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Julia Bartoli, Audrey C Tempier, Noa L Guzzi, Chloé M Piras, Eric Cascales, et al.. Characterization of a (p)ppApp Synthetase Belonging to a New Family of Polymorphic Toxin Associated with Temperate Phages. *Journal of Molecular Biology*, 2023, 435 (21), pp.168282. 10.1016/j.jmb.2023.168282. hal-04231945

HAL Id: hal-04231945

<https://hal.science/hal-04231945>

Submitted on 9 Oct 2023

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1 **Characterization of a (p)ppApp synthetase belonging to a new family of polymorphic**
2 **toxin associated with temperate phages**

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1 **ABSTRACT**

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

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18 Keywords: MuF, Apk2, IapK, Aph1, modified nucleotide

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

25

1 INTRODUCTION

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

17 Bioinformatic analyses identified a new family of PTs, associated with temperate
18 phages [3,4]. The conserved N-terminal domain of these PTs corresponds to MuF, which shares
19 homologies with the F protein of the Mu phage that infects *Escherichia coli*. MuF proteins
20 categorize into two length variants: short proteins containing only the MuF domain, and long
21 proteins that include a C-terminal extension either of unknown function or with a predicted
22 toxic activity [4]. The MuF long versions with a C-terminal toxic domain thereby define this
23 new family of PTs, for which a variety of toxic activities can be predicted: nuclease,
24 metallopeptidase, ADP-ribosyl transferase and RelA_SpoT domains.

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

1 synthesis and degradation domains are characterized by the presence of conserved sequence
2 motifs. The (p)ppGpp synthetase domain is comprised of five motifs, Syn1-5, involved in the
3 coordination of magnesium, GDP/GTP and ATP. The (p)ppGpp HD hydrolase domain bears
4 six conserved catalytic motifs, HD1-6, involved in the coordination of manganese and the
5 guanine base [17].

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

15 Here, we show that the C-terminal RelA_SpoT domain of the *smu* MuF PT is bactericidal
16 when produced in *E. coli*, and that it carries a (p)ppApp synthetase activity. Its toxicity is
17 counteracted by two immunity proteins encoded by genes immediately downstream of the toxin
18 gene. While the first immunity binds to and inhibits the toxin, the second candidate immunity
19 protein harbors a functional (p)ppApp hydrolase domain similar to SpoT HD. We finally
20 compare (p)ppApp and (p)ppGpp synthetase sequences and identify conserved amino acid
21 positions in the Syn2 and Syn4 motifs that might serve as signature of (p)ppApp or (p)ppGpp
22 synthetases. While we were conducting our study, Ahmad et al. published a study on the same
23 enzyme encoded by a prophage of the Gram negative bacterium *Bacteroides caccae* [19]. As
24 our work corroborates their results, we adopted the same nomenclature to avoid confusion.
25 (p)ppApp synthetase toxic domains were named Apk (adenosine 3'-pyrophosphokinase). Apk

1 domains associated with N-terminal PAAR and MuF were named Apk1 and Apk2,
2 respectively. The (p)ppApp hydrolase enzyme was named Aph1 for adenosine 3'-
3 pyrophosphohydrolase. However, taking into consideration the change of nomenclature for the
4 Tas1 effector domain, we propose to name the immunity proteins that bind and inhibit Apk1
5 and Apk2 domains IapK (immunity of adenosine 3'-pyrophosphokinase).

6

7 RESULTS

8 **Apk2_{tox-snu} is a bactericidal toxin in *E. coli***

9 In addition to genes encoding phage components, the *S. pneumoniae* SPNA45 (snu) large
10 prophage comprises the *apk2*, *iapK* and *aph1* genes located between the portal and scaffold
11 genes (Fig. 1a). Apk2 is composed of an N-terminal MuF domain fused to a C-terminal
12 RelA_SpoT domain [4]. To evaluate the toxicity of the C-terminal domain, hereafter named
13 Apk2_{tox-snu}, the corresponding coding sequence was cloned into the pBAD33 plasmid under the
14 control of the P_{BAD} promoter. While *E. coli* MG1655 cells producing the putative toxin grew
15 similarly to those bearing the empty parental plasmid in repression conditions, no colony was
16 growing when Apk2_{tox-snu} expression was induced (Fig. 1b). This toxicity was due to the
17 enzymatic activity of Apk2_{tox-snu} since the substitution of the conserved Syn2 aspartate 72
18 residue, involved in Mg²⁺ binding in (p)ppGpp synthetases, abolished Apk2_{tox-snu} toxicity
19 (Fig. 1b).

20 To determine whether Apk2_{tox-snu} has bacteriostatic or bactericidal impacts on cell
21 growth and viability, *E. coli* cells were grown in liquid medium to mid-exponential phase,
22 Apk2_{tox-snu} expression was induced, and cells harvested at different time post-induction were
23 washed and spotted on a repressive LB agar medium. Growth monitoring in liquid medium
24 showed that bacteria stopped growing from the moment Apk2_{tox-snu} was produced (Fig. 1c), and
25 they were not able to resume growth when washed and spotted on a repressive LB agar medium

1 (Fig. 1d). In comparison, cells producing a constitutively active truncated RelA variant (RelA
2 Δ Ct 288) [20] stopped growing upon induction in liquid medium but formed colonies on
3 repressive LB agar medium, whereas cells carrying the empty parental plasmid or producing
4 an inactive version of RelA (RelA Δ Ct 412) [20] grew normally upon induction (Fig. 1c and
5 1d). Taken together, these results indicate that the activity of $\text{Apk2}_{\text{tox-snu}}$ is bactericidal.

6 It has been proposed that the bactericidal effect associated with the production of Apk
7 domains results from the depletion of ADP and ATP and hence alters essential metabolism [18].
8 In agreement with this hypothesis, an *in vitro* coupled transcription/translation assay attempting
9 to produce $\text{Apk2}_{\text{tox-snu}}\text{-Strep}_{\text{tag}}$ ($\text{Apk2}_{\text{tox-snu}}^{\text{St}}$) did not provide any product that could be
10 immunodetected (Fig. 1e). While GFP- $\text{Strep}_{\text{tag}}$ (GFP^{St}) could be produced with such an assay,
11 no GFP^{St} could be detected if the template to produce $\text{Apk2}_{\text{tox-snu}}^{\text{St}}$ was also added (Fig. 1e). We
12 suggest that nucleotide di- or tri-phosphate consumption by $\text{Apk2}_{\text{tox-snu}}$ leads to
13 transcription/translation inhibition. Although *in vitro* production can be a solution when
14 working with toxic proteins, this experiment further shows that it is unlikely an option when
15 trying to produce Apk.

16

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

18 The fact that $\text{Apk2}_{\text{tox-snu}}$ shares homologies with RelA_SpoT domains prompted us to test
19 whether it synthesizes (p)ppGpp. We therefore used a genetic approach by complementation of
20 an *E. coli* strain unable to produce (p)ppGpp (ppGpp°). While a wild-type strain grows on
21 minimal media, the ppGpp° strain cannot since it does not synthesize (p)ppGpp to activate
22 amino acid biosynthesis pathways [21]. As expected, our control experiment showed that the
23 production of SpoT complemented the ppGpp° strain, demonstrating that (p)ppGpp is
24 synthesized (Fig. 2a). By contrast, the production of $\text{Apk2}_{\text{tox-snu}}$ did not complement the ppGpp°
25 strain, suggesting that no (p)ppGpp is synthesized (Fig. 2a). Though, this experiment is tricky

1 to interpret, as it is difficult to distinguish between the protein's toxicity and the absence of
2 production of (p)ppGpp. Indeed, the absence of complementation could be due to the toxicity
3 of Apk2_{tox-snu} although the strain was grown in glucose minimal medium, which is a repressive
4 condition for pBAD-driven expression, as the wild-type strain carrying *apk2*_{tox-snu} is slightly
5 intoxicated (Fig. 2a). In addition, if the protein is too active, too much (p)ppGpp would inhibit
6 the growth of the ppGpp^o strain that is deleted of both *relA* and *spoT*.

