Research Article

Characterization of a (p)ppApp Synthetase Belonging to a New Family of Polymorphic Toxin Associated with Temperate Phages

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Abstract

Polymorphic toxins (PTs) are a broad family of toxins involved in interbacterial competition and pathogenesis. PTs are modular proteins that are comprised of a conserved N-terminal domain responsible for its transport, and a variable C-terminal domain bearing toxic activity. Although the mode of transport has yet to be elucidated, a new family of putative PTs containing an N-terminal MuF domain, resembling the Mu coliphage F protein, was identified in prophage genetic elements. The C-terminal toxin domains of these MuF PTs are predicted to bear nuclease, metallopeptidase, ADP-ribosyl transferase and RelA_SpoT activities. In this study, we characterized the MuF-RelA_SpoT toxin associated with the temperate phage of *Streptococcus pneumoniae* SPNA45. We show that the RelA_SpoT domain has (p)ppApp synthetase activity, which is bactericidal under our experimental conditions. We further determine that the two genes located downstream encode two immunity proteins, one binding to and inactivating the toxin and the other detoxifying the cell via a pppApp hydrolase activity. Finally, based on protein sequence alignments, we propose a signature for (p)ppApp synthetases that distinguishes them from (p)ppGpp synthetases.

Introduction

Polymorphic toxins (PTs) belong to a broad family of toxins involved in interbacterial competition and pathogenesis.[1,2] These modular proteins are comprised of one or more conserved N-terminal domains usually involved in a transport step fused to a variable C-terminal domain corresponding to a toxic activity of variable nature. Each class of PT is characterized by a conserved N-terminal domain that directs the toxin to a specific mode of transport for its delivery into the target cell. Typical classes of PT include colicins, passengers of Type 5 secretion systems (T5SS) involved in contact-dependent inhibition (CDI), evolved effectors of Type 6 secretion system (T6SS), neisserial MafB effectors, and LXG/WXG effectors of Esx-like secretion systems (or Type 7 secretion system, T7SS). The C-terminal domain is very diverse and bears the toxic activity, such as nuclease, deaminase, ADP-rybosyl transferase, phospholipase, phosphatase, amidase, etc. The gene encoding the PT is immediately followed by an immunity gene which protects the cell from the toxin it produces and/or from that which may be injected by its siblings. Immunity proteins are of small size, generally do not contain typical domain and usually bind to their cognate enzymatic toxins to occlude the active sites.

Bioinformatic analyses identified a new family of PTs, associated with temperate phages.[3,4] The

conserved N-terminal domain of these PTs corresponds to MuF, which shares homologies with the F protein of the Mu phage that infects *Escherichia coli*. MuF proteins categorize into two length variants: short proteins containing only the MuF domain, and long proteins that include a C-terminal extension either of unknown function or with a predicted toxic activity.[4] The MuF long versions with a C-terminal toxic domain thereby define this new family of PTs, for which a variety of toxic activities can be predicted: nuclease, metallopeptidase, ADP-ribosyl transferase and RelA_SpoT domains.

In this study, we characterize a member of the new MuF PT family belonging to a large prophage from *Streptococcus pneumoniae* SPNA45 (snu) with a predicted RelA_SpoT C-terminal domain (see Accession Numbers hereinafter for genome and protein ID). In *E. coli,* the RelA and SpoT proteins govern the stringent response, a regulatory program occurring during nutritional starvation. This program consists of switching off the cell's macromolecule biosynthesis pathways while switching on the stress response and amino acid biosynthesis pathways to replenish the cell.[5–7] The stringent response is mediated by the accumulation of a modified nucleotide, the (p)ppGpp alarmone, which results from the transfer of a pyrophosphate group from ATP to the 3′-OH of a GDP or GTP. In *E. coli*, (p)ppGpp could bind to some fifty proteins.[8,9] Notably, (p)ppGpp binds to two distinct RNA polymerase sites,[10,11] thereby modifying the transcription initiation step according to the kinetic properties of the promoters concerned, and more generally the cell's transcriptional program.[12] Although RelA is only capable of synthesizing (p)ppGpp (RelA_SpoT domain), SpoT can synthesize (RelA_SpoT domain) and degrade (HD domain) this nucleotide. The ability to degrade (p)ppGpp is necessary to halt the program and to promote growth when conditions are better. The enzymatic activities of RelA and SpoT are carried by N-terminal domains and controlled by the C-terminal region of the protein, depending on its interaction with partners. For example, RelA associates with the ribosome and synthesizes (p)ppGpp when it detects uncharged tRNA.[13] Thus in *E. coli*, RelA responds specifically to amino acid starvation. By contrast, SpoT is responsive to carbon, fatty acid, phosphate, or iron starvation.[5,14] Hence, two (p)ppGpp synthetases exist in *E. coli* and some other γ- and β-Pseudomonata. In other organisms, such as Bacillota, α-,δ- and ε-Pseudomonata, RelA SpoT Homologs (RSH) carry both synthesis and degradation activities as well as a regulatory C-terminal domain. Finally, in some organisms (p)ppGpp levels are additionally controlled by small alarmone synthetases (SAS) and hydrolases (SAH), which consist only of a catalytic domain.[15,16] The (p)ppGpp synthesis and degradation domains are characterized by the presence of conserved sequence motifs. The (p)ppGpp synthetase domain is comprised of five motifs, Syn1-5, involved in the coordination of magnesium, GDP/GTP and ATP. The (p)ppGpp HD hydrolase domain bears six conserved catalytic motifs, HD1-6, involved in the coordination of manganese and the guanine base.[17]

Recently a novel nucleotide synthetase, responsible for the production of (p)ppApp was identified. (p)ppApp results from the transfer of a pyrophosphate group from ATP to the 3′-OH of an ADP or ATP. The structure of this enzyme showed that it displays a similar fold to (p)ppGpp synthetase domains. This (p)ppApp synthetase domain lies in the C-terminal region of the T6SS Tas1 effector from *Pseudomonas aeruginosa* PA14.[18] Tas1 also includes a N-terminal PAAR domain, which associates with the VgrG spike of the T6SS needle. The (p)ppApp synthetase activity of Tas1 is bactericidal, likely due to the depletion of the ADP /ATP pool. Cells producing Tas1 protect themselves with the Tis1 immunity protein encoded immediately after *tas1*.

Here, we show that the C-terminal RelA_SpoT domain of the *snu* MuF PT is bactericidal when produced in *E. coli*, and that it carries a (p)ppApp synthetase activity. Its toxicity is counteracted by two immunity proteins encoded by genes immediately downstream of the toxin gene. While the first immunity binds to and inhibits the toxin, the second candidate immunity protein harbors a functional (p)ppApp hydrolase domain similar to SpoT HD. We finally compare (p)ppApp and (p)ppGpp synthetase sequences and identify conserved amino acid positions in the Syn2 and Syn4 motifs that might serve as signature of (p)ppApp or (p)ppGpp synthetases. While we were conducting our study, Ahmad et al. published a study on the same enzyme encoded by a prophage of the Gram negative bacterium *Bacteroides caccae*.[19] As our work corroborates their results, we adopted the same nomenclature to avoid confusion. (p)ppApp synthetase toxic domains were named Apk (adenosine 3′-pyrophosphokinase). Apk domains associated with N-terminal PAAR and MuF were named Apk1 and Apk2, respectively. The (p)ppApp hydrolase enzyme was named Aph1 for adenosine 3′-pyrophosphohydrolase. However, taking into consideration the change of nomenclature for the Tas1 effector domain, we propose to name the immunity proteins that bind and inhibit Apk1 and Apk2 domains IapK (immunity of adenosine 3′-pyrophosphokinase).

