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A eukaryotic-like Serine/Threonine kinase protects Staphylococci against viruses

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Summary

Organisms from all domains of life are infected by viruses, the most abundant biological entities on earth. In eukaryotes, Serine/Threonine kinases play a central role in antiviral response. Bacteria, however, are not commonly known to use protein phosphorylation as part of their defense against phages. Here we identify Stk2, a Serine/Threonine kinase that provides efficient immunity against bacteriophages in staphylococci through abortive infection. A phage protein of unknown function activates the kinase activity of Stk2 when expressed in the cell. This leads to the phosphorylation of several proteins involved in translation, global transcription control, cell cycle control, stress response, DNA topology, DNA repair and central metabolism. Cells die as a consequence of Stk2 activation, thereby preventing phage propagation to the rest of the bacterial population. Our work shows that mechanisms of viral defense that rely on protein phosphorylation constitute an antiviral strategy conserved across domains of life.

1 Introduction

2 The arms race between bacteria and phages has led to the evolution of many bacterial defense
3 systems that can act at every stage of the phage life cycle, blocking phage adsorption, DNA
4 injection, degrading phage DNA, and interfering with phage replication or the production of
5 phage proteins (Labrie et al., 2010). These defense systems are mechanistically diverse and
6 can vary considerably among bacterial species or even among different isolates of a particular
7 species. At a glance, bacterial defense against phages has little in common with eukaryotic
8 antiviral systems. In plants, defense is primarily conducted via RNA-mediated interference
9 (RNAi), while in vertebrates, pattern-recognition receptors (PRR) recognize nucleic acids and
10 proteins from pathogens and activate the interferon, proinflammatory and adaptive immune
11 responses (Kanneganti, 2010; Palm and Medzhitov, 2009; Pumplin and Voinnet, 2013; Sadler
12 and Williams, 2008). Serine/Threonine kinases (STKs) play a critical role at different stages
13 of the antiviral response in both plants and vertebrates. They behave as switches that are
14 activated by phosphorylation of one or several residues in an activation loop (Huse and
15 Kuriyan, 2002). Some STKs, such as the interferon-induced, double-stranded RNA-activated
16 protein kinase (PKR) in mammals, can also directly sense and interfere with viruses (Yan and
17 Chen, 2012). Upon activation by dsRNA, PKR phosphorylates the translation initiation factor
18 eIF2 α , blocking translation and viral protein synthesis. A similar mechanism was also recently
19 described in plants. The NIK1 STK of *Arabidopsis* was shown to phosphorylate the ribosomal
20 protein L10 and globally suppress translation as an antiviral immunity strategy (Zorzatto et
21 al., 2015).

22
23 STKs were assumed for a long time to exist only in eukaryotes, but Eukaryotic-like STKs
24 (eSTKs) have now been found in most bacterial clades where they have been implicated in a
25 variety of functions including cell cycle control, exit of dormancy, cell wall synthesis, cell
26 division, control of the central metabolism and virulence (Pereira et al., 2011). Unlike
27 eukaryotes, bacteria are generally not known to use STKs in viral defense. An exception to
28 this is the *pgl* phage defense system from *Streptomyces coelicolor* and the related BREX
29 (Bacteriophage Exclusion) systems, which are thought to exist in many unrelated bacteria
30 (Goldfarb et al., 2015; Hoskisson et al., 2015; Sumbly and Smith, 2002). These systems
31 include a STK known as PglW for which kinase activity was confirmed *in vitro*; however, its
32 exact role in the defense pathway is not yet understood (Hoskisson et al., 2015). Also of note
33 is the discovery of a prophage-encoded tyrosine kinase which excludes superinfection by
34 phage HK97 in *Escherichia coli* (Friedman et al., 2011).

35
36 Here we report the discovery of an eSTK involved in phage defense in *Staphylococci*. The
37 Stk2 protein is activated when a specific phage protein, PacK, is present in the cell. The
38 activation of Stk2 results in cell death through phosphorylation of proteins involved in essential
39 cellular processes, including translation, transcription, control of cell cycle and others.
40 Infected cells die before releasing infectious phages thereby protecting neighboring bacteria.
41 This altruistic defense strategy is known as abortive infection (Abi) and can be performed by
42 mechanically diverse systems (Chopin et al., 2005). *Staphylococci* carry another STK known as
43 Stk1 or PknB, which is important for cell-wall structure, antimicrobial resistance, and
44 virulence (Beltramini et al., 2009; Debarbouille et al., 2009; Donat et al., 2009; Truong-
45 Bolduc et al., 2008). The existence of a second STK, known as Stk2, present in only some
46 isolates of *S. aureus* was noted in a few studies but its function remained
47 mysterious (Debarbouille et al., 2009; Didier et al., 2010). We now demonstrate that Stk2
48 provides immunity against bacteriophages through an Abi mechanism. Interestingly, the Stk1
49 kinase is also involved in this defense pathway, suggesting a phosphorylation cascade
50 reminiscent of Eukaryotic viral defense pathways.

