro. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, 1861, 731–742. <https://doi.org/10.1016/j.bbagrm.2018.07.005>
- Castang, S., McManus, H.R., Turner, K.H. & Dove, S.L. (2008) H-NS family members function coordinately in an opportunistic pathogen. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 18947–18952. <https://doi.org/10.1073/pnas.0808215105>
- Costerton, J.W., Irvin, R.T. & Cheng, K.J. (1981) The bacterial glycocalyx in nature and disease. *Annual Review of Microbiology*, 35, 299–324. <https://doi.org/10.1146/annurev.mi.35.100181.001503>
- Dalebroux, Z.D. & Swanson, M.S. (2012) ppGpp: Magic beyond RNA polymerase. *Nature Reviews Microbiology*, 10, 203–212.
- de la Fuente-Nunez, C., Reffuveille, F., Haney, E.F., Straus, S.K. & Hancock, R.E. (2014) Broad-spectrum anti-biofilm peptide that targets a cellular stress response. *PLoS Pathogens*, 10, e1004152.
- Dedrick, R.M., Jacobs-Sera, D., Bustamante, C.A., Garlena, R.A., Mavrich, T.N., Pope, W.H., et al. (2017) Prophage-mediated defence against viral attack and viral counter-defence. *Nature Microbiology*, 2, 16251. <https://doi.org/10.1038/nmicrobiol.2016.251>
- Diaz-Salazar, C., Calero, P., Espinosa-Portero, R., Jimenez-Fernandez, A., Wirebrand, L., Velasco-Dominguez, M.G., et al. (2017) The stringent response promotes biofilm dispersal in *Pseudomonas putida*. *Scientific Reports*, 7, 18055. <https://doi.org/10.1038/s41598-017-18518-0>
- Ding, C.C., Rose, J., Sun, T., Wu, J., Chen, P.H., Lin, C.C., et al. (2020) MESH1 is a cytosolic NADPH phosphatase that regulates ferroptosis. *Nature Metabolism*, 2, 270–277. <https://doi.org/10.1038/s42255-020-0181-1>
- Emsley, P. & Cowtan, K. (2004) Coot: Model-building tools for molecular graphics. *Acta crystallographica Section D, Biological Crystallography*, 60, 2126–2132.

- Esyunina, D., Turtola, M., Pupov, D., Bass, I., Klimasauskas, S., Belogurov, G., et al. (2016) Lineage-specific variations in the trigger loop modulate RNA proofreading by bacterial RNA polymerases. *Nucleic Acids Research*, 44, 1298–1308. <https://doi.org/10.1093/nar/gkv1521>
- Foster, A.W., Osman, D. & Robinson, N.J. (2014) Metal preferences and metallation. *The Journal of Biological Chemistry*, 289, 28095–28103. <https://doi.org/10.1074/jbc.R114.588145>
- Gaca, A.O., Kudrin, P., Colomer-Winter, C., Beljantseva, J., Liu, K., Anderson, B., et al. (2015) From (p)ppGpp to (pp)pGpp: Characterization of regulatory effects of pGpp synthesized by the small alarmone synthetase of *Enterococcus faecalis*. *Journal of Bacteriology*, 197, 2908–2919. <https://doi.org/10.1128/JB.00324-15>
- Goodman, A.L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R.S. & Lory, S. (2004) A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Developmental Cell*, 7, 745–754. <https://doi.org/10.1016/j.devcel.2004.08.020>
- Gourse, R.L., Chen, A.Y., Gopalkrishnan, S., Sanchez-Vazquez, P., Myers, A. & Ross, W. (2018) Transcriptional responses to ppGpp and DksA. *Annual Review of Microbiology*, 72, 163–184. <https://doi.org/10.1146/annurev-micro-090817-062444>
- Hachani, A., Allsopp, L.P., Oduko, Y. & Filloux, A. (2014) The VgrG proteins are "à la carte" delivery systems for bacterial type VI effectors. *The Journal of Biological Chemistry*, 289, 17872–17884.
- Harms, A., Brodersen, D.E., Mitarai, N. & Gerdes, K. (2018) Toxins, targets, and triggers: An overview of toxin-antitoxin biology. *Molecular Cell*, 70, 768–784. <https://doi.org/10.1016/j.molcel.2018.01.003>
- Hauryliuk, V., Atkinson, G.C., Murakami, K.S., Tenson, T. & Gerdes, K. (2015) Recent functional insights into the role of (p)ppGpp in bacterial physiology. *Nature Reviews. Microbiology*, 13, 298–309.