Results

$Apk2_{tox\text{-}snu}$ is a bactericidal toxin in E. coli

In addition to genes encoding phage components, the *S. pneumoniae* SPNA45 (snu) large prophage comprises the *apk2*, *iapK* and *aph1* genes located between the portal and

scaffold genes (Figure 1(a)). Apk2 is composed of an N-terminal MuF domain fused to a C-terminal RelA_SpoT domain.[4] To evaluate the toxicity of the C-terminal domain, hereafter named Apk2$_{tox-snu}$, the corresponding coding sequence was cloned into the pBAD33 plasmid under the control of the P_{BAD} promoter. While *E. coli* MG1655 cells producing the putative toxin grew similarly to those bearing the empty parental plasmid in repression conditions, no colony was growing when Apk2$_{tox-snu}$

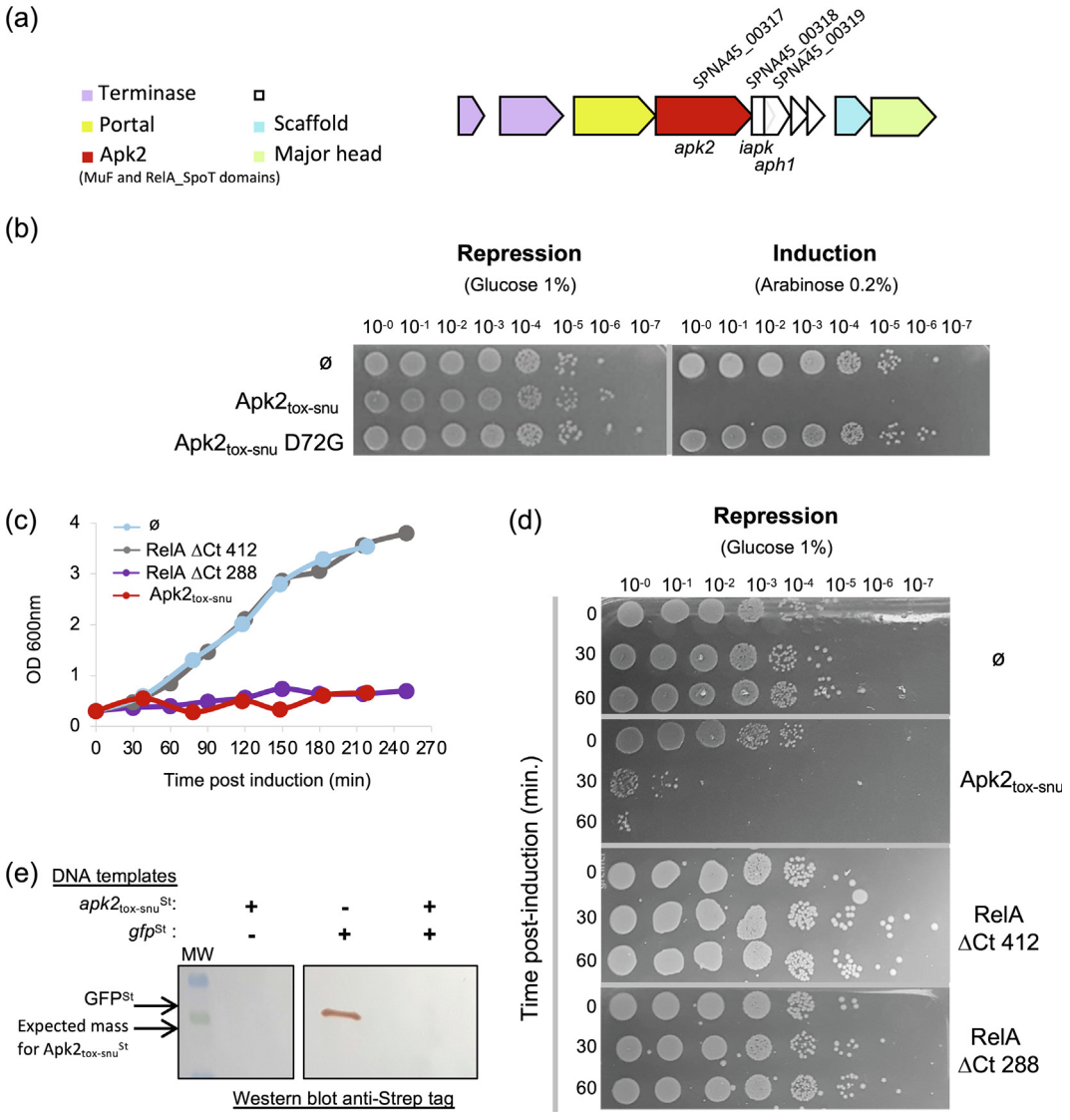
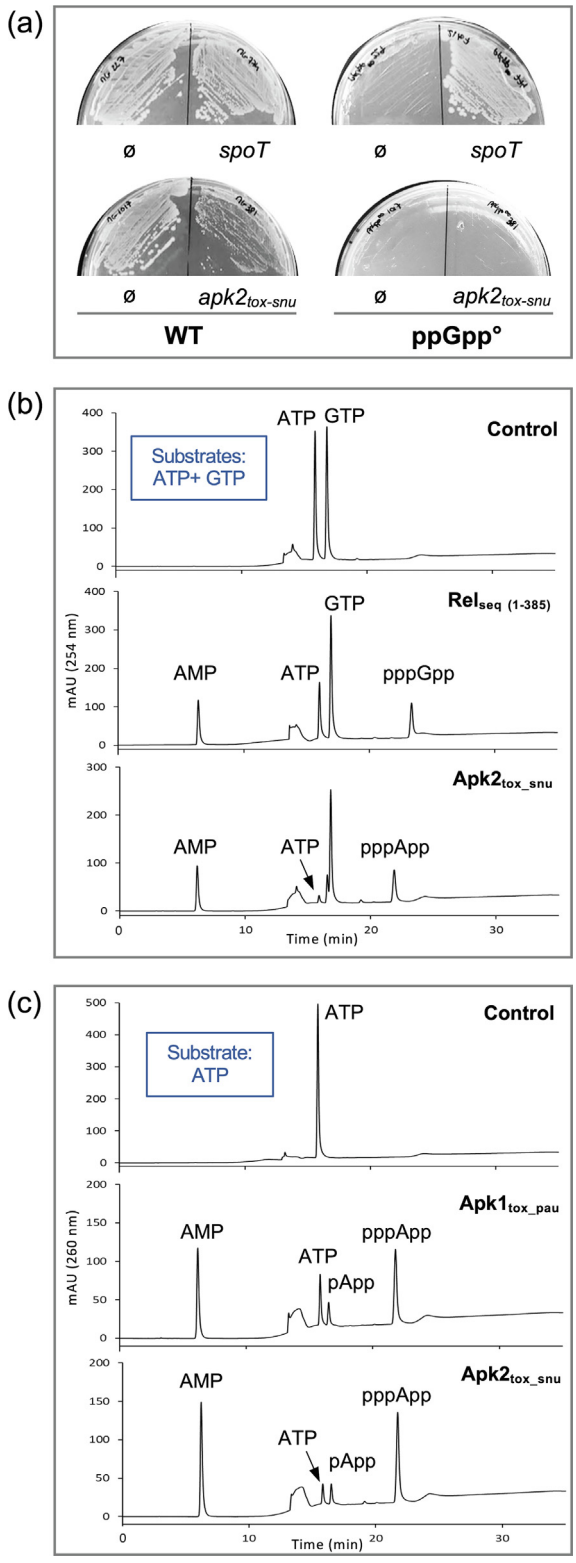


Figure 1. *S. pneumoniae* Apk2$_{tox-snu}$ is a bactericidal toxin. (a) Schematic representation of the genetic environment of the *apk2* gene found within the large prophage of *S. pneumoniae* SPNA45. Genes encoding proteins associated with capsid formation are shown in color and genes shown in white correspond to proteins with not yet defined functions. Locus tags are indicated above *apk2*, *iapK* and *aph1*. (b) Toxicity assays. *E. coli* MG1655 cells carrying the pBAD33 empty plasmid (ø) or pBAD33 expressing the wild-type Apk2$_{tox-snu}$ or the D72G catalytic-null variant (Apk2$_{tox-snu}$ D72G), were grown to an OD_{600} 0.5, serially diluted, and spotted on LB agar containing glucose 1% or arabinose 0.2% to repress or induce the production of the Apk2$_{tox}$ domain, respectively. (c) Growth inhibition in liquid medium. *E. coli* MG1655 cells carry the pBAD33 empty plasmid (ø) or pBAD33 expressing wild-type Apk2$_{tox}$, or plasmids expressing constitutively-active (RelA ΔCt 288) or inactive (RelA ΔCt 412) RelA C-terminal truncated variants.[20] Cell growth in liquid medium was monitored upon induction of the production of the indicated protein. (d) Bactericidal effect associated with the production of Apk2$_{tox-snu}$. *E. coli* MG1655 cells from panel (c) were harvested 0, 30, and 60 min post-induction, washed, serially diluted and spotted on LB agar containing glucose to repress the production of the indicated protein. (e) *In vitro* transcription/translation assays with indicated DNA templates coding Apk2$_{tox-snu}$-Strep$_{tag}$ (Apk2$^{St}_{tox-snu}$) or GFP-Strep$_{tag}$ (GFPSt). Products of the assays were separated on SDS-PAGE and immunodetected with an antibody against the Strep tag. MW: molecular weight.



expression was induced (Figure 1(b)). This toxicity was due to the enzymatic activity of $Apk2_{tox\text{-}snu}$ since the substitution of the conserved Syn2 aspartate 72 residue, involved in Mg^{2+} binding in (p)ppGpp synthetases, abolished $Apk2_{tox\text{-}snu}$ toxicity (Figure 1(b)).