51

52 **Results**

53

54 **Discovery of *stk2*, a bacteriophage defense kinase.** We isolated a novel temperate phage of
55 *Staphylococcus epidermidis*, CNP_x a 43kb Siphoviridae with 90.2% overall nucleotide
56 identity to phage CNPH82 (Daniel et al., 2007). CNP_x was isolated on strain LM1680, a
57 derivative of *S. epidermidis* RP62A carrying a large deletion that includes a type III-A
58 CRISPR system and a type I restriction modification (RM) system (Hatoum-Aslan et al.,
59 2014). Interestingly, CNP_x does not infect strain RP62A, suggesting that the region deleted in
60 LM1680 contains a defense system, possibly the CRISPR or the type I RM, that blocks
61 infection by this phage. To narrow down the position of the defense system, we tested the
62 ability of CNP_x to infect a collection of RP62A derivatives, obtained by Marraffini and
63 colleagues, that carry various deletions of this region (Jiang et al., 2013). This analysis
64 allowed us to identify a ~16kb candidate region that carries the phage defense system (Figure
65 1A). To our surprise, this region did not include the type I RM system or the CRISPR system;
66 instead, it contained a number of hypothetical proteins as well as an operon involved in
67 potassium transport and associated regulatory genes (Table S1). Genes coding for the
68 hypothetical proteins were cloned either alone or two at a time on plasmid pC194 which is
69 present in ~15 copies in the cell (Novick, 1989). The resulting plasmids (pDB31, pDB32,
70 pDB33 and pDB34) were introduced by electrotransformation in strain LM1680, and the
71 bacteria were tested for sensitivity to phage CNP_x. The pDB31 plasmid carrying gene
72 SERP2479 provided strong resistance (EOP < 10⁻⁵) while the other genes carried by plasmids
73 pDB32, pDB33 and pDB34 did not have any effect on the susceptibility of the strain to the
74 phage (Figure 1B).

75

76 SERP2479 contains a serine/threonine kinase domain that is easily identified by prediction
77 algorithm such as hhmer or CD-search (Figure 2A) (Finn et al., 2011; Marchler-Bauer and
78 Bryant, 2004). Proteins identical to SERP2479 are found in several strains of *Staphylococcus*
79 *aureus* (Figure 1C). In particular, protein SA0077 whose sequence is 100% identical to
80 SERP2479 was previously described in *S. aureus* strain N315. It was named Stk2 and shown
81 to phosphorylate the virulence regulator SarA *in vitro*, but could not be assigned a
82 physiological role (Didier et al., 2010). Closely related proteins are also present in more
83 distantly related Firmicutes, including some *Bacilli* and *Streptococci* (Table S2). In addition
84 to the kinase domain, a distinctive feature of these proteins is the presence of large N-term
85 and C-term domains of unidentified fold or function.

86

87 **Stk2 provides phage resistance in staphylococci.** To investigate the scope and significance
88 of our finding, we decided to test whether *stk2* could also block phage infection in *S. aureus*.
89 The pDB31 plasmid, carrying *stk2* under the control of its wild-type promoter, was introduced
90 by electrotransformation in several *S. aureus* strains (TB4, NCTC8325-4 and RN4220) which
91 do not carry *stk2* in their chromosome (Bae et al., 2006; Nair et al., 2011). Stk2
92 provided strong resistance to phage phiNM1 in all backgrounds (Table S3). Strain RN4220 is
93 a derivative of *S. aureus* strain NCTC8325 that is easy to manipulate but is generally not
94 considered to be a good model strain for the study of bacterial virulence (Nair et al., 2011).
95 Nonetheless, since the phage resistance phenotype provided by *stk2* was identical in all tested
96 strains, we decided to use RN4220 for the remaining of this study. We tested the ability of
97 plasmid pDB31 to confer resistance to infection by different phages in the RN4220
98 background, including five Siphoviridae (phage 80alpha, phage 85, phiNM1, phiNM2 and
99 phiNM4) and one Twort-like Myoviridae (phage Staph1N) (Bae et al., 2006; Lobočka et al.,

100 2012). Stk2 provided resistance against all tested Siphoviridae but not against the Twort-like
101 phage (Table S3).