- He, H., Cooper, J.N., Mishra, A. & Raskin, D.M. (2012) Stringent response regulation of biofilm formation in *Vibrio cholerae*. *Journal of Bacteriology*, 194, 2962–2972. <https://doi.org/10.1128/JB.00014-12>
- Hmelo, L.R., Borlee, B.R., Almblad, H., Love, M.E., Randall, T.E., Tseng, B.S., et al. (2015) Precision-engineering the *Pseudomonas aeruginosa* genome with two-step allelic exchange. *Nature Protocols*, 10, 1820–1841. <https://doi.org/10.1038/nprot.2015.115>
- Hoang, T.T., Kutchma, A.J., Becher, A. & Schweizer, H.P. (2000) Integration-proficient plasmids for *Pseudomonas aeruginosa*: Site-specific integration and use for engineering of reporter and expression strains. *Plasmid*, 43, 59–72. <https://doi.org/10.1006/plas.1999.1441>
- Hogg, T., Mechold, U., Malke, H., Cashel, M. & Hilgenfeld, R. (2004) Conformational antagonism between opposing active sites in a bifunctional RelA/SpoT homolog modulates (p)ppGpp metabolism during the stringent response. *Cell*, 117, 57–68. [https://doi.org/10.1016/S0092-8674\(04\)00260-0](https://doi.org/10.1016/S0092-8674(04)00260-0)
- Jenal, U., Reinders, A. & Lori, C. (2017) Cyclic di-GMP: Second messenger extraordinaire. *Nature Reviews Microbiology*, 15, 271–284.
- Jiang, M., Sullivan, S.M., Wout, P.K. & Maddock, J.R. (2007) G-protein control of the ribosome-associated stress response protein SpoT. *Journal of Bacteriology*, 189, 6140–6147. <https://doi.org/10.1128/JB.00315-07>
- Jimmy, S., Saha, C.K., Kurata, T., Stavropoulos, C., Oliveira, S.R.A., Koh, A., et al. (2020) A widespread toxin-antitoxin system exploiting growth control via alarmone signaling. *Proceedings of the National Academy of Sciences of the United States of America*, 117(19), 10500–10510.
- Kabsch, W. (2010) Xds. *Acta crystallographica Section D, Biological Crystallography*, 66, 125–132.
- Kim, M., Kim, S. & Ryu, S. (2012) Complete genome sequence of bacteriophage SSU5 specific for *Salmonella enterica* serovar Typhimurium rough strains. *Journal of Virology*, 86, 10894. <https://doi.org/10.1128/JVI.01796-12>
- Kriel, A., Bittner, A.N., Kim, S.H., Liu, K., Tehranchi, A.K., Zou, W.Y., et al. (2012) Direct regulation of GTP homeostasis by (p)ppGpp: A critical component of viability and stress resistance. *Molecular Cell*, 48, 231–241. <https://doi.org/10.1016/j.molcel.2012.08.009>
- Kriel, A., Brinsmade, S.R., Tse, J.L., Tehranchi, A.K., Bittner, A.N., Sonenshein, A.L., et al. (2014) GTP dysregulation in *Bacillus subtilis* cells lacking (p)ppGpp results in phenotypic amino acid auxotrophy and failure to adapt to nutrient downshift and regulate biosynthesis genes. *Journal of Bacteriology*, 196, 189–201. <https://doi.org/10.1128/JB.00918-13>
- Lee, J.W., Park, Y.H. & Seok, Y.J. (2018) Rsd balances (p)ppGpp level by stimulating the hydrolase activity of SpoT during carbon source downshift in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America*, 115, E6845–E6854.
- Liu, H., Xiao, Y., Nie, H., Huang, Q. & Chen, W. (2017) Influence of (p)ppGpp on biofilm regulation in *Pseudomonas putida* KT2440. *Microbiological Research*, 204, 1–8. <https://doi.org/10.1016/j.micres.2017.07.003>
- Liu, K., Bittner, A.N. & Wang, J.D. (2015) Diversity in (p)ppGpp metabolism and effectors. *Current Opinion in Microbiology*, 24, 72–79. <https://doi.org/10.1016/j.mib.2015.01.012>
- Loveland, A.B., Bah, E., Madireddy, R., Zhang, Y., Brilot, A.F., Grigorieff, N., et al. (2016) Ribosome*RelA structures reveal the mechanism of stringent response activation. *eLife*, 5, e17029.