To determine whether $Apk2_{tox\text{-}snu}$ has bacteriostatic or bactericidal impacts on cell growth and viability, *E. coli* cells were grown in liquid medium to mid-exponential phase, $Apk2_{tox\text{-}snu}$ expression was induced, and cells harvested at different time post-induction were washed and spotted on a repressive LB agar medium. Growth monitoring in liquid medium showed that bacteria stopped growing from the moment $Apk2_{tox\text{-}snu}$ was produced (Figure 1(c)), and they were not able to resume growth when washed and spotted on a repressive LB agar medium (Figure 1(d)). In comparison, cells producing a constitutively active truncated RelA variant (RelA ΔCt 288)[20] stopped growing upon induction in liquid medium but formed colonies on repressive LB agar medium, whereas cells carrying the empty parental plasmid or producing an inactive version of RelA (RelA ΔCt 412)[20] grew normally upon induction (Figure 1(c) and 1(d)). Taken together, these results indicate that the activity of $Apk2_{tox\text{-}snu}$ is bactericidal.

It has been proposed that the bactericidal effect associated with the production of Apk domains results from the depletion of ADP and ATP and hence alters essential metabolism.[18] In agreement with this hypothesis, an *in vitro* coupled transcription/translation assay attempting to produce $Apk2_{tox\text{-}snu}$-$Strep_{tag}$ ($Apk2^{St}_{tox\text{-}snu}$) did not provide any

Figure 2. $Apk2_{tox\text{-}snu}$ has (p)ppApp synthetase activity. (a) Complementation assays. Wild-type and ppGpp° *E. coli* strains carrying the empty plasmid (ø) or containing the *spoT* or $apk2_{tox\text{-}snu}$ genes were streaked on minimal medium agar. (b, c) *In vitro* synthesis assay of modified nucleotides. The indicated domains were purified and incubated in the presence of ATP/GTP (b) or ATP alone (c). The reaction products were separated by anion exchange HPLC. In the control panel, no enzyme was mixed with the nucleotides. Rel_{seq} is the catalytic N-terminal fragment (residues 1 to 385) of the bifunctional Rel/Spo homolog from *S. dysgalactiae subsp. equisimilis*.[22] $Apk1_{tox\_pau}$ is the (p)ppApp synthetase domain from the formerly named T6SS-effector Tas1 from *Pseudomonas aeruginosa* PA14.[18,19]

product that could be immunodetected (Figure 1(e)). While GFP-Strep$_{tag}$ (GFPSt) could be produced with such an assay, no GFPSt could be detected if the template to produce Apk2$^{St}_{tox\text{-}snu}$ was also added (Figure 1(e)). We suggest that nucleotide di- or triphosphate consumption by Apk2$_{tox\text{-}snu}$ leads to transcription/translation inhibition. Although *in vitro* production can be a solution when working with toxic proteins, this experiment further shows that it is unlikely an option when trying to produce Apk.

Apk2$_{tox\text{-}snu}$ is a (p)ppApp synthetase

The fact that Apk2$_{tox\text{-}snu}$ shares homologies with RelA_SpoT domains prompted us to test whether it synthesizes (p)ppGpp. We therefore used a genetic approach by complementation of an *E. coli* strain unable to produce (p)ppGpp (ppGpp°). While a wild-type strain grows on minimal media, the ppGpp° strain cannot since it does not synthesize (p)ppGpp to activate amino acid biosynthesis pathways.[21] As expected, our control experiment showed that the production of SpoT complemented the ppGpp° strain, demonstrating that (p)ppGpp is synthesized (Figure 2(a)). By contrast, the production of Apk2$_{tox\text{-}snu}$ did not complement the ppGpp° strain, suggesting that no (p)ppGpp is synthesized (Figure 2(a)). Though, this experiment is tricky to interpret, as it is difficult to distinguish between the protein's toxicity and the absence of production of (p)ppGpp. Indeed, the absence of complementation could be due to the toxicity of Apk2$_{tox\text{-}snu}$ although the strain was grown in glucose minimal medium, which is a repressive condition for pBAD-driven expression, as the wild-type strain carrying *apk2*$_{tox\text{-}snu}$ is slightly intoxicated (Figure 2(a)). In addition, if the protein is too active, too much (p)ppGpp would inhibit the growth of the ppGpp° strain that is deleted of both *relA* and *spoT*.

To better define the activity of Apk2$_{tox\text{-}snu}$, the protein was purified (see Material and Methods) and its activity on nucleotide phosphate was assayed using an *in vitro* assay. As controls, we also purified and assayed Rel$_{seq}$ (1–385) and Apk1$_{tox}$ (Tas1$_{tox}$). Rel$_{seq}$ (1–385) is the catalytic N-terminal fragment (residues 1 to 385) of the bifunctional Rel/Spo homolog from *S. dysgalactiae subsp. equisimilis* that displays (p)ppGpp synthetase activity,[22] while Apk1$_{tox}$ corresponds to the C-terminal domain of the *P. aeruginosa* PA14 T6SS Tas1 PT that displays (p)ppApp synthetase activity.[18] *In vitro* assays were first performed by incubating both ATP and GTP with purified protein domains, before separation of the reaction products by Strong Anion Exchange (SAX) High Performance Liquid Chromatography (HPLC). As expected, Rel$_{seq}$ (1–395) synthesized pppGpp (Figure 2(b)). By contrast Apk2$_{tox\text{-}snu}$ was responsible for the synthesis of a different molecule (Figure 2(b)), which one was also detected when ATP was provided as only substrate for the *in vitro* assay (Figure 2(c)). As previously shown, ATP was converted into AMP, pApp and pppApp when incubated with the *P. aeruginosa* Apk1 toxin (Apk1$_{tox\text{-}pau}$) (Figure 2(c)). An identical profile was obtained when ATP was incubated with Apk2tox-snu (Figure 2(c)), demonstrating that Apk2$_{tox\text{-}snu}$ is a (p)ppApp synthetase.

IapK and Aph1 are two distinct immunity proteins rescuing from Apk2$_{tox\text{-}snu}$ toxicity

The *apk2* gene is followed by 4 open reading frames encoding < 150-residue proteins with no assigned function (Figure 1(a)). The first ORF is the best candidate for serving as immunity protein, as they are usually encoded directly downstream the gene encoding the toxin. The second ORF harbors a HD domain, found in a superfamily of metal-dependent phosphohydrolases, enzymes that cleave phosphoester bonds of phosphorylated compounds. We suspected that the second ORF could thus protect from the toxicity associated with the production of Apk2$_{tox\text{-}snu}$. The genes corresponding to these two ORFs, that we named *iapK* and *aph1*, were cloned together or independently into the pASK-IBA37+ vector, under the control of the P$_{TET}$ promoter. Co-production of Mesh1, a *Drosophila melanogaster* hydrolase, which has been shown to hydrolyze both (p)ppGpp and (p)ppApp,[23] with Apk2$_{tox\text{-}snu}$ shows that the *E. coli* cell viability can be partly rescued (Figure 3(a)), likely by the partial hydrolysis of the (p)ppApp pool. The co-production of both candidate immunity proteins IapK and Aph1 fully rescued *E. coli* and this rescue could only rely on IapK since its production alone provided full protection (Figure 3(a)). Still, Aph1 was able to provide partial protection, comparable to that provided by Mesh1. This partial protection is likely due to its phosphohydrolase activity as a substitution of the predicted D48 catalytic residue did not confer protection (Figure 3(a)). The third and fourth ORFs downstream *apk2* were also tested for their ability to protect the cell against Apk2$_{tox\text{-}snu}$ but no rescue was observed (data not shown).

An AlphaFold2 structural model suggested that IapK binds to Apk2$_{tox\text{-}snu}$ (Figure 3(b)). Interestingly, in this model the IapK protein interacts with a groove of the Apk2$_{tox\text{-}snu}$ toxin and occludes the catalytic pocket (Figure 3(b)). The interaction between IapK and a catalytic-null variant of Apk2$_{tox\text{-}snu}$ (carrying the D72G substitution to avoid cell toxicity, see Figure 1(b)) was experimentally validated by a bacterial adenylate cyclase two-hybrid (BACTH) assay (Figure 3(c)). The IapK-Apk2$_{tox\text{-}snu}$ interaction was also detected by co-purification upon co-production of the two partners from a pET-Duet vector (Figure 3(d)). Taken together, these results demonstrate that IapK inhibits Apk2$_{tox\text{-}snu}$ toxicity via protein–protein interaction, likely by occlusion of its active site.