102

103 **Characterization of the Stk2 kinase activity.** The *stk2* gene was cloned with a 6x His N-
104 terminal tag in plasmid pET15b and introduced in *E. coli* BL21 (DE3). Upon induction with
105 IPTG, efficient overproduction of His6-Stk2 fusion protein was obtained, though in the form
106 of inclusion bodies. The His6-Stk2 product was then purified by denaturation/renaturation
107 method using guanidinium chloride, followed by a step of purification on an affinity column.
108 Finally, the linked His6 was removed through proteolysis by thrombin (Figure
109 2B). Autophosphorylation activity was tested in the presence of various divalent cations:
110 Mn²⁺, Mg²⁺, Ca²⁺, Zn²⁺ and Co²⁺ (Figure 2C). It was observed that purified Stk2 was
111 significantly labelled *in vitro* in the presence of [γ -³²P] ATP and Mn²⁺ (Figure 2C, lane 4).
112 The ability of Stk2 to autophosphorylate in these conditions indicates that it displays intrinsic
113 kinase activity. To exclude the possibility of contamination by an exogenous kinase that
114 might interfere with the phosphorylation of Stk2, the invariant lysine 152 involved in the
115 binding of the ATP phosphoryl donor, was mutated to isoleucine. As expected Stk2-
116 K152I could no longer autophosphorylate (Figure 2B, lane 5).

117

118 The phosphoamino acid content of the labelled protein was determined after acid hydrolysis
119 and two-dimensional analysis (Duclos et al., 1991). Both phosphoserine and
120 phosphothreonine were revealed on the corresponding autoradiogram (Figure 2D), indicating
121 that Stk2 was modified exclusively on these two types of residues. NanoLC/nano-
122 spray/tandem mass spectrometry was then used for the identification of phosphorylated
123 peptides and for the localization of the phosphorylation sites in Stk2 (Molle et al., 2006). Nine
124 phosphorylation sites could be identified including three sites in the activation loop of Stk2
125 (S272, T275 and T278) (Figure 2A). Various mutated proteins were produced and purified,
126 and the effect of substitution of the different residues to alanine on kinase activity was
127 analyzed by measuring autophosphorylation activity (Figure 2E). Kinase activity was
128 completely abolished when the substitution was on T147, S272, T275, and T278. In contrast,
129 substitution of S246 had no effect on the activity of Stk2, and replacement of S163, S337 and
130 S360 unexpectedly increased Stk2 activity. Altogether these results show that Stk2 is indeed
131 an active STK.

132

133 To confirm *in vivo* that the kinase activity of Stk2 is required for the phage defense phenotype,
134 we mutated conserved residues: K152 in the ATP-binding loop, the predicted catalytic
135 aspartate D243, and the T275 autophosphorylation residue in the activation loop (Figure 2A).
136 All alleles were introduced in *S. aureus* strain RN4220 and tested for sensitivity to phage
137 phiNM1 (Figure 3A). As expected, all mutants showed sensitivity to the phage.

138

139 **Stk2 triggers cell death.** Several assays were performed to understand the mechanism of
140 protection provided by Stk2. We first tested whether it could affect phage adsorption. Cells
141 carrying plasmid pDB31 or the control pC194 were both able to adsorb 99% of the phiNM1
142 phage particles. However, infection of growing cells carrying *stk2*(pDB31) by phage phiNM1
143 led to an interruption in the growth of the culture (Figure 3B). This result stands in clear
144 contrast to the lysis observed for cells that lack *stk2* and are sensitive to the phage. Consistent
145 with this observation, ~3000x fewer phage particles are recovered after infection of cells
146 carrying *stk2* relative to cells without *stk2* (Figure 3C). Also, measurement of the efficiency of
147 center of infection (ECOI) in the presence of *stk2* indicates that only 0.4±0.08% of cells
148 receiving the phiNM1 phage are able to release functional phiNM1 particles. Plating a culture
149 of cells carrying *stk2* after infection reveals that most cells are dead as only ~5% of cells form

150 colonies (Figure 3C). This shows that *stk2* mediates cell death and acts as an abortive infection
151 (Abi) system, killing bacteria upon infection to prevent phage propagation (Abedon, 2012).

152

153 It is worth noting that the number of bacteria that survive phiNM1 infection is similar in the
154 presence and absence of *stk2* (Figure 3C). Wild-type RN4220 cells can survive phiNM1
155 infection when the phage enters lysogeny and integrates in the genome. Interestingly, analysis
156 of cells that survive phiNM1 infection in the presence of *stk2* revealed that some (3/8) had
157 lysogenized phage phiNM1, while the remainder (5/8) most likely did not receive a phiNM1
158 phage particle, eliminated the phage without dying or mutated its receptor (Figure S1A).