7 To better define the activity of Apk2_{tox-snu}, the protein was purified (see Material and Methods)
8 and its activity on nucleotide phosphate was assayed using an *in vitro* assay. As controls, we
9 also purified and assayed Rel_{seq} (1-385) and Apk1_{tox} (Tas1_{tox}). Rel_{seq} (1-385) is the catalytic N-
10 terminal fragment (residues 1 to 385) of the bifunctional Rel/Spo homolog from *S. dysgalactiae*
11 *subsp. equisimilis* that displays (p)ppGpp synthetase activity [22], while Apk1_{tox} corresponds
12 to the C-terminal domain of the *P. aeruginosa* PA14 T6SS Tas1 PT that displays (p)ppApp
13 synthetase activity [18]. *In vitro* assays were first performed by incubating both ATP and GTP
14 with purified protein domains, before separation of the reaction products by Strong Anion
15 Exchange (SAX) High Performance Liquid Chromatography (HPLC). As expected, Rel_{seq} (1-
16 395) synthesized pppGpp (Fig. 2b). By contrast Apk2_{tox-snu} was responsible for the synthesis of
17 a different molecule (Fig. 2b), which one was also detected when ATP was provided as only
18 substrate for the *in vitro* assay (Fig. 2c). As previously shown, ATP was converted into AMP,
19 pApp and pppApp when incubated with the *P. aeruginosa* Apk1 toxin (Apk1_{tox-pau}) (Fig. 2c).
20 An identical profile was obtained when ATP was incubated with Apk2_{tox-snu} (Fig. 2c),
21 demonstrating that Apk2_{tox-snu} is a (p)ppApp synthetase.

22

23 **IapK and Aph1 are two distinct immunity proteins rescuing from Apk2_{tox-snu} toxicity**

24 The *apk2* gene is followed by 4 open reading frames encoding <150-residue proteins with no
25 assigned function (Fig. 1a). The first ORF is the best candidate for serving as immunity protein,

1 as they are usually encoded directly downstream the gene encoding the toxin. The second ORF
2 harbors a HD domain, found in a superfamily of metal-dependent phosphohydrolases, enzymes
3 that cleave phosphoester bonds of phosphorylated compounds. We suspected that the second
4 ORF could thus protect from the toxicity associated with the production of $Apk2_{tox-snu}$. The
5 genes corresponding to these two ORFs, that we named *iapK* and *aph1*, were cloned together
6 or independently into the pASK-IBA37+ vector, under the control of the P_{TET} promoter. Co-
7 production of Mesh1, a *Drosophila melanogaster* hydrolase, which has been shown to
8 hydrolyze both (p)ppGpp and (p)ppApp [23], with $Apk2_{tox-snu}$ shows that the *E. coli* cell
9 viability can be partly rescued (Fig. 3a), likely by the partial hydrolysis of the (p)ppApp pool.
10 The co-production of both candidate immunity proteins IapK and Aph1 fully rescued *E. coli*
11 and this rescue could only rely on IapK since its production alone provided full protection (Fig.
12 3a). Still, Aph1 was able to provide partial protection, comparable to that provided by Mesh1.
13 This partial protection is likely due to its phosphohydrolase activity as a substitution of the
14 predicted D48 catalytic residue did not confer protection (Fig. 3a). The third and fourth ORFs
15 downstream *apk2* were also tested for their ability to protect the cell against $Apk2_{tox-snu}$ but no
16 rescue was observed (data not shown).

17 An AlphaFold2 structural model suggested that IapK binds to $Apk2_{tox-snu}$ (Fig. 3b).
18 Interestingly, in this model the IapK protein interacts with a groove of the $Apk2_{tox-snu}$ toxin and
19 occludes the catalytic pocket (Fig. 3b). The interaction between IapK and a catalytic-null
20 variant of $Apk2_{tox-snu}$ (carrying the D72G substitution to avoid cell toxicity, see Fig. 1b) was
21 experimentally validated by a bacterial adenylate cyclase two-hybrid (BACTH) assay (Fig. 3c).
22 The IapK- $Apk2_{tox-snu}$ interaction was also detected by co-purification upon co-production of the
23 two partners from a pET-Duet vector (Fig. 3d). Taken together, these results demonstrate that
24 IapK inhibits $Apk2_{tox-snu}$ toxicity via protein-protein interaction, likely by occlusion of its active
25 site.

1 In contrast, no interaction was detected between $\text{Apk2}_{\text{tox-snu}}$ and Aph1 (Fig. 3c). Aph1
2 also partly rescued $\text{Apk2}_{\text{tox-snu}}$ toxicity and phenocopied the Mesh1 phosphohydrolase, in
3 agreement with the observation that Aph1 carries a phosphohydrolase HD motif. We therefore
4 conducted *in vitro* assays by co-incubating the purified Aph1 protein and pppApp or ppGpp
5 nucleotides. Mesh1 was used as control, as it was previously shown that it is a versatile
6 phosphohydrolase able to cleave the 3'-pyrophosphate group from (p)ppGpp and (p)ppApp
7 [23]. Indeed, the *in vitro* assay shows that Mesh1 hydrolyzed both pppApp and ppGpp (Fig. 3e).
8 In contrast, Aph1 only cleaved the 3'-pyrophosphate group from pppApp (Fig. 3e).

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

14

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

16 Classical protein sequence alignment tools, such as Blastp, do not distinguish (p)ppApp
17 synthetase and (p)ppGpp synthetase domains. As our work and the study of Ahmad et al.
18 identified three members of (p)ppApp synthetases, we carried out a protein multiple sequence
19 alignment (MSA) with representative members of well-known (p)ppGpp synthetases [24,25]
20 and newly experimentally characterized (p)ppApp synthetases [18,19]. We also included
21 homologs of Apk1_{tox} and Apk2_{tox} identified by Ahmad and collaborators and Jamet and
22 collaborators [4, 19], which belong to different bacterial genera and share between 18 and 64%
23 identity (Fig. 4). This MSA shows that the Syn1 motif is relatively well conserved between the
24 two sub-families, including the conserved Arg, Lys, Ser and Lys residues of the RxKxxxSxxxK
25 consensus. However, while the conserved Syn2 Asp and Syn4 Glu residues involved in Mg^{2+}

1 coordination and ATP binding [17] are perfectly conserved in both (p)ppApp and (p)ppGpp
2 synthetase domains, we noticed significant differences in these two motifs (Fig. 4). In Syn2,
3 the alignment shows that residue at positions +3 with respect to the conserved Asp residue
4 corresponds to short non-polar side-chain residue (Ala or Gly) in (p)ppGpp synthetase domains
5 while an Arg residue is found at this position in (p)ppApp synthetase domains. This Arg residue
6 is positioned at the +5 position in (p)ppGpp synthetases, where a Thr residue is found in
7 (p)ppApp synthetases. Thus, while the motif Syn2 is defined as DxxxxR in (p)ppGpp
8 synthetases, a conserved DxxRxT motif is found in (p)ppApp synthetases (Fig. 4 and 5a). In
9 Syn4, the residue at position +4 with respect to the conserved Glu residue corresponds to an
10 Arg residue in (p)ppGpp synthetases and His or Asn residue in (p)ppApp synthetases. Thus,
11 while the motif Syn4 is defined as ExQIRT in (p)ppGpp synthetases, a conserved ExQxH/N T
12 is associated with (p)ppApp synthetases (Fig. 4 and 5a). Finally, in the Syn3 motif that
13 coordinates GDP/GTP substrate in (p)ppGpp synthetase domains, the position +3 of the YxxxH
14 motif corresponds to a Ser and Gly residues in (p)ppGpp and (p)ppApp synthetases,
15 respectively, while the +5 His residue is not conserved in (p)ppApp synthetases and replaced
16 by a Ser or Asn residue (Fig. 4 and 5a). Interestingly, all the side chains of the Syn2, -3 and -4
17 residues that differ between (p)ppGpp and (p)ppApp synthetases locate on the same side of the
18 β -sheet of the *Apk2*_{tox-snu} AlphaFold2 model (Fig. 5b).