- Manav, M.C., Beljantseva, J., Bojer, M.S., Tenson, T., Ingmer, H., Hauryliuk, V., et al. (2018) Structural basis for (p)ppGpp synthesis by the *Staphylococcus aureus* small alarmone synthetase RelP. *The Journal of Biological Chemistry*, 293, 3254–3264. <https://doi.org/10.1074/jbc.RA117.001374>
- Marden, J.N., Diaz, M.R., Walton, W.G., Gode, C.J., Betts, L., Urbanowski, M.L., et al. (2013) An unusual CsrA family member operates in series with RsmA to amplify posttranscriptional responses in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the United States of America*, 110, 15055–15060. <https://doi.org/10.1073/pnas.1307217110>
- Mechold, U., Murphy, H., Brown, L. & Cashel, M. (2002) Intramolecular regulation of the opposing (p)ppGpp catalytic activities of Rel(Seq), the Rel/Spo enzyme from *Streptococcus equisimilis*. *Journal of Bacteriology*, 184, 2878–2888. <https://doi.org/10.1128/JB.184.11.2878-2888.2002>
- Molina-Henares, M.A., Ramos-Gonzalez, M.I., Daddaoua, A., Fernandez-Escamilla, A.M. & Espinosa-Urgel, M. (2017) FleQ of *Pseudomonas putida* KT2440 is a multimeric cyclic diguanylate binding protein that differentially regulates expression of biofilm matrix components. *Research in Microbiology*, 168, 36–45. <https://doi.org/10.1016/j.resmic.2016.07.005>
- O'Sullivan, W.J. & Cohn, M. (1966) Magnetic resonance investigations of the metal complexes formed in the manganese-activated creatine kinase reaction. *The Journal of Biological Chemistry*, 241, 3104–3115. [https://doi.org/10.1016/S0021-9258\(18\)96504-X](https://doi.org/10.1016/S0021-9258(18)96504-X)
- Pausch, P., Abdelshahid, M., Steichen, W., Schäfer, H., Gratani, F.L., Freibert, S.A., et al. (2020) Structural basis for regulation of the opposing (p)ppGpp synthetase and hydrolase within the stringent response orchestrator rel. *Cell Reports*, 32, 108157. <https://doi.org/10.1016/j.celrep.2020.108157>
- Pausch, P., Steichen, W., Wieland, M., Klaus, T., Freibert, S.A., Altegoer, F., et al. (2018) Structural basis for (p)ppGpp-mediated inhibition of the GTPase RbgA. *The Journal of Biological Chemistry*, 293, 19699–19709. <https://doi.org/10.1074/jbc.RA118.003070>
- Potrykus, K. & Cashel, M. (2008) (p)ppGpp: Still magical? *Annual Review of Microbiology*, 62, 35–51. <https://doi.org/10.1146/annurev.micro.62.081307.162903>
- Potrykus, K., Thomas, N.E., Bruhn-Olszewska, B., Sobala, M., Dylewski, M., James, T., et al. (2020) Estimates of Rel(Seq), Mesh1, and SAH(Mex) hydrolysis of (p)ppGpp and (p)ppApp by thin layer chromatography and NADP/NADH coupled assays. *Frontiers in Microbiology*, 11, 581271. <https://doi.org/10.3389/fmicb.2020.581271>

- Rao, F., See, R.Y., Zhang, D., Toh, D.C., Ji, Q. & Liang, Z.X. (2010) YybT is a signaling protein that contains a cyclic dinucleotide phosphodiesterase domain and a GGDEF domain with ATPase activity. *The Journal of Biological Chemistry*, 285, 473–482. <https://doi.org/10.1074/jbc.M109.040238>