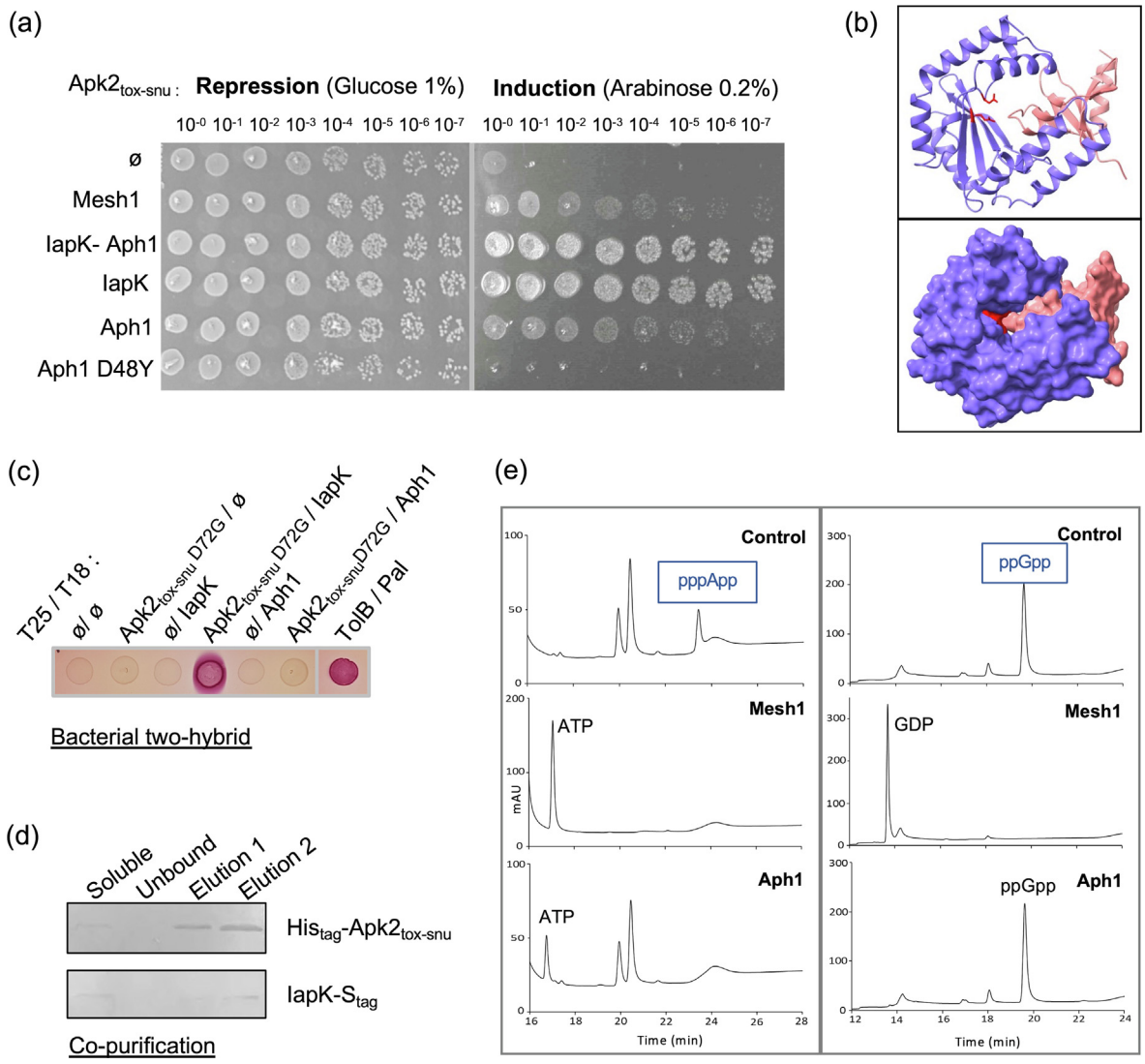


Figure 3. Two distinct immunity proteins confer protection against Apk2$_{tox-snu}$. (a) Toxicity neutralization assays. *E. coli* MG1655 cells producing Apk2$_{tox-snu}$ and the HD domain *Drosophila melanogaster* Mesh1 protein, or one or the two ORFs following the *apk2* gene: *iapK* and *aph1*, or the *aph1* D48Y variant were grown to OD_{600} 0.5, serially diluted and spotted on LB agar containing anhydrotetracycline to induce the production of the proteins for which immune function was tested, and glucose 1% or arabinose 0.2% to repress or induce the production of the Apk2$_{tox-snu}$, respectively. (b) Ribbon (top) and surface (bottom) representations of the Apk2$_{tox-snu}$- IapK complex AlphaFold2 structural model. Apk2$_{tox-snu}$ and IapK are shown in purple and pink, respectively. The side chains of the two conserved Syn2 Asp and Syn4 Glu residues, involved in Mg^{2+} binding in (p)ppGpp synthetases, are highlighted in red. (c) BACTH experiment. *E. coli* BTH101 cells producing the indicated proteins fused to the T18 or T25 domains of the *Bordetella pertussis* adenylate cyclase were spotted on MacConkey agar (Ø, no protein fused to the T18 or T25 domain). The T25_TolB / T18_Pal pair serves as a positive control of protein–protein interaction. Due to high toxicity of the Apk2$_{tox-snu}$ toxin, the inactivated version D72G was used in these experiments. (d) Co-purification. Cell lysates of *E. coli* BL21(DE3) cells producing His$_{tag}$-Apk2$_{tox-snu}$ and IapK-S$_{tag}$ were subjected to immobilized metal affinity chromatography on cobalt beads. Fractions corresponding to the protein extract, unbound proteins and two successive elutions were separated by SDS-PAGE, transferred onto nitrocellulose membranes and immunodetected using anti-His (upper blot) and anti-S-tag (lower blot) antibodies. (e) Chromatograms of the *in vitro* degradation assay of modified nucleotides. The indicated proteins were purified and their activity on the nucleotides indicated in the frames was tested *in vitro* (control, no protein). The products of the reaction were separated by anion exchange HPLC.

In contrast, no interaction was detected between $Apk2_{tox\text{-}snu}$ and Aph1 (Figure 3(c)). Aph1 also partly rescued $Apk2_{tox\text{-}snu}$ toxicity and phenocopied the Mesh1 phosphohydrolase, in agreement with the observation that Aph1 carries a phosphohydrolase HD motif. We therefore conducted *in vitro* assays by co-incubating the purified Aph1 protein and pppApp or ppGpp nucleotides. Mesh1 was used as control, as it was previously shown that it is a versatile phosphohydrolase able to cleave the 3′-pyrophosphate group from (p)ppGpp and (p)ppApp.[23] Indeed, the *in vitro* assay shows that Mesh1 hydrolyzed both pppApp and ppGpp (Figure 3(e)). In contrast, Aph1 only cleaved the 3′-pyrophosphate group from pppApp (Figure 3(e)).

Overall, these results show that the gene encoding the Apk2 MuF PT is followed by two ORFS encoding two proteins that confer protection against $Apk2_{tox\text{-}snu}$ toxicity via two distinct mechanisms. The first and most protective one, IapK, inhibits $Apk2_{tox\text{-}snu}$ toxicity most probably by occlusion of the active site, while the second, Aph1, partly rescues the cell from $Apk2_{tox\text{-}snu}$ action by detoxification through its pyrophosphohydrolase activity.

Attempts to identify signatures of (p)ppGpp and (p)ppApp synthetases

Classical protein sequence alignment tools, such as Blastp, do not distinguish (p)ppApp synthetase and (p)ppGpp synthetase domains. As our work and the study of Ahmad et al. identified three members of (p)ppApp synthetases, we carried out a protein multiple sequence alignment (MSA) with representative members of well-known (p)ppGpp synthetases[24,25] and newly experimentally characterized (p)ppApp synthetases.[18,19] We also included homologs of $Apk1_{tox}$ and $Apk2_{tox}$ identified by Ahmad and collaborators and Jamet and collaborators,[4,19] which belong to different bacterial genera and share between 18 and 64% identity (Figure 4). This MSA shows that the Syn1 motif is relatively well conserved between the two subfamilies, including the conserved Arg, Lys, Ser and Lys residues of the RxKxxxSxxxK consensus. However, while the conserved Syn2 Asp and