19

20 **DISCUSSION**

21 In this study, we demonstrated that the RelA_SpoT domain of *Apk2* from the large prophage
22 of the Gram-positive bacterium *S. pneumoniae* SPNA45 has a (p)ppApp synthetase activity.
23 With *Apk1* (or *Tas1*) from *P. aeruginosa* PA14 and *Apk2* from *B. caccae* temperate phage
24 [18,19], the *S. pneumoniae* *Apk2* protein is the third member of a family of strict (p)ppApp
25 synthetases. With the experimental set-up used in this study, i.e. heterologous expression of a

1 synthetic gene from the pBAD33 vector in *E. coli* MG1655, the activity of the (p)ppApp
2 synthetase domain is toxic and bactericidal. Apk2_{tox-snu} toxicity is abolished by the co-
3 production of IapK, which is encoded downstream of *apk2* on the *S. pneumoniae* SPNA45
4 prophage. IapK binds Apk2 and likely occlude its active site. The ORF downstream *iapK*
5 encodes Aph1, a protein with the HD motif specific to the superfamily of metal-dependent
6 phosphohydrolases. When co-produced with Apk2_{tox-snu}, Aph1 partially rescues cell survival.
7 Our *in vitro* results demonstrated that Aph1 cleaves pppApp to regenerate ATP, suggesting that
8 Aph1 detoxifies the cell from the accumulation of pppApp or from ATP depletion. It is not yet
9 clear why there are two immunity proteins. Possibly a cumulative effect would be required for
10 full protection, but this would have to be explored in physiological conditions of expression
11 and in the natural host. What is remarkable is that these two immunity proteins confer protection
12 through two distinct modes of action. IapK seems specific of the toxic partner protein since the
13 immunity protein from *B. caccae* is unable to protect from *P. aeruginosa* Apk1_{tox} toxicity [19].
14 In contrast, because the activity of Aph1 is directed toward the product of the (p)ppApp
15 synthetase and not toward the enzyme, its protection is “universal”. Indeed, a similar level of
16 protection was conferred by the *Drosophila* Mesh1 phosphohydrolase. As a result, Aph1 may
17 still offer a minimal level of protection to the bacterial host in case the latter would be
18 intoxicated by a variant form of Apk2_{tox} for which it would not have the specific IapK
19 immunity protein.

20 Our results also showed that Apk2_{tox} does not have (p)ppGpp synthesis activity. A
21 multiple sequence alignment with members of the (p)ppApp or (p)ppGpp synthetase domain
22 families showed that there are significant differences at some positions of the conserved and
23 functional Syn motifs. We therefore propose signatures that could distinguish (p)ppApp and
24 (p)ppGpp synthetases in Syn2 and Syn4 motifs: DxxxxR and ExQIRT, and DxxRxT and
25 ExQxH/N T in (p)ppGpp and (p)ppApp synthetases, respectively (Fig. 5a). Interestingly, recent

1 studies mention the ability of the *Methylobacterium extorquens* RSH, *B. subtilis* SasA and
2 *Treponema denticola* SAS proteins to synthesize both (p)ppGpp and (p)ppApp [26–28].
3 However, in agreement with the observation that the predominant activity of these enzymes is
4 (p)ppGpp synthesis, the examination of their sequences revealed Syn2 and Syn4 motifs typical
5 of (p)ppGpp synthetases. Conversely, the *Cellulomonas marina* FaRel, described to synthesize
6 both ppGpp and ppApp, has a sequence corresponding to a possible (p)ppApp synthetase
7 signature. Only the FaRel ppApp synthesis activity was confirmed *in vitro* [16].

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

17

18 Given the definition of polymorphic toxins and the involvement of conserved N-
19 terminal domains in a transport step, it is tempting to propose that the MuF domain of Apk2
20 serves to transport the toxin. The *muf* genes are generally located close to the genes encoding
21 the portal and terminase proteins and therefore belong to the head morphogenesis and DNA
22 packaging modules of the phage [4]. The portal protein i) acts as a nucleation site to initiate
23 capsid assembly, ii) forms a channel for the bidirectional passage of viral DNA, and iii) acts as
24 an attachment point for the tail of the phage. The portal protein also works in concert with the
25 TerSL terminase complex to translocate viral DNA. The TerS protein recognizes the DNA

1 based on a packaging signal and addresses it to the TerL protein, which is organized as a
2 pentameric ring interacting with a dodecamer of the portal protein [30]. Little is known about
3 the MuF protein and most studies have been carried out on the short MuF protein (Gp7) from
4 the *Bacillus subtilis* SPP1 virulent phage. A series of *in vitro* experiments proposed that Gp7
5 binds the Gp6 portal protein, and this interaction is necessary to locate Gp7 to the procapsid.
6 One or two copies of Gp7 would be therefore present in proheads and phages. Then, either
7 because the passage of DNA entering the capsid displaces the interaction between the portal
8 and Gp7 proteins, or because this entry triggers a conformational change in the portal protein,
9 Gp7 detaches and instead binds viral DNA. Although it is tempting to hypothesize that Gp7 is
10 co-ejected with viral DNA, this is not yet supported by experimental data. Instead, what has
11 been shown is that Gp7 would slow down the release of viral DNA by keeping it anchored into
12 the capsid by one of its extremities during the ejection. The absence of Gp7 does not prevent
13 the formation of virions, but these are 5 to 10 times less infectious [31–33]. However, recent
14 studies on staphylococcal temperate bacteriophage 80 α suggest that gp44, which belongs to the
15 (short) MuF family, has a post-injection role and is therefore co-injected with phage DNA.
16 These conclusions are based on a trans-complementation experiment in which the production
17 of gp44 in recipient cells restores cycles of infection of a $\Delta gp44$ phage lysate [34]. The authors
18 further suggest that gp44 would protect the phage DNA from degradation post-injection [35].

19 The presence of a toxin on a continuous polypeptide with MuF in the phage head and
20 the perspective that the toxin could be delivered to a recipient cell upon infection by
21 bacteriophage raises the question of the biological role. This question must be considered in the
22 context of infection by temperate phages since those are the ones associated with this novel PT
23 family. From the point of view of interbacterial competition, the injection of a toxin would be
24 beneficial to lysogenic bacteria already equipped with the immunity protein. However, this
25 immunity protein would have to be produced by the lysogen at the time of infection and, as it

1 is encoded within the module of the phage head morphogenesis, it is not clear why it should be
2 unless the phage is in lytic mode. Otherwise, PT and immunity genes, which together appear
3 as an operonic organization, would have to be independently regulated. Alternatively,
4 regardless of when the immunity protein is produced, the prophage could confer an advantage
5 on its host if it avoids poisoning thanks to a superinfection exclusion system. Other hypothesis
6 could be that the activity of the toxin favors the bacteriophage lysogenic cycle. For example,
7 one possibility could be that the products of the enzymatic activity regulate the expression of
8 genes that are important for lysogeny. Precisely, ppApp has been shown to bind to the RNA
9 polymerase, albeit at different sites compared to ppGpp, and an *in vitro* study showed a positive
10 regulation by ppApp on *rrnB* P1 activity, unlike ppGpp, highlighting the possibility of a distinct
11 impact for these two modified nucleotides [36]. A study comparing the cellular targets and
12 global effects of these two nucleotides would be of great interest. Otherwise, the enzymatic
13 activity could impact the state of the recipient cell and influence the lytic/lysogenic decision.
14 Indeed, although the activity of $\text{Apk2}_{\text{tox-snu}}$ was shown to be bactericidal in this study, the level
15 of intoxication might be lower with only a bacteriostatic effect in physiological conditions,
16 considering that only one or two copies of the polypeptide might be present in the phage head
17 as it has been estimated for the short MuF protein from the SPP1 bacteriophage from *B. subtilis*
18 [31]. In this case, can the arrest of host cell growth processes be unfavorable to the lytic cycle
19 option and lead to a lysogenic decision? In the context of the arms race between bacteria and
20 phages, another suggestion could be that the toxin's activity counteracts a possible anti-phage
21 mechanism. But why such a defense process would be more beneficial to temperate phages
22 than lytic ones?