- Raskin, D.M., Judson, N. & Mekalanos, J.J. (2007) Regulation of the stringent response is the essential function of the conserved bacterial G protein CgtA in *Vibrio cholerae*. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 4636–4641. <https://doi.org/10.1073/pnas.0611650104>
- Rhaese, H.J. & Groscurth, R. (1976) Control of development: Role of regulatory nucleotides synthesized by membranes of *Bacillus subtilis* in initiation of sporulation. *Proceedings of the National Academy of Sciences of the United States of America*, 73, 331–335. <https://doi.org/10.1073/pnas.73.2.331>
- Rhaese, H.J., Hoch, J.A. & Groscurth, R. (1977) Studies on the control of development: Isolation of *Bacillus subtilis* mutants blocked early in sporulation and defective in synthesis of highly phosphorylated nucleotides. *Proceedings of the National Academy of Sciences of the United States of America*, 74, 1125–1129. <https://doi.org/10.1073/pnas.74.3.1125>
- Ronneau, S., Caballero-Montes, J., Coppine, J., Mayard, A., Garcia-Pino, A. & Hallez, R. (2019) Regulation of (p)ppGpp hydrolysis by a conserved archetypal regulatory domain. *Nucleic Acids Research*, 47, 843–854. <https://doi.org/10.1093/nar/gky1201>
- Ronneau, S. & Hallez, R. (2019) Make and break the alarmone: Regulation of (p)ppGpp synthetase/hydrolase enzymes in bacteria. *FEMS Microbiology Reviews*, 43, 389–400. <https://doi.org/10.1093/femsr/e/fuz009>
- Ronneau, S., Petit, K., De Bolle, X. & Hallez, R. (2016) Phosphotransferase-dependent accumulation of (p)ppGpp in response to glutamine deprivation in *Caulobacter crescentus*. *Nature Communications*, 7, 11423. <https://doi.org/10.1038/ncomms11423>
- Ruwe, M., Ruckert, C., Kalinowski, J. & Persicke, M. (2018) Functional characterization of a small alarmone hydrolase in *Corynebacterium glutamicum*. *Frontiers in Microbiology*, 9, 916. <https://doi.org/10.3389/fmicb.2018.00916>
- Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular cloning: A laboratory manual*, 2nd edition. Cold Spring Harbor Laboratory Press.
- Santos, S.B., Kropinski, A.M., Ceyssens, P.J., Ackermann, H.W., Villegas, A., Lavigne, R., et al. (2011) Genomic and proteomic characterization of the broad-host-range Salmonella phage PVP-SE1: Creation of a new phage genus. *Journal of Virology*, 85, 11265–11273. <https://doi.org/10.1128/JVI.01769-10>
- Singal, B., Balakrishna, A.M., Nartey, W., Manimekalai, M.S.S., Jeyakanthan, J. & Gruber, G. (2017) Crystallographic and solution structure of the N-terminal domain of the Rel protein from *Mycobacterium tuberculosis*. *FEBS Letters*, 591, 2323–2337.
- Sobala, M., Bruhn-Olszewska, B., Cashel, M. & Potrykus, K. (2019) Methylobacterium extorquens RSH enzyme synthesizes (p)ppGpp and pppApp in vitro and in vivo, and leads to discovery of pppApp synthesis in *Escherichia coli*. *Frontiers in Microbiology*, 10, 859. <https://doi.org/10.3389/fmicb.2019.00859>
- Stanisich, V.A. & Holloway, B.W. (1972) A mutant sex factor of *Pseudomonas aeruginosa*. *Genetical Research*, 19, 91–108.