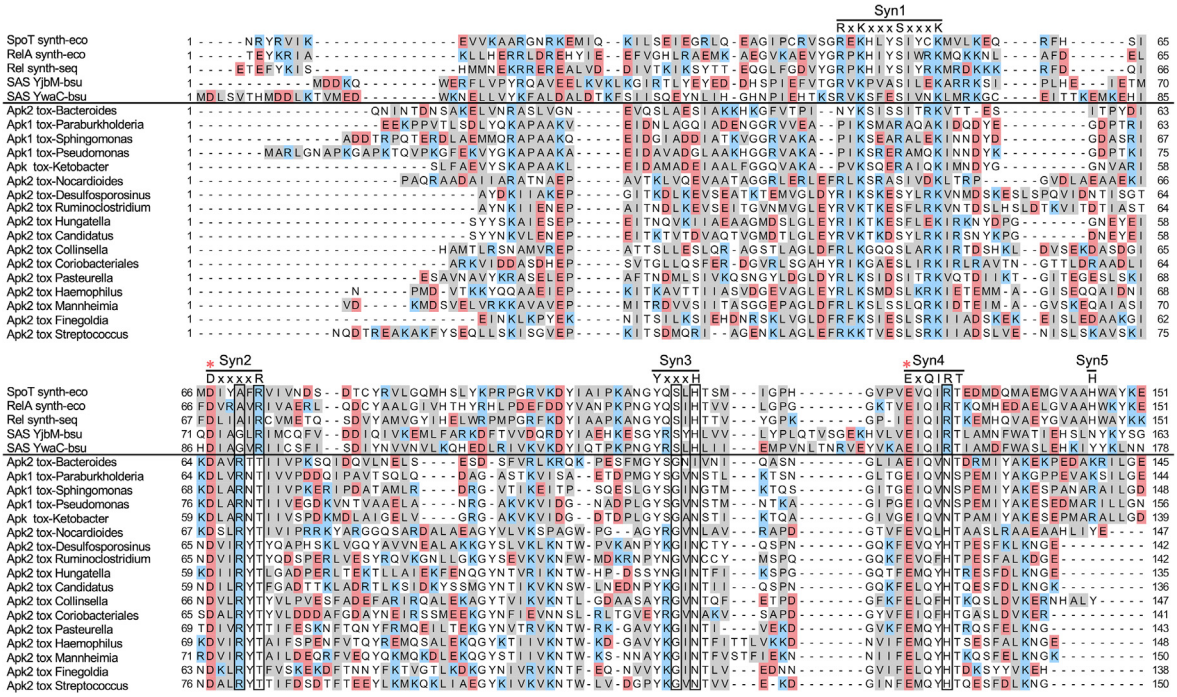


Figure 4. Multiple sequence alignment of (p)ppGpp and (p)ppApp synthetase domains. Clustal Omega protein multiple sequence alignment of synthesis domains from (p)ppGpp synthetases (RelA and SpoT from *Escherichia coli* (eco), Rel from *Streptococcus dysgalactiae subsp. equisimilis* (seq), and small alarmone synthetases YjbM and YwaC from *Bacillus subtilis* (bsu)) and from Apk1 and Apk2 (p)ppApp synthetase domains from different bacterial genera (see Accession Numbers hereinafter for protein ID). Members of the two synthetase families are separated by a black line. Amino acids are colored according to Clustal omega grouping (red, acidic residues; blue, basic residues except His; grey, hydrophobic and aromatic residues except Tyr; the other amino-acids were left uncolored). The 5 conserved motifs (Syn1-5) of the (p)ppGpp synthetase domains are indicated. Catalytic Asp (Syn2) and Glu (Syn4) residues, involved in Mg^{2+} binding, are indicated by red stars. Residues that are conserved but different in each synthetase family are boxed. MSA was shorten after syn5 motif, most of Apk2 synthetase domain sequences were already ended.

Syn4 Glu residues involved in Mg^{2+} coordination and ATP binding[17] are perfectly conserved in both (p)ppApp and (p)ppGpp synthetase domains, we noticed significant differences in these two motifs (Figure 4). In Syn2, the alignment shows that residue at positions +3 with respect to the conserved Asp residue corresponds to short non-polar side-chain residue (Ala or Gly) in (p)ppGpp synthetase domains while an Arg residue is found at this position in (p)ppApp synthetase domains. This Arg residue is positioned at the +5 position in (p)ppGpp synthetases, where a Thr residue is found in (p) ppApp synthetases. Thus, while the motif Syn2 is defined as DxxxxR in (p)ppGpp synthetases, a conserved DxxRxT motif is found in (p)ppApp synthetases (Figures 4 and 5(a)). In Syn4, the residue at position +4 with respect to the conserved Glu residue corresponds to an Arg residue in (p)ppGpp synthetases and His or Asn residue in (p)ppApp synthetases. Thus, while the motif Syn4 is defined as ExQIRT in (p)ppGpp synthetases, a conserved ExQxH/N T is associated with (p)ppApp synthetases (Figures 4 and 5(a)). Finally, in the Syn3 motif that coordinates GDP/GTP substrate in (p) ppGpp synthetase domains, the position +3 of the YxxxH motif corresponds to a Ser and Gly residues in (p)ppGpp and (p)ppApp synthetases, respectively, while the +5 His residue is not conserved in (p)ppApp synthetases and replaced by a Ser or Asn residue (Figures 4 and 5(a)). Interestingly, all the side chains of the Syn2, -3 and -4 residues that differ between (p)ppGpp and (p)ppApp synthetases locate on the same side of the β -sheet of the $Apk2_{tox-snu}$ AlphaFold2 model (Figure 5(b)).

Discussion

In this study, we demonstrated that the RelA_SpoT domain of Apk2 from the large prophage of the Gram-positive bacterium *S. pneumoniae* SPNA45 has a (p)ppApp synthetase activity. With Apk1 (or Tas1) from *P. aeruginosa* PA14 and Apk2 from *B. caccae* temperate phage,[18,19] the *S. pneumoniae* Apk2 protein is the third member of a family of strict (p)ppApp synthetases. With the experimental set-up used in this study, i.e. heterologous expression of a synthetic gene from the pBAD33 vector in *E. coli* MG1655, the activity of the (p)ppApp synthetase domain is toxic and bactericidal. $Apk2_{tox-snu}$ toxicity is abolished by the co-production of IapK, which is encoded downstream of *apk2* on the *S. pneumoniae* SPNA45 prophage. IapK binds Apk2 and likely occlude its active site. The ORF downstream *iapK* encodes Aph1, a protein with the HD motif specific to the superfamily of metal-dependent phosphohydrolases. When co-produced with $Apk2_{tox-snu}$, Aph1 partially rescues cell survival. Our *in vitro* results demonstrated that Aph1 cleaves pppApp to regenerate ATP, suggesting that Aph1 detoxifies the cell from the accumulation of pppApp or from ATP depletion. It is not yet clear why there are two immunity proteins. Possibly a cumulative effect would be required for full protection, but this would have to be explored in physiological conditions of expression and in the natural host. What is remarkable is that these two immunity proteins confer protection through two distinct modes of action. IapK seems specific of the toxic partner protein since

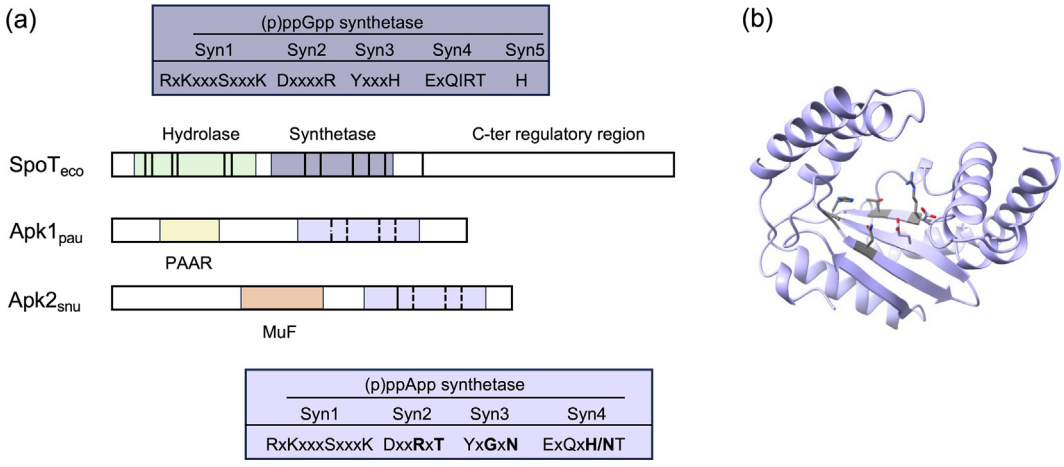


Figure 5. Distinction between (p)ppGpp and (p)ppApp synthetases. (a)Domain architecture of enzymes involved in (p)ppGpp and (p)ppApp metabolism (domains found in the C-terminal regulatory region of SpoT are not detailed). Catalytic motifs are shown as black lines. Dashed lines symbolized slightly different motif from the (p)ppGpp synthetase reference. Scheme inspired form Steinchen and Bange 2016.[17] (b) Ribbon representation of the $Apk2_{tox-snu}$ AlphaFold2 structural model. The side chains of residues conserved in the syn motifs of (p)ppApp synthetases, but which are different from those found in (p)ppGpp synthetases (in bold in the (p)ppApp synthetase box, a) are colored grey and heteroatom. The side chain colored heteroatom of the conserved catalytic Asp (Syn2) and Glu (Syn4) residues are also shown.

the immunity protein from *B. caccae* is unable to protect from *P. aeruginosa* $Apk1_{tox}$ toxicity.[19] In contrast, because the activity of Aph1 is directed toward the product of the (p)ppApp synthetase and not toward the enzyme, its protection is "universal". Indeed, a similar level of protection was conferred by the *Drosophila* Mesh1 phosphohydrolase. As a result, Aph1 may still offer a minimal level of protection to the bacterial host in case the latter would be intoxicated by a variant form of $Apk2_{tox}$ for which it would not have the specific IapK immnunity protein.