23 Interesting new avenues of research aimed at understanding the biological role of these toxin-
24 antitoxin systems in the life cycle of temperate phages and their bacterial host are therefore

1 open. And more discoveries are yet to come with the investigation of MuF proteins that display
2 C-terminal domains of unknown functions.

3

4 **MATERIALS AND METHODS**

5 **Bacterial strains and media**

6 *E. coli* strains used in this study are described in Table S1. Bacteria were grown in 2YT, Luria-
7 Bertani (LB) (Sigma-Aldrich), MacConkey agar (BD), M9 minimal medium (1× M9 salts,
8 1 mM MgSO₄, 0.1 mM CaCl₂, 2 μg/mL vitamin B12, 0.2% glucose). Plasmids were maintained
9 by the addition of antibiotics (ampicillin 100 μg/mL, kanamycin 50 μg/mL or chloramphenicol
10 50 μg/mL).

11

12 **Plasmid construction and site-directed mutagenesis**

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

19

20 **Toxicity and toxicity neutralization assays**

21 For toxicity assays, *E. coli* MG1655 was transformed with pBAD33 encoding the Apk2_{tox-snu}
22 domain and transformants were selected on LB agar plate containing the appropriate antibiotic
23 and 1% glucose for toxin repression. Stationary phase overnight cultures were diluted to an
24 OD₆₀₀ of 0.05 in fresh LB medium supplemented with 1% glucose. Bacteria were cultivated at
25 37°C to exponential phase (OD₆₀₀ ≈ 0.5). An aliquot was washed twice with LB and cultures

1 were normalized to $OD_{600} = 0.5$. Serial dilutions in sterile PBS were performed and spotted on
2 LB agar containing 1% glucose for P_{BAD} promoter repression or 0.2% arabinose for induction.
3 For toxicity neutralization assays, *E. coli* MG1655 was co-transformed with pBAD33 encoding
4 the $Apk2_{tox-snu}$ domain and pASK-IBA37+ encoding the candidate immunity proteins. Co-
5 transformants were selected on LB agar containing the appropriate antibiotic and 1% glucose.
6 Experiments were carried out as described above and dilutions were spotted on LB agar plates
7 containing appropriate antibiotics, anhydrotetracycline 200 ng/mL to induce the P_{TET} promoter
8 from pASK-IBA37+, and either 1% glucose or 0.2% arabinose to repress or induce the P_{BAD}
9 promoter from pBAD33.

10

11 **Bacteriostatic or bactericidal effect of the toxin**

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

23

24 **In vitro transcription-translation assays**

1 Coupled *in vitro* transcription-translation assays were performed with the PURExpress® *In*
2 *vitro* Protein synthesis kit (NEB) supplemented with murine RNase inhibitor (NEB) as
3 recommended by the manufacturer. DNA templates encoding the $\text{Apk2}_{\text{tox-snu}}\text{-Strep}_{\text{tag}}$ and the
4 GFP- $\text{Strep}_{\text{tag}}$ proteins, were amplified using the primer pairs ebm2109/2110 and
5 ebm2120/2121, respectively. These templates were added to the reactions (3ng/ μL), which
6 were performed for 2 h at 37°C. Proteins were separated by SDS-PAGE, transferred onto
7 nitrocellulose membranes and *in vitro* synthesized proteins were detected by immunoblotting
8 with antibodies against Strep tag (Classic, BioRad).

9

10 **Bacterial two-hybrid**

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

15

16 **Protein production, purification or co-purification**

- 17 • Toxins purification or co-purification

18 6 \times His- $\text{Apk1}_{\text{tox-pau}}$ and 6 \times His- $\text{Apk2}_{\text{tox-snu}}$ were co-produced with their cognate IapK-S-tag
19 immunity proteins from the pET-Duet1 plasmid, using *E. coli* BL21 DE3 (pLys). For this, 1 L
20 of culture was grown at 30°C until $\text{OD}_{600} = 0.5$ and protein production was induced with
21 500 μM IPTG at 25°C for 4h. Cells were harvested by centrifugation at 4,000g for 20 min,
22 washed with PBS, and resuspended in 20 mL lysis buffer (20 mM Tris-HCl pH 8, 200 mM
23 NaCl, 10 mM imidazole 2 mM β -mercaptoethanol, 0.2% NP40) supplemented with DNase 0.1
24 mg/mL, MgCl_2 10 mM and protease inhibitor (PMSF, 0.5 mM). Cells were disrupted using a
25 high-pressure homogeniser (Emulsiflex) and unbroken cells or fragments were eliminated by

1 centrifugation at 15,000g for 30 min. His-tagged proteins were purified by affinity
2 chromatography on metal/cobalt affinity resin (Takara). Unbound fraction was recovered by
3 gravity using a Poly-Prep Chromatography column (Bio-rad) and the resin was washed with
4 15 mL of lysis buffer.

5 For purification of the $\text{Apk1}_{\text{tox-pau}}$ and $\text{Apk2}_{\text{tox-snu}}$ domains alone, inspired by the protocol of
6 Ahmad *et al.*, 2019, the immunity protein (IapK) was dissociated by denaturing the protein-
7 protein complex using 10 mL of lysis buffer supplemented with 8 M urea. Renaturation of the
8 toxin was then performed by washing the resin with 20 mL of lysis buffer. Finally, proteins
9 were eluted using lysis buffer containing 300 mM imidazole.

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

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

15 • Immunity-protein purification

16 6×His-IapK, 6×His-Aph1 and 6×His-Mesh1 were produced from pASK-IBA37+ in *E. coli*
17 MG1655. The experimental protocol for protein production and purification was the same as
18 described above except that only 100 mL of culture were necessary, that cells were disrupted
19 by sonication and buffer exchange was carried out by dialysis (Side-A-lyzer dialysis cassette,
20 3.500 MWCO; Thermo Scientific).

21

22 **Western Blot**

23 Western blot analyses of protein samples were performed using mouse anti-Strep-tag (Biorad),
24 mouse anti-His-tag (Proteintech) or mouse anti-S-tag (Sigma) and detected with anti-mouse

1 horseradish peroxidase-conjugated secondary antibodies (SantaCruz) or with anti-mouse
2 phosphatase alkaline-conjugated secondary antibodies (Sigma).

3

4 ***In vitro* synthesis or hydrolysis of (p)ppGpp/(p)ppApp coupled with HPLC analysis or** 5 **purification**

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

8 For testing pppApp synthetase activity, a 20- μ L reaction containing 5 mM ATP, used as both
9 the phosphate donor and acceptor, and 1 μ M of purified enzyme (Apk2_{tox-snu} or Apk1_{tox-pau}) in
10 10 mM Tris-HCl pH 8, 100 mM NaCl and 15 mM MgCl₂ was incubated at 37°C for 2 h. After
11 15-fold dilution in HPLC solvent A (KH₂PO₄ 50 mM pH 3.4), the enzyme was eliminated by
12 passing the reaction mixture through a spin filter column (Nanosep 10K Omega, Pall
13 Corporation).

14 For analytical purpose, 20 μ L of reaction was injected on an Agilent 1260 Infinity HPLC
15 system equipped with a SAX 5 μ m 4.6 \times 250 mm Waters Spherisorb analytical column.
16 Nucleotides separation was carried out over 35 min using an ionic strength gradient from
17 solvent A to solvent B (KH₂PO₄ 1 M pH 3.4) at a flow rate of 1 mL.min⁻¹ [38]. The nucleotides
18 were monitored at 254 nm (max absorption for guanosine nucleotide) and/or 260 nm (max
19 absorption for adenosine nucleotide).