159 These results suggest that *stk2* kills staphylococci only if the phage enters its lytic cycle. To
160 confirm this, we sought to induce the lytic cycle of phage phiNM1 lysogenized in cells
161 carrying *stk2* or a control plasmid (Figure 3D). Prophages were induced with mitomycin C. In
162 the presence of *stk2*, the culture stopped growing but did not lyse. In agreement with this
163 observation, 3×10^4 -fold fewer phage particles were recovered after induction of cells carrying
164 *stk2* compared to cells carrying the control plasmid (Figure 3D). Note that in the absence of
165 mitomycin C, phage phiNM1 is spontaneously induced at a lower rate. Under these
166 conditions, the presence of *stk2* also limits the number of phages released. Altogether, these
167 experiments demonstrate that presence of phage DNA is not recognized by Stk2; instead, the
168 Abi phenotype of Stk2 is only triggered during the lytic cycle of the phage.

169

170 **Identification of the Stk2 activation factor.** In the pDB31 plasmid used here, *stk2* is
171 expressed under the control of its WT promoter. To understand whether the transcriptional
172 control of *stk2* is important for the Abi phenotype, we cloned *stk2* under the control of a
173 Ptet promoter (Table S5). Resistance to phage was only observed upon induction of Stk2, and
174 overexpression of Stk2 on its own did not lead to any growth defect (Figure S1B). These
175 results clearly show that the Abi phenotype is only induced in the presence of the phage, and
176 that the natural transcriptional control of *stk2* is not required. Thus, as expected, Stk2 likely
177 behaves as a protein switch that is activated upon phage infection. While Stk2 is able to
178 rapidly auto-phosphorylate *in vitro*, we believe that it is not active in the absence of phage
179 infection *in vivo*.

180

181 To understand what might activate Stk2, we isolated phage mutants capable of
182 infecting *S. aureus* strain RN4220 carrying pDB31. The efficiency of plaquing (EOP) of phage
183 phiNM1 on cells carrying *stk2* is only 5×10^{-7} (Table S3). Nonetheless, some plaques can be
184 recovered and propagated on cells expressing *stk2* (Figure S2A). Phages isolated in this way
185 retain the ability to infect cells carrying *stk2* even after being passaged on cells lacking *stk2*,
186 suggesting that the new phenotype is the result of mutation and not epigenetic variation.
187 These phiNM1 mutants form small plaques and are harder to propagate than the wild-type
188 phage (data not shown). Two mutant phages were sequenced (phiNM1k1 and phiNM1k2), and
189 both carried independent miss-sense mutations (V200M and K30E) in the same gene,
190 SAPPV1_GP14. These results indicate that this protein likely activates Stk2. SAPPV1_GP14
191 contains a P-loop NTPase domain frequently found in proteins involved in molecular motion.
192 An interesting observation enabled us to link the function of this gene to either DNA
193 replication or packaging. When sequencing phage phiNM1k1 and phiNM1k2 we observed
194 that coverage was highest shortly after the packaging site of the phage and then slowly
195 dropped over the rest of the sequence (Figure 4A). This contrasts sharply with the wild-type
196 phage which shows almost uniform coverage throughout the sequence. A possible explanation
197 for this skewed coverage is that the capsids from which DNA was purified do not all contain
198 the full phage genome; instead, most capsids only contain the part of the phage genome that is
199 packaged first. Phage particles which contain only part of the genome would likely not be

200 functional and might even lack the tail. As we did not purify intact phage particles before
201 DNA extraction, we recovered DNA from both functional phage particles and any
202 incompletely assembled particles present in our samples. Thus, the skewed coverage could be
203 explained by random premature termination of phage DNA packaging. Such premature
204 termination could either be due to a defect of the packaging machinery itself, or a defect of
205 the phage DNA replication or concatemer formation. The position of SAPPV1_GP14 in the
206 replication cluster of the phage supports the later hypothesis.

207
208 Because of its phenotype in DNA packaging, we decided to call the SAPPV1_GP14 gene
209 *pacK*. To confirm that this phage protein is sufficient to trigger Stk2, we cloned *pacK* under
210 the control of an inducible Ptet promoter on plasmid pE194, giving plasmid pAS10 (Figure
211 4B). Upon induction with anhydrotetracycline, cell death was observed only when *stk2* was
212 present in the cells (Figure 4C). The V200M and K30E mutations identified in the mutant
213 phages were also tested in this assay and abolished the Abi phenotype (Figure S2B). These
214 experiments confirm that PacK is sufficient to trigger Stk2-mediated cell death.

215
216 It is interesting to note that Stk2 can provide resistance to phages which do not carry PacK
217 (see Tables S3 and S4), suggesting that it can be activated by other phage proteins. In
218 particular, this is true for *S. epidermidis* phage CNP_x which was used in this study to first
219 identify Stk