- Steinchen, W. & Bange, G. (2016) The magic dance of the alarmones (p)ppGpp. *Molecular Microbiology*, 101, 531–544. <https://doi.org/10.1111/mmi.13412>
- Steinchen, W., Schuhmacher, J.S., Altegoer, F., Fage, C.D., Srinivasan, V., Linne, U., et al. (2015) Catalytic mechanism and allosteric regulation of an oligomeric (p)ppGpp synthetase by an alarmone. *Proceedings of the National Academy of Sciences of the United States of America*, 112, 13348–13353. <https://doi.org/10.1073/pnas.1505271112>
- Steinchen, W., Vogt, M.S., Altegoer, F., Giammarinaro, P.I., Horvatek, P., Wolz, C., et al. (2018) Structural and mechanistic divergence of the small (p)ppGpp synthetases RelP and RelQ. *Scientific Reports*, 8, 2195. <https://doi.org/10.1038/s41598-018-20634-4>
- Sun, D., Lee, G., Lee, J.H., Kim, H.Y., Rhee, H.W., Park, S.Y., et al. (2010) A metazoan ortholog of SpoT hydrolyzes ppGpp and functions in starvation responses. *Nature Structural & Molecular Biology*, 17, 1188–1194. <https://doi.org/10.1038/nsmb.1906>
- Sy, J. & Lipmann, F. (1973) Identification of the synthesis of guanosine tetraphosphate (MS I) as insertion of a pyrophosphoryl group into the 3'-position in guanosine 5'-diphosphate. *Proceedings of the National Academy of Sciences of the United States of America*, 70, 306–309. <https://doi.org/10.1073/pnas.70.2.306>
- Takada, H., Roghanian, M., Caballero-Montes, J., Van Nerom, K., Jimmy, S., Kudrin, P., et al. (2020) Ribosome association primes the stringent factor Rel for tRNA-dependent locking in the A-site and activation of (p)ppGpp synthesis. *Nucleic Acids Research*, 49, 444–457.
- Tamman, H., Van Nerom, K., Takada, H., Vandenberg, N., Scholl, D., Polikanov, Y., et al. (2020) A nucleotide-switch mechanism mediates opposing catalytic activities of Rel enzymes. *Nature Chemical Biology*, 16, 834–840. <https://doi.org/10.1038/s41589-020-0520-2>
- Valentini, M., Gonzalez, D., Mavridou, D.A. & Filloux, A. (2018) Lifestyle transitions and adaptive pathogenesis of *Pseudomonas aeruginosa*. *Current Opinion in Microbiology*, 41, 15–20. <https://doi.org/10.1016/j.mib.2017.11.006>
- Van Nerom, K., Tamman, H., Takada, H., Hauryliuk, V. & Garcia-Pino, A. (2019) The Rel stringent factor from *Thermus thermophilus*: Crystallization and X-ray analysis. *Acta Crystallographica Section F: Structural Biology Communications*, 75, 561–569.
- Vashishtha, A.K. & Konigsberg, W.H. (2016) Effect of different divalent cations on the kinetics and fidelity of RB69 DNA polymerase. *Biochemistry*, 55, 2661–2670. <https://doi.org/10.1021/acs.biochem.5b01350>
- Voisard, C., Bull, C.T., Keel, C., Laville, J., Maurhofer, M., Schnider, U., et al. (1994) Biocontrol of root diseases by *Pseudomonas fluorescens* CHA0: Current concepts and experimental approaches. : VCH.
- Wout, P., Pu, K., Sullivan, S.M., Reese, V., Zhou, S., Lin, B., et al. (2004) The *Escherichia coli* GTPase CgtAE cofractionates with the 50S ribosomal subunit and interacts with SpoT, a ppGpp synthetase/hydrolase. *Journal of Bacteriology*, 186, 5249–5257. <https://doi.org/10.1128/JB.186.16.5249-5257.2004>
- Xu, X., Yu, H., Zhang, D., Xiong, J., Qiu, J., Xin, R., et al. (2016) Role of ppGpp in *Pseudomonas aeruginosa* acute pulmonary infection and virulence regulation. *Microbiological Research*, 192, 84–95. <https://doi.org/10.1016/j.micres.2016.06.005>
- Yang, N., Xie, S., Tang, N.Y., Choi, M.Y., Wang, Y. & Watt, R.M. (2019) The Ps and Qs of alarmone synthesis in *Staphylococcus aureus*. *PLoS One*, 14, e0213630. <https://doi.org/10.1371/journal.pone.0213630>
- Ye, R.W., Haas, D., Ka, J.O., Krishnapillai, V., Zimmermann, A., Baird, C. & et al. (1995) Anaerobic activation of the entire denitrification pathway in *Pseudomonas aeruginosa* requires Anr, an analog of Fnr. *Journal of Bacteriology*, 177, 3606–3609. <https://doi.org/10.1128/JB.177.12.3606-3609.1995>

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Steinchen W, Ahmad S, Valentini M, et al. Dual role of a (p)ppGpp- and (p)ppApp-degrading enzyme in biofilm formation and interbacterial antagonism. *Mol Microbiol*. 2021;115:1339–1356. <https://doi.org/10.1111/mmi.14684>