20 For pppApp purification, the initial *in vitro* reaction was performed in 120 μ L, followed by
21 dilution and passing through the spin filter column, and 15 injections of 100 μ L of reaction
22 mixture were repeated during which the nucleotide was collected. The collected fractions were
23 pooled and purified using Oasis WAX SPE Cartridges (6 cc Vac Cartridge ; Waters) and

1 lyophilized as described before [38]. pppApp was then resuspended in 5 mM Tris-HCl pH 8
2 before verification by HPLC and quantification by spectrophotometry.

3 For testing ppGpp and pppApp hydrolysis, 20- μ L reaction mixtures containing either 3 mM
4 ppGpp (Jena Bioscience) or 180 μ M pppApp and 7 μ M 6 \times His-Mesh1 or 6 \times His-Aph1 in
5 10 mM Tris-HCl pH 8, 100 mM NaCl and 15 mM MgCl₂ were incubated at 37 °C for 2 h. The
6 following steps and the separation using HPLC were performed as described above.

7

8 **ACCESSION NUMBERS**

9 Proteins that were analyzed in this study were encoded by genes belonging to a prophage carried
10 by *Streptococcus pneumoniae* SPNA45, whose organism code in Kyoto Encyclopedia of Genes
11 and Genomes (<https://www.genome.jp/kegg/>) is snu and GenBank HE983624. The start and
12 end limits of the prophage are 294 427 and 329 589, respectively (Jamet 2017). The locus tags
13 and NCBI-protein ID were respectively SPNA45_00317 and CCM07607 for Apk2,
14 SPNA45_00318 and CCM07608 for IapK and SPNA45_00319 and CCM07609 for Aph1.

15 UniProt ID used to collect the protein sequences, whose (p)ppApp synthetase motifs have been
16 aligned, are the following (the organism from which they originate is indicated in brackets):

17 TAS1_PSEAB (*Pseudomonas aeruginosa*), A5ZE37 (*Bacteroides caccae*) A0A7Y7QXY9
18 (*Sphingomonas sanguinis*), A0A3L8C885 (*Ketobacter sp.*), A0A1I3VM54 (*Paraburkholderia*
19 *megapolitana*), A0A7W4VSZ0 (*Nocardioides soli*), A0A2S6HSH1 (*Hungatella xylanolytica*),
20 A0A502JM48 (*Haemophilus haemolyticus*), A0A7V7UC92 (*Candidatus Galacturonibacter*
21 *soehngeni*), H5Y2L1 (*Desulfosporosinus youngiae* DSM 17734), A0A096KKG4 (*Collinsella*
22 *sp.* 4_8_47FAA), A0A437UU02 (*Coriobacteriales bacterium* OH1046), A0A2N6SUF2
23 (*Finegoldia magna*), B8I908 (*Ruminoclostridium cellulolyticum*), V4NCR8 (*Pasteurella*
24 *multocida*), A0A547E9T1 (*Mannheimia haemolytica*).

25

1 **ACKNOWLEDGEMENTS**

2 We thank Emmanuelle Bouveret (Institut Pasteur, Paris France) for sharing materials and
3 knowledge. We are grateful to people from Cascales group and to Dukas Jurénas (ULB,
4 Belgium), a former post-doctoral fellow, for fruitful discussions. This work was funded by the
5 CNRS and the Aix-Marseille Université.

6

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19

1 Table S1 -Bacterial strains

Lab code	Name	Genotype	Reference
EB3	BTH101	F-, <i>cya</i> -99, <i>araD</i> 139, <i>galE</i> 15, <i>galK</i> 16, <i>rpsL</i> 1 (Str r), <i>hsdR</i> 2, <i>mcrA</i> 1, <i>mcrB</i> 1	1
EB70	DH5 α	<i>fhuA</i> 2 Δ (<i>argF-lacZ</i>)U169 <i>phoA glnV</i> 44 Φ 80 Δ (<i>lacZ</i>)M15 <i>gyrA</i> 96 <i>recA</i> 1 <i>relA</i> 1 <i>endA</i> 1 <i>thi</i> -1 <i>hsdR</i> 17	Lab stock
EB72	BL21(DE3) pLys	F- <i>ompT gal dcm lon hsdSB</i> (rB- mB-) λ (DE3) pLysS(cmR)	Lab stock
EB944	MG1655	F ⁻ λ : <i>ilvG- rfb</i> -50 <i>rph</i> -1	Lab stock
EB421	Δ <i>relA</i>	MG1655 Δ <i>relA</i> without antibiotic resistance	2
EB544	ppGpp ⁺	MG1655 Δ <i>relA spoT</i> 203	3
EB1080	ppGpp ^o	MG1655 Δ <i>relA \Delta</i> <i>spoT</i> without antibiotic resistance	This study

2

1 Table S2- Plasmids

Lab code	Description	Reference
pEB1017	pBAD33	4
pJV381	pBAD33- <i>apk2</i> _{tox-snu} (PCR product w/ primers ebm 2104/2095 cloned in pEB1017 at restriction sites KpnI/SalI)	This study
pJV390	pBAD33- <i>apk2</i> _{tox-snu} D72G (mutagenesis w/ primers ebm 2118/2119 on pJV381)	This study
pEB698	pSM11 harbors a truncated version of <i>relA</i> 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 <i>relA</i> 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} <i>iapK</i> (PCR product w/ primers ebm 2083/2084 cloned in pEB1242 at restriction sites EcoRI-XhoI)	This study
pJV375	pP _{tet} <i>aph1</i> (PCR product w/ primers ebm 2085/2086 cloned in pEB1242 at restriction sites EcoRI-XhoI)	This study
pJV377	pP _{tet} <i>mesh1</i> (<i>mesh1</i> cloned in pEB1242 at restriction sites EcoRI-HindIII)	This study
pJV378	pP _{tet} <i>iapK aph1</i>	This study
pJV390	pP _{tet} <i>aph1</i> 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- <i>Apk2</i> _{tox-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- <i>iapK</i> (PCR product w/ primers ebm 2083/2084 cloned in pEB355 at restriction sites EcoRI/XhoI)	This study
pJV398	pT18- <i>Aph1</i> (PCR product w/ ebm 2085/2086 cloned in pEB355 at restriction sites EcoRI/XhoI)	This study
pEB1520	pETDuet-1	Novagen
pJV403	pETDuet-1 <i>apk2_{tox-snu} iapK</i> (PCR product w/ primers ebm 2129/2082 and ebm 2130/2131 cloned in pEB1520 at restriction sites EcoRI/Sall and NdeI/XhoI)	This study
pJV405	pETDuet-1- <i>apk1_{tox-pau} iapK pau</i> (PCR product w/ primers ebm 2124/2125 and ebm 2126/2127 cloned in pEB1520 at restriction sites KpnI/Sall and NdeI/XhoI) (<i>Apk1_{tox-pau}</i> contains mutation R206S)	This study
pEB1886	Rel _{seq} (1-385) production plasmid	7