Our results also showed that $Apk2_{tox}$ does not have (p)ppGpp synthesis activity. A multiple sequence alignment with members of the (p)ppApp or (p)ppGpp synthetase domain families showed that there are significant differences at some positions of the conserved and functional Syn motifs. We therefore propose signatures that could distinguish (p)ppApp and (p)ppGpp synthetases in Syn2 and Syn4 motifs: DxxxxR and ExQIRT, and DxxRxT and ExQxH/N T in (p)ppGpp and (p)ppApp synthetases, respectively (Figure 5(a)). Interestingly, recent studies mention the ability of the *Methylobacterium extorquens* RSH, *B. subtilis* SasA and *Treponema denticola* SAS proteins to synthesize both (p)ppGpp and (p)ppApp.[26–28] However, in agreement with the observation that the predominant activity of these enzymes is (p)ppGpp synthesis, the examination of their sequences revealed Syn2 and Syn4 motifs typical of (p)ppGpp synthetases. Conversely, the *Cellulomona marina* FaRel, described to synthesize both ppGpp and ppApp, has a sequence corresponding to a possible (p)ppApp synthetase signature. Only the FaRel ppApp synthesis activity was confirmed *in vitro*.[16]

Modified nucleotides (p)ppGpp and (p)ppApp appear to play a role in phage cycle, given the distribution of (p)ppGpp and (p)ppApp Toxin-Antitoxin systems encoded by phage and prophage genomes.[4,16,19,29] While this co-occurrence is not yet understood, one study suggests that it could provide protection against superinfection. Hence, the mycobacterial Phrann prophage Gp29 (p)ppGpp synthetase is proposed to be maintained inactive by an interaction with the Gp30 membrane protein and that infection by lytic phages would induce their dissociation and the consequent activation of Gp29.[29] Accumulation of (p)ppGpp would turns the host bacterium in a dormancy state that would be unfavorable to virulent bacteriophages.

Given the definition of polymorphic toxins and the involvement of conserved N-terminal domains in a transport step, it is tempting to propose that the MuF domain of Apk2 serves to transport the toxin. The *muf* genes are generally located close to the genes encoding the portal and terminase proteins and therefore belong to the head morphogenesis and DNA packaging modules of the phage.[4] The portal protein i) acts as a nucleation site to initiate capsid assembly, ii) forms a channel for the bidirectional passage of viral DNA, and iii) acts as an attachment point for the tail of the phage. The portal protein also works in concert with the TerSL terminase complex to translocate viral DNA. The TerS protein recognizes the DNA based on a packaging signal and addresses it to the TerL protein, which is organized as a pentameric ring interacting with a dodecamer of the portal protein.[30] Little is known about the MuF protein and most studies have been carried out on the short MuF protein (Gp7) from the *Bacillus subtilis* SPP1 virulent phage. A series of *in vitro* experiments proposed that Gp7 binds the Gp6 portal protein, and this interaction is necessary to locate Gp7 to the procapsid. One or two copies of Gp7 would be therefore present in proheads and phages. Then, either because the passage of DNA entering the capsid displaces the interaction between the portal and Gp7 proteins, or because this entry triggers a conformational change in the portal protein, Gp7 detaches and instead binds viral DNA. Although it is tempting to hypothesize that Gp7 is co-ejected with viral DNA, this is not yet supported by experimental data. Instead, what has been shown is that Gp7 would slow down the release of viral DNA by keeping it anchored into the capsid by one of its extremities during the ejection. The absence of Gp7 does not prevent the formation of virions, but these are 5 to 10 times less infectious.[31–33] However, recent studies on staphylococcal temperate bacteriophage 80α suggest that gp44, which belongs to the (short) MuF family, has a post-injection role and is therefore co-injected with phage DNA. These conclusions are based on a trans-complementation experiment in which the production of gp44 in recipient cells restores cycles of infection of a Δ*gp44* phage lysate.[34] The authors further suggest that gp44 would protect the phage DNA from degradation post-injection.[35]

The presence of a toxin on a continuous polypeptide with MuF in the phage head and the perspective that the toxin could be delivered to a recipient cell upon infection by bacteriophage raises the question of the biological role. This question must be considered in the context of infection by temperate phages since those are the ones associated with this novel PT family. From the point of view of interbacterial competition, the injection of a toxin would be beneficial to lysogenic bacteria already equipped with the immunity protein. However, this immunity protein would have to be produced by the lysogen at the time of infection and, as it is encoded within the module of the phage head morphogenesis, it is not clear why it should be unless the phage is in lytic mode. Otherwise, PT and immunity genes, which together appear as an operonic organization, would have to be independently regulated. Alternatively, regardless of when the immunity protein is produced, the prophage could confer an

advantage on its host if it avoids poisoning thanks to a superinfection exclusion system. Other hypothesis could be that the activity of the toxin favors the bacteriophage lysogenic cycle. For example, one possibility could be that the products of the enzymatic activity regulate the expression of genes that are important for lysogeny. Precisely, ppApp has been shown to bind to the RNA polymerase, albeit at different sites compared to ppGpp, and an *in vitro* study showed a positive regulation by ppApp on *rrnB* P1 activity, unlike ppGpp, highlighting the possibility of a distinct impact for these two modified nucleotides.[36] A study comparing the cellular targets and global effects of these two nucleotides would be of great interest. Otherwise, the enzymatic activity could impact the state of the recipient cell and influence the lytic/lysogenic decision. Indeed, although the activity of $Apk2_{tox\text{-}snu}$ was shown to be bactericidal in this study, the level of intoxication might be lower with only a bacteriostatic effect in physiological conditions, considering that only one or two copies of the polypeptide might be present in the phage head as it has been estimated for the short MuF protein from the SPP1 bacteriophage from *B. subtilis*.[31] In this case, can the arrest of host cell growth processes be unfavorable to the lytic cycle option and lead to a lysogenic decision? In the context of the arms race between bacteria and phages, another suggestion could be that the toxin's activity counteracts a possible anti-phage mechanism. But why such a defense process would be more beneficial to temperate phages than lytic ones?

Interesting new avenues of research aimed at understanding the biological role of these toxin-antitoxin systems in the life cycle of temperate phages and their bacterial host are therefore open. And more discoveries are yet to come with the investigation of MuF proteins that display C-terminal domains of unknown functions.

Materials and Methods

Bacterial strains and media

E. coli strains used in this study are described in Table S1. Bacteria were grown in 2YT, Luria-Bertani (LB) (Sigma-Aldrich), MacConkey agar (BD), M9 minimal medium (1 × M9 salts, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 2 μg/ml vitamin B12, 0.2% glucose). Plasmids were maintained by the addition of antibiotics (ampicillin 100 μg/ml, kanamycin 50 μg/ml or chloramphenicol 50 μg/ml).

Plasmid construction and site-directed mutagenesis

Plasmids and primers used in this study are described in Tables S2 and S3, respectively. PCR amplifications were performed with Phusion High-Fidelity DNA Polymerase (Finnzymes). Site-directed mutagenesis was performed on plasmids following the instructions of the QuickChange site-directed mutagenesis kit (Stratagene). DNA template corresponding to a portion of a *Streptococcus pneumoniae* SPNA45 prophage genomic region was sequence-optimized for *E. coli* and synthesized by IDT.

Toxicity and toxicity neutralization assays

For toxicity assays, *E. coli* MG1655 was transformed with pBAD33 encoding the $Apk2_{tox\text{-}snu}$ domain and transformants were selected on LB agar plate containing the appropriate antibiotic and 1% glucose for toxin repression. Stationary phase overnight cultures were diluted to an OD_{600} of 0.05 in fresh LB medium supplemented with 1% glucose. Bacteria were cultivated at 37 °C to exponential phase ($OD_{600} \approx 0.5$). An aliquot was washed twice with LB and cultures were normalized to OD_{600} = 0.5. Serial dilutions in sterile PBS were performed and spotted on LB agar containing 1% glucose for P_{BAD} promoter repression or 0.2% arabinose for induction.

For toxicity neutralization assays, *E. coli* MG1655 was co-transformed with pBAD33 encoding the $Apk2_{tox\text{-}snu}$ domain and pASK-IBA37 + encoding the candidate immunity proteins. Co-transformants were selected on LB agar containing the appropriate antibiotic and 1% glucose. Experiments were carried out as described above and dilutions were spotted on LB agar plates containing appropriate antibiotics, anhydrotetracycline 200 ng/ml to induce the P_{TET} promoter from pASK-IBA37+, and either 1% glucose or 0.2% arabinose to repress or induce the P_{BAD} promoter from pBAD33.