1

1 Table S3- Primers

Lab code	Sequence	Usage
ebm 2082	ctctcgagTCATTTAACACGCTCAATGTTTT	Cloning <i>apk2</i> _{tox-snu}
ebm 2083	gaagaattcAGCGTGTTAAATGATATGAAAGAC	Cloning <i>iapK</i> _{snu}
ebm 2084	ctctcgagTCAAGCTGCCACCATGCGG	Cloning <i>iapK</i> _{snu}
ebm 2085	gaagaattcATTGATATTGCACTTGCAATCG	Cloning <i>aph1</i> _{snu}
ebm 2086	ctctcgagTTATGTGGATAAATAATAAAATCGCG	Cloning <i>aph1</i> _{snu}
ebm 2095	ccagtgaattcctcgagcagctgTCATTTAACACGCTCAATGTTTTTTG	Cloning <i>apk2</i> _{tox-snu}
ebm 2104	ggtggtaccGGGGGcgtctg gatg GCGAAAGCTAAATTCTATAGTGAA	Cloning <i>apk2</i> _{tox-snu}
ebm 2109	gcgaattaatac gactcactatagggccttaagtataaggaggaaaaaatatgGCGAAAGCTAAATTCTATAGTGAA	Fwd IVT <i>apk2</i> _{tox-snu}
ebm 2110	aaaccctccgttagagaggggtatgctagtaTTAttttgaactcgggtggtccaTTTAAACACGCTCAATGTTTTTT GGAA	Rev IVT <i>apk2</i> _{tox-snu}
ebm 2118	AAAGCAGTTAGCAAAATTAACGgCGCTTACGTTATACAACTATCTTT	Mutagenesis <i>apk2</i> _{tox-snu}
ebm 2119	AAAGATAGTTGTATAACGTAAAGCGcCGTTAATTTTGCTAACTGCTTT	Mutagenesis <i>apk2</i> _{tox-snu}
ebm 2120	GCGAATTAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGAGTAAAG GAGAAGAACTTTTCAC	Fwd IVT <i>gfp</i>
ebm 2121	AAACCCTCCGTTTAGAGAGGGGTTATGCTAGTTATTATTTTCGAACTGCGGGTGGCTCC ATTT	Rev IVT <i>gfp</i>
ebm 2122	gaagaattcatgGCGAAAGCTAAATTCTATAGTGAA	Cloning <i>apk2</i> _{tox-snu}
ebm 2124	gtcgtcgac TC AATTGCCATTGCCTTTGCGC	Cloning <i>apk1</i> _{tox-pau}
ebm 2125	gaagaattcg AT GGCACGGCTCGGCAACG	Cloning <i>apk1</i> _{tox-pau}
ebm 2126	cat cat AT GGCAATTGAAAAGGGCGAAG	Cloning <i>iapK</i> _{pau}

ebm 2127	ctcctcgagGCCCTTGGGAAAGCCCGTC	Cloning <i>iapK</i> _{pau}
ebm 2129	gaagaattc <u>g</u> ATGGCGAAAGCTAAATTCTATAGTGAA	Cloning <i>apk2</i> _{tox-snu}
ebm 2130	catcat <u>ATG</u> AAAGACATTAAGTATTACCGTAC	Cloning <i>iapK</i> _{snu}
ebm 2131	ctcctcgagAGCTGCCACCATGCGGTCAA	Cloning <i>iapK</i> _{snu}
ebm 2156	gaagaattcATGAAATATCGCAAAAAGCCCG	Cloning SPNA45_00320
ebm 2157	ctcctcgagTTATTCCTCGGTCTTTCATAA	Cloning SPNA45_00320
ebm 2158	gaagaattcATGCTTGAAAAGGCTAAGCAAT	Cloning SPNA45_00321
ebm 2159	ctcctcgagCTAATCCTTAATTGCGCGGTT	Cloning SPNA45_00321

1

2

1 **References Supplementary Tables**

2

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26

1 **FIGURE CAPTIONS**

2 **Figure 1 *S. pneumoniae* Apk2_{tox-snu} is a bactericidal toxin**

3 (a) Schematic representation of the genetic environment of the *apk2* gene found within the large
4 prophage of *S. pneumoniae* SPNA45. Genes encoding proteins associated with capsid
5 formation are shown in color and genes shown in white correspond to proteins with not yet
6 defined functions. Locus tags are indicated above *apk2*, *iapK* and *aph1*. (b) Toxicity assays.
7 *E. coli* MG1655 cells carrying the pBAD33 empty plasmid (\emptyset) or pBAD33 expressing the wild-
8 type (Apk2_{tox-snu}) or the D72G catalytic-null variant of Apk2_{tox-snu} D72G, were grown to an
9 OD₆₀₀ 0.5, serially diluted, and spotted on LB agar containing glucose 1% or arabinose 0.2%
10 to repress or induce the production of the Apk2_{tox} domain, respectively. (c) Growth inhibition
11 in liquid medium. *E. coli* MG1655 cells carry the pBAD33 empty plasmid (\emptyset) or pBAD33
12 expressing wild-type Apk2_{tox}, or plasmids expressing constitutively-active (RelA Δ Ct 288) or
13 inactive (RelA Δ Ct 412) RelA C-terminal truncated variants [20]. Cell growth in liquid medium
14 was monitored upon induction of the production of the indicated protein. (d) Bactericidal effect
15 associated with the production of Apk2_{tox-snu}. *E. coli* MG1655 cells from panel (c) were
16 harvested 0, 30, and 60 min post-induction, washed, serially diluted and spotted on LB agar
17 containing glucose to repress the production of the indicated protein. (e) *In vitro*
18 transcription/translation assays with indicated DNA templates coding Apk2_{tox-snu}-Strep_{tag}
19 (Apk2_{tox-snu}St) or GFP-Strep_{tag} (GFPSt). Products of the assays were separated on SDS-PAGE
20 and immunodetected with an antibody against the Strep tag. MW: molecular weight.

21

22 **Figure 2 Apk2_{tox-snu} has (p)ppApp synthetase activity**

23 (a) Complementation assays. Wild-type and ppGpp^o *E. coli* strains carrying the empty plasmid
24 (\emptyset) or containing the *spoT* or *apk2*_{tox-snu} genes were streaked on minimal medium agar. (b, c)
25 *In vitro* synthesis assay of modified nucleotides. The indicated domains were purified and

1 incubated in the presence of ATP/GTP (b) or ATP alone (c). The reaction products were
2 separated by anion exchange HPLC. In the control panel, no enzyme was mixed with the
3 nucleotides. Rel_{seq} is the catalytic N-terminal fragment (residues 1 to 385) of the bifunctional
4 Rel/Spo homolog from *S. dysgalactiae subsp. equisimilis* [22]. Apk1_{tox__pau} is the (p)ppApp
5 synthetase domain from the formerly named T6SS-effector Tas1 from *Pseudomonas*
6 *aeruginosa* PA14 [18,19].

7

8 Figure 3 **Two distinct immunity proteins confer protection against Apk2_{tox-snu}**

9 (a) Toxicity neutralization assays. *E. coli* MG1655 cells producing Apk2_{tox-snu} and the HD
10 domain *Drosophila melanogaster* Mesh1 protein, or one or the two ORFs following the *apk2*
11 gene: *iapK* and *aph1*, or the *aph1* D48Y variant were grown to OD₆₀₀ 0.5, serially diluted and
12 spotted on LB agar containing anhydrotetracycline to induce the production of the proteins for
13 which immune function was tested, and glucose 1% or arabinose 0.2% to repress or induce the
14 production of the Apk2_{tox-snu}, respectively. (b) Ribbon (top) and surface (bottom)
15 representations of the Apk2_{tox-snu}- IapK complex AlphaFold2 structural model. Apk2_{tox-snu} and
16 IapK are shown in purple and pink, respectively. The side chains of the two conserved Syn2
17 Asp and Syn4 Glu residues, involved in Mg²⁺ binding in (p)ppGpp synthetases, are highlighted
18 in red. (c) BACTH experiment. *E. coli* BTH101 cells producing the indicated proteins fused to
19 the T18 or T25 domains of the *Bordetella pertussis* adenylate cyclase were spotted on
20 MacConkey agar (Ø, no protein fused to the T18 or T25 domain). The T25_TolB / T18_Pal
21 pair serves as a positive control of protein-protein interaction. Due to high toxicity of the
22 Apk2_{tox-snu} toxin, the inactivated version D72G was used in these experiments. (d) Co-
23 purification. Cell lysates of *E. coli* BL21(DE3) cells producing His_{tag}-Apk2_{tox-snu} and IapK-S_{tag}
24 were subjected to immobilized metal affinity chromatography on cobalt beads. Fractions
25 corresponding to the protein extract, unbound proteins and two successive elutions were

1 separated by SDS-PAGE, transferred onto nitrocellulose membranes and immunodetected
2 using anti-His (upper blot) and anti-S-tag (lower blot) antibodies. (e) Chromatograms of the *in*
3 *vitro* degradation assay of modified nucleotides. The indicated proteins were purified and their
4 activity on the nucleotides indicated in the frames was tested *in vitro* (control, no protein). The
5 products of the reaction were separated by anion exchange HPLC.

6

7 **Figure 4 Multiple sequence alignment of (p)ppGpp and (p)ppApp synthetase domains**

8 Clustal Omega protein multiple sequence alignment of synthesis domains from (p)ppGpp
9 synthetases (RelA and SpoT from *Escherichia coli* (eco), Rel from *Streptococcus dysgalactiae*
10 *subsp. equisimilis* (seq), and small alarmone synthetases YjbM and YwaC from *Bacillus subtilis*
11 (bsu)) and from Apk1 and Apk2 (p)ppApp synthetase domains from different bacterial genera
12 (see Accession Numbers hereinafter for protein ID). Members of the two synthetase families
13 are separated by a black line. Amino acids are colored according to Clustal omega grouping
14 (red, acidic residues; blue, basic residues except His; grey, hydrophobic and aromatic residues
15 except Tyr; the other amino-acids were left uncolored). The 5 conserved motifs (Syn1-5) of the
16 (p)ppGpp synthetase domains are indicated. Catalytic Asp (Syn2) and Glu (Syn4) residues,
17 involved in Mg²⁺ binding, are indicated by red stars. Residues that are conserved but different
18 in each synthetase family are boxed. MSA was shorten after syn5 motif, most of Apk2
19 synthetase domain sequences were already ended.

20

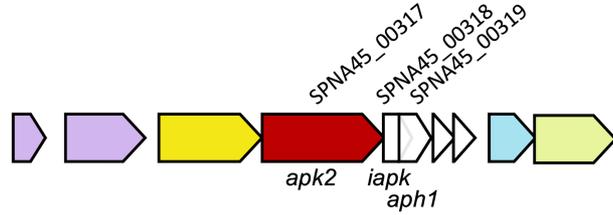
21 **Figure 5 Distinction between (p)ppGpp and (p)ppApp synthetases**

22 **(a)** Domain architecture of enzymes involved in (p)ppGpp and (p)ppApp metabolism (domains
23 found in the C-terminal regulatory region of SpoT are not detailed). Catalytic motifs are shown
24 as black lines. Dashed lines symbolized slightly different motif from the (p)ppGpp synthetase
25 reference. Scheme inspired form Steinchen and Bange 2016 [17]. (b) Ribbon representation of

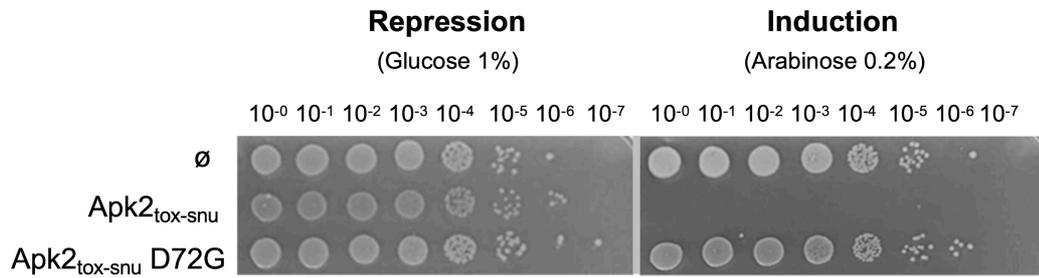
1 the *Apk2*_{tox-snu} AlphaFold2 structural model. The side chains of residues conserved in the syn
2 motifs of (p)ppApp synthetases, but which are different from those found in (p)ppGpp
3 synthetases (in bold in the (p)ppApp synthetase box, Fig. 5a) are colored grey and heteroatom.
4 The side chain colored heteroatom of the conserved catalytic Asp (Syn2) and Glu (Syn4)
5 residues are also shown.

(a)

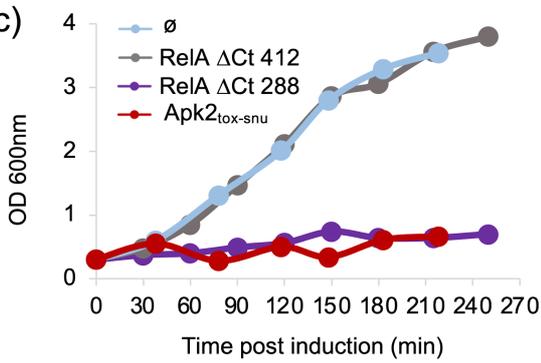
■ Terminase
■ Portal
■ Apk2
 (MuF and RelA_SpoT domains)



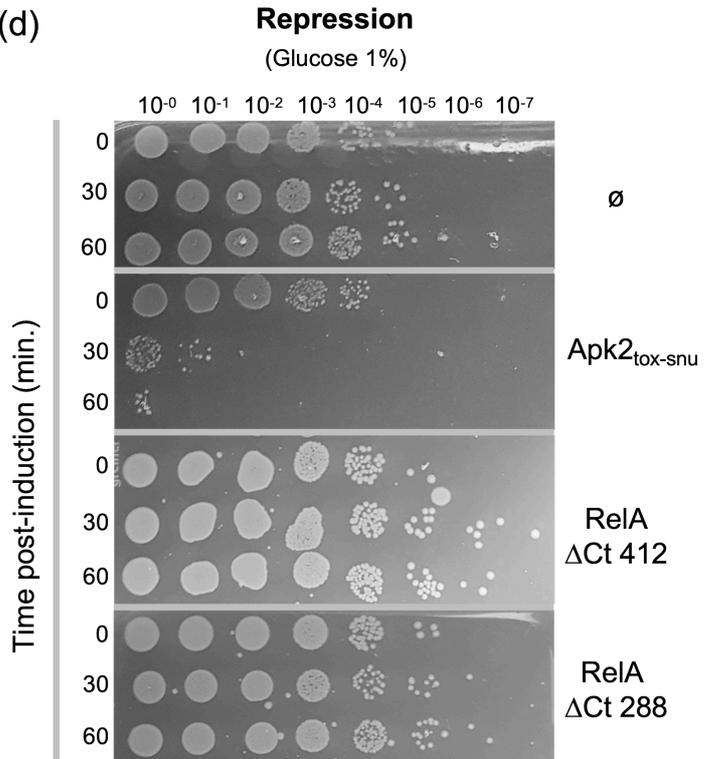
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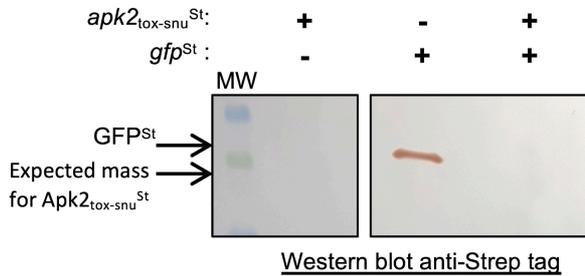
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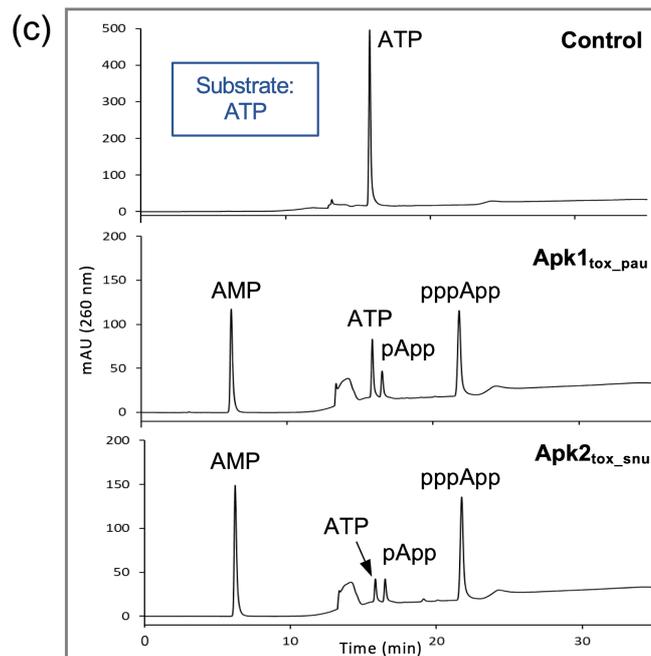
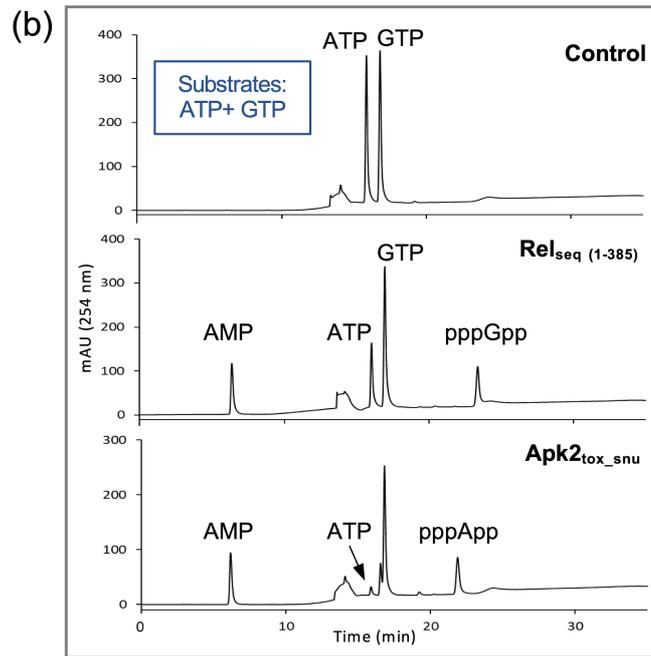
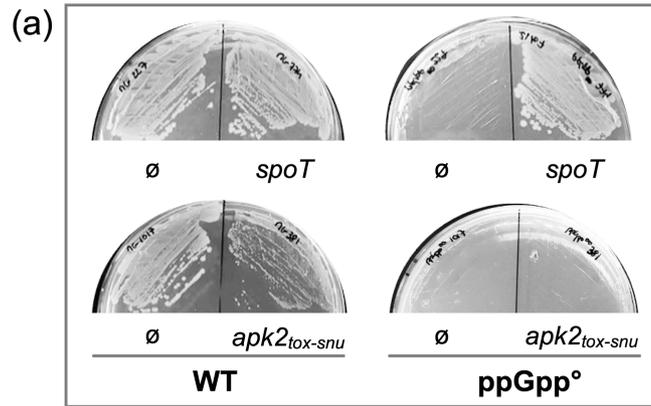