Bacteriostatic or bactericidal effect of the toxin

To examine the bacteriostatic or bactericidal effect associated with the production of the toxin, *E. coli* MG1655 was transformed with plasmids encoding the $Apk2_{tox\text{-}snu}$ domain, or a constitutively active (RelA ΔCt 288) or an unactive (RelA ΔCt 412) truncated variant of RelA as controls.[20] From stationary-phase overnight cultures, fresh LB medium containing 1% glucose was inoculated to an OD_{600} of 0.01. Bacteria were cultivated at 37 °C to OD_{600} = 0.3, washed twice with LB before induction of P_{BAD} with 0.2% arabinose for pBAD33 plasmids, or induction of P_{tac} with 500 μM Isopropyl ß-D-thiogalactopyranoside (IPTG) for RelA-containing plasmids. At time 0, 30 and 60 min post-induction, an aliquot was recovered and chilled in ice water for 2 min. Cells were pelleted at 6,000 g at 4 °C and re-suspended in ice-cold fresh LB. Serial dilutions were done in sterile PBS and spotted on LB agar plates containing appropriate antibiotics and 1% glucose.

In vitro transcription-translation assays

Coupled *in vitro* transcription-translation assays were performed with the PURExpress® *In vitro* Protein synthesis kit (NEB) supplemented with murine RNase inhibitor (NEB) as recommended by the manufacturer. DNA templates encoding the $Apk2_{tox\text{-}snu}$-$Strep_{tag}$ and the GFP-$Strep_{tag}$ proteins, were amplified using the primer pairs ebm2109/2110 and ebm2120/2121, respectively. These templates were added to the reactions (3 ng/μl), which were performed for 2 h at 37 °C. Proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes and *in vitro* synthesized proteins were detected by immunoblotting with antibodies against Strep tag (Classic, BioRad).

Bacterial two-hybrid

Plasmids allowing the production of proteins fused to the T18 or T25 domains of the *Bordetella pertussis* adenylate cyclase were co-transformed in *E. coli* BTH101. Bacteria were grown overnight in LB supplemented with 0.5 mM IPTG, and 2 μl were spotted on McConkey agar medium containing 1% maltose.

Protein production, purification or co-purification

- Toxins purification or co-purification

6 × His-$Apk1_{tox\text{-}pau}$ and 6 × His-$Apk2_{tox\text{-}snu}$ were co-produced with their cognate IapK-S-tag immunity proteins from the pET-Duet1 plasmid, using *E coli* BL21 DE3 (pLys). For this, 1 L of culture was grown at 30 °C until OD_{600} = 0.5 and protein production was induced with 500 μM IPTG at 25 °C for 4 h. Cells were harvested by centrifugation at 4,000g for 20 min, washed with PBS, and resuspended in 20 ml lysis buffer (20 mM Tris-HCl pH 8, 200 mM NaCl, 10 mM imidazole, 2 mM β -mercaptoethanol, 0.2% NP40) supplemented with DNase 0.1 mg/ml, $MgCl_2$ 10 mM and protease inhibitor (PMSF, 0.5 mM). Cells were disrupted using a high-pressure homogeniser (Emulsiflex) and unbroken cells or fragments were eliminated by centrifugation at 15,000*g* for 30 min. His-tagged proteins were purified by affinity chromatography on metal/cobalt affinity resin (Takara). Unbound fraction was recovered by gravity using a Poly-Prep Chromatography column (Bio-rad) and the resin was washed with 15 ml of lysis buffer.

For purification of the $Apk1_{tox\text{-}pau}$ and $Apk2_{tox\text{-}snu}$ domains alone, inspired by the protocol of Ahmad *et al.*, 2019[18], the immunity protein (IapK) was dissociated by denaturing the protein–protein complex using 10 ml of lysis buffer supplemented with 8 M urea. Renaturation of the toxin was then performed by washing the resin with 20 ml of lysis buffer. Finally, proteins were eluted using lysis buffer containing 300 mM imidazole.

Rel_{seq} (1–385) has been produced and purified as previously published.[37,38]

Protein concentration and buffer exchange was performed using centrifugal filter (Amicon Ultra-4, 10 MWCO, Millipore). Glycerol was finally added for long-term −80 °C conservation, resulting in a final protein buffer composition of 10 mM Tris-HCl pH 8, 75 mM NaCl, and 40% Glycerol.

- Immunity-protein purification

6 × His-IapK, 6 × His-Aph1 and 6 × His-Mesh1 were produced from pASK-IBA37+ in E. coli MG1655. The experimental protocol for protein production and purification was the same as described above except that only 100 ml of culture were necessary, that cells were disrupted by sonication and buffer exchange was carried out by dialysis (Side-A-lyzer dialysis cassette, 3.500 MWCO; Thermo Scientific).

Western blot

Western blot analyses of protein samples were performed using mouse anti-Strep-tag (Biorad), mouse anti-His-tag (Proteintech) or mouse anti-S-tag (Sigma) and detected with anti-mouse horseradish peroxidase-conjugated secondary antibodies (SantaCruz) or with anti-mouse phosphatase alkaline-conjugated secondary antibodies (Sigma).

In vitro synthesis or hydrolysis of (p)ppGpp/(p)ppApp coupled with HPLC analysis or purification

In vitro synthesis of guanosine pentaphosphate nucletotide followed by analytical separation or purification using HPLC was performed as previously described.[38]

For testing pppApp synthetase activity, a 20-μl reaction containing 5 mM ATP, used as both the phosphate donor and acceptor, and 1 μM of purified enzyme ($Apk2_{tox\text{-}snu}$ or $Apk1_{tox\text{-}pau}$) in 10 mM Tris-HCl pH 8, 100 mM NaCl and 15 mM $MgCl_2$ was incubated at 37 °C for 2 h. After 15-fold dilution in HPLC solvent A (KH_2PO_4 50 mM pH 3.4), the enzyme was eliminated by passing the reaction mixture through a spin filter column (Nanosep 10 K Omega, Pall Corporation).

For analytical purpose, 20 μl of reaction was injected on an Agilent 1260 Infinity HPLC system equipped with a SAX 5 μm 4.6 × 250 mm Waters Spherisorb analytical column. Nucleotides separation was carried out over 35 min using an ionic strength gradient from solvent A to solvent B (KH_2PO_4 1 M pH 3.4) at a flow rate of 1 ml. min^{-1}.[38] The nucleotides were monitored at 254 nm (max absorption for guanosine nucleotide)

and/or 260 nm (max absorption for adenosine nucleotide).

For pppApp purification, the initial *in vitro* reaction was performed in 120 μl, followed by dilution and passing through the spin filter column, and 15 injections of 100 μl of reaction mixture were repeated during which the nucleotide was collected. The collected fractions were pooled and purified using Oasis WAX SPE Cartridges (6 cc Vac Cartridge; Waters) and lyophilized as described before.[38] pppApp was then resuspended in 5 mM Tris-HCl pH 8 before verification by HPLC and quantification by spectrophotometry.

For testing ppGpp and pppApp hydrolysis, 20-μl reaction mixtures containing either 3 mM ppGpp (Jena Bioscience) or 180 μM pppApp and 7 μM 6× His-Mesh1 or 6× His-Aph1 in 10 mM Tris-HCl pH 8, 100 mM NaCl and 15 mM $MgCl_2$ were incubated at 37 °C for 2 h. The following steps and the separation using HPLC were performed as described above.

Accession Numbers

Proteins that were analyzed in this study were encoded by genes belonging to a prophage carried by *Streptococcus pneumoniae* SPNA45, whose organism code in Kyoto Encyclopedia of Genes and Genomes (https://www.genome.jp/kegg/) is snu and GenBank HE983624. The start and end limits of the prophage are 294 427 and 329 589, respectively.[4] The locus tags and NCBI-protein ID were respectively SPNA45_00317 and CCM07607 for Apk2, SPNA45_00318 and CCM07608 for IapK and SPNA45_00319 and CCM07609 for Aph1.

UniProt ID used to collect the protein sequences, whose (p)ppApp synthetase motifs have been aligned, are the following (the organism from which they originate is indicated in brackets): TAS1_PSEAB (*Pseudomonas aeruginosa*), A5ZE37 (*Bacteroides caccae*) A0A7Y7QXY9 (*Sphingomonas sanguinis*), A0A3L8C885 (*Ketobacter sp.*), A0A1I3VM54 (*Paraburkholderia megapolitana*), A0A7W4VSZ0 (*Nocardioides soli*), A0A2S6HSH1 (*Hungatella xylanolytica*), A0A502JM48 (*Haemophilus haemolyticus*), A0A7V7UC92 (*Candidatus Galacturonibacter soehngenii*), H5Y2L1 (*Desulfosporosinus youngiae* DSM 17734), A0A096KKG4 (*Collinsella sp.* 4_8_47FAA), A0A437UU02 (*Coriobacteriales bacterium* OH1046), A0A2N6SUF2 (*Finegoldia magna*), B8I908 (*Ruminoclostridium cellulolyticum*), V4NCR8 (*Pasteurella multocida*), A0A547E9T1 (*Mannheimia haemolytica*).