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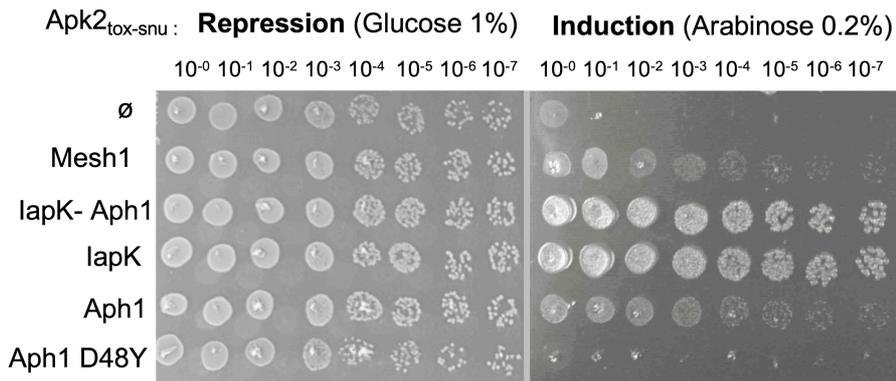


(e) DNA templates

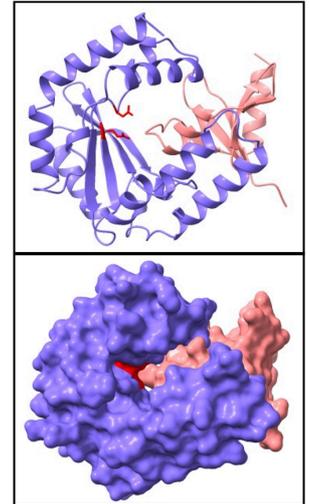




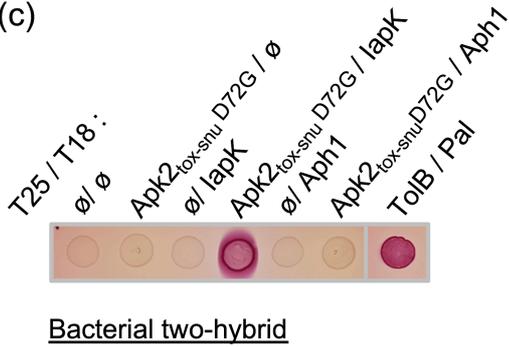
(a)



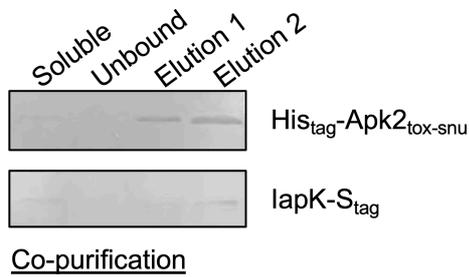
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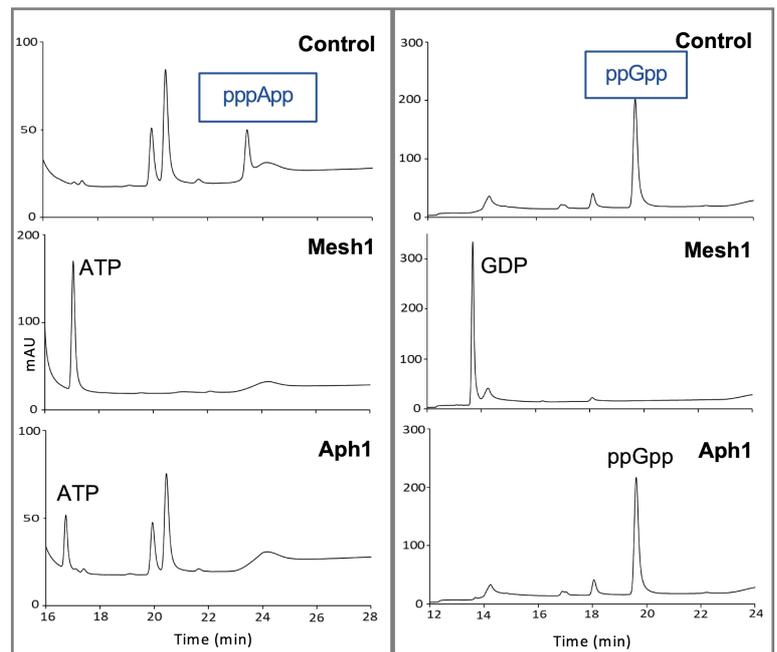
(c)



(d)

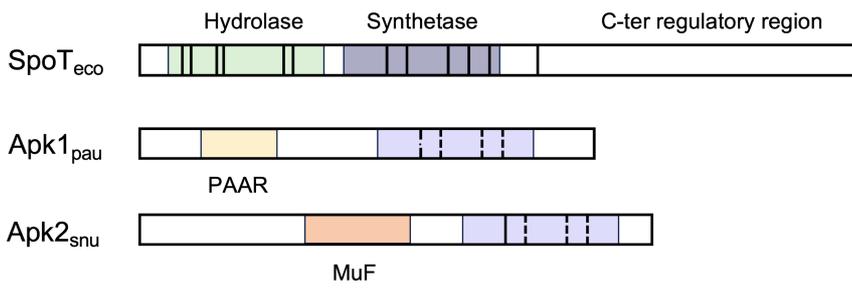


(e)



(a)

(p)ppGpp synthetase				
Syn1	Syn2	Syn3	Syn4	Syn5
RxKxxxSxxxK	DxxxxR	YxxxH	ExQIRT	H



(p)ppApp synthetase			
Syn1	Syn2	Syn3	Syn4
RxKxxxSxxxK	DxxRxT	YxGxN	ExQxH/NT

(b)