CRediT authorship contribution statement

Julia Bartoli: Conceptualization, Methodology. **Audrey C. Tempier:** Methodology. **Noa L. Guzzi:** Methodology. **Chloé M. Piras:** Methodology. **Eric Cascales:** Writing – review & editing. **Julie P.M. Viala:** Conceptualization, Supervision, Writing – review & editing.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2023.168282.

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Abbreviations:
CDI, contact dependent inhibition; MSA, multiple sequence alignment; PT, polymorphic toxin; RSH, RelA SpoT homolog; SAH, small alarmone hydrolase; SAS, small alarmone synthetase; TxSS, type x secretion system

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Table S1 -Bacterial strains

Lab code	Name	Genotype	Reference
EB3	BTH101	F-, *cya*-99, *araD*139, *galE*15, *galK*16, *rpsL*1 (Str r), *hsdR*2, *mcrA*1, *mcrB*1	1
EB70	DH5α	*fhuA*2 Δ(*argF*-*lacZ*)U169 *phoA gln*V44 Φ80 Δ(*lacZ*)M15 *gyrA*96 *recA*1 *relA*1 *endA*1 *thi*-1 *hsdR*17	Lab stock
EB72	BL21(DE3) pLys	F- *ompT gal dcm lon hsdSB* (rB- mB-) λ(DE3) pLysS(cmR)	Lab stock
EB944	MG1655	F^- λ^- ilvG- rfb-50 rph-1	Lab stock
EB421	Δ*relA*	MG1655 Δ*relA* without antibiotic resistance	2
EB544	$ppGpp^+$	MG1655 Δ*relA spoT*203	3
EB1080	ppGpp°	MG1655 Δ*relA* Δ*spoT* without antibiotic resistance	This study

Table S2- Plasmids

Lab code	Description	Reference
pEB1017	pBAD33	4
pJV381	pBAD33-*apk2*$_{tox\text{-}snu}$ (PCR product w/ primers ebm 2104/2095 cloned in pEB1017 at restriction sites KpnI/SalI)	This study
pJV390	pBAD33-*apk2*$_{tox\text{-}snu}$ D72G (mutagenesis w/ primers ebm 2118/2119 on pJV381)	This study
pEB698	pSM11harbors a truncated version of *relA* coding for a constitutively active protein of 455 amino acids out of 742. Expression is driven by P_{tac}.	5
pEB699	pSM12 harbors a truncated version of *relA* coding for an unactive protein of 331 amino acids out of 742. Expression is driven by P_{tac}.	5
pEB227	pBAD24	4
pEB774	pBAD24_SpoT (SpoT contains mutation Y190H)	Generous gift from E. Bouveret
pEB1242	pASK-IBA37plus	IBA
pJV374	pP_{tet} *iapK* (PCR product w/ primers ebm 2083/2084 cloned in pEB1242 at restriction sites EcoRI-XhoI)	This study
pJV375	pP_{tet} *aph1* (PCR product w/ primers ebm 2085/2086 cloned in pEB1242 at restriction sites EcoRI-XhoI)	This study
pJV377	pP_{tet} *mesh1* (mesh1 cloned in pEB1242 at restriction sites EcoRI-HindIII)	This study
pJV378	pP_{tet} *iapK aph1*	This study
pJV390	pP_{tet} *aph1* D48Y	This study
pJV417	pP_{tet} SPNA45_00320 (PCR product w/ primers ebm 2156/2157 cloned in pEB1242 at restriction sites EcoRI/XhoI)	This study
pJV418	pP_{tet} SPNA45_00321 (PCR product w/ ebm 2158/2159 cloned in pEB1242 at restriction sites EcoRI/XhoI)	This study
pEB354	pKT25linker	6
pEB362	TolB	Generous gift from E. Bouveret
pJV396	pT25-Apk2$_{tox\text{-}snu}$ D72G (PCR product w/ primers ebm 2122/2082 cloned in pEB354 at restriction sites EcoRI/XhoI)	This study
pEB355	pUT18Clinker	6
pEB356	Pal	Generous gift from E. Bouveret

pJV397	pT18-IapK (PCR product w/ primers ebm 2083/2084 cloned in pEB355 at restriction sites EcoRI/XhoI)	This study
pJV398	pT18-Aph1 (PCR product w/ ebm 2085/2086 cloned in pEB355 at restriction sites EcoRI/XhoI)	This study
pEB1520	pETDuet-1	Novagen
pJV403	pETDuet-1 *apk2*$_{tox\text{-}snu}$ *iapK* (PCR product w/ primers ebm 2129/2082 and ebm 2130/2131 cloned in pEB1520 at restriction sites EcoRI/SalI and NdeI/XhoI)	This study
pJV405	pETDuet-1- *apk1*$_{tox}$pau *iapK pau* (PCR product w/ primers ebm 2124/2125 and ebm 2126/2127 cloned in pEB1520 at restriction sites KpnI/SalI and NdeI/XhoI) (Apk1$_{tox\text{-}pau}$ contains mutation R206S)	This study
pEB1886	Rel$_{seq}$ (1-385) production plasmid	7

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1 Table S3- Primers

Lab code	Sequence	Usage
ebm 2082	ctcctcgagTCATTTAACACGCTCAATGTTTTT	Cloning *apk2*$_{tox\text{-}snu}$
ebm 2083	gaagaattcAGCGTGTTAAATGATATGAAAGAC	Cloning *iapK*$_{snu}$
ebm 2084	ctcctcgagTCAAGCTGCCACCATGCGG	Cloning *iapK*$_{snu}$
ebm 2085	gaagaattcATTGATATTGCACTTGCAATCG	Cloning *aph1*$_{snu}$
ebm 2086	ctcctcgagTTATGTGGATAAATAATAAATCGCG	Cloning *aph1*$_{snu}$
ebm 2095	ccagtgaattcctcgagcacgtgTCATTTAACACGCTCAATGTTTTTTG	Cloning *apk2*$_{tox\text{-}snu}$
ebm 2104	ggtggtaccGGGGGcgtctgg**atg**GCGAAAGCTAAATTCTATAGTGAA	Cloning *apk2*$_{tox\text{-}snu}$
ebm 2109	gcgaattaatacgactcactatagggcttaagtataaggaggaaaaaatatgGCGAAAGCTAAATTCTATAGTGAA	Fwd IVT *apk2*$_{tox\text{-}snu}$
ebm 2110	aaacccctccgtttagagaggggttatgctagttaTTAtttttcgaactgcgggtggctccaTTTAACACGCTCAATGTTTTTT GGAA	Rev IVT *apk2*$_{tox\text{-}snu}$
ebm 2118	AAAGCAGTTAGCAAAATTAACGgCGCTTTACGTTATACAACTATCTTT	Mutagenesis *apk2*$_{tox}$-*snu*
ebm 2119	AAAGATAGTTGTATAACGTAAAGCGcCGTTAATTTTGCTAACTGCTTT	Mutagenesis *apk2*$_{tox\text{-}snu}$
ebm 2120	GCGAATTAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGAGTAAAG GAGAAGAACTTTTCAC	Fwd IVT *gfp*
ebm 2121	AAACCCCTCCGTTTAGAGAGGGGTTATGCTAGTTATTATTTTTCGAACTGCGGGTGGCTCC ATTT	Rev IVT *gfp*
ebm 2122	gaagaattcatgGCGAAAGCTAAATTCTATAGTGAA	Cloning *apk2*$_{tox\text{-}snu}$
ebm 2124	gtcgtcgac**TCA**ATTGCCATTGCCTTTGCGC	Cloning *apk1*$_{tox\text{-}pau}$
ebm 2125	gaagaattcg**ATG**GCACGGCTCGGCAACG	Cloning *apk1*$_{tox\text{-}pau}$
ebm 2126	catcat**ATG**GCAATTGAAAAGGGCGAAG	Cloning *iapK*$_{pau}$

ebm 2127	ctcctcgagGCCCTTGGGAAAGCCCGTC	Cloning *iapK*$_{pau}$
ebm 2129	gaagaattcg**ATG**GCGAAAGCTAAATTCTATAGTGAA	Cloning *apk2*$_{tox\text{-}snu}$
ebm 2130	catcat**ATG**AAAGACATTAAGTATTACCGTAC	Cloning *iapK*$_{snu}$
ebm 2131	ctcctcgagAGCTGCCACCATGCGGTCAA	Cloning *iapK*$_{snu}$
ebm 2156	gaagaattcATGAAATATCGCAAAAAGCCCG	Cloning SPNA45_00320
ebm 2157	ctcctcgagTTATTCCTCGGTCTTCTCATAA	Cloning SPNA45_00320
ebm 2158	gaagaattcATGCTTGAAAAGGCTAAGCAAT	Cloning SPNA45_00321
ebm 2159	ctcctcgagCTAATCCTTAATTGCGCGGTT	Cloning SPNA45_00321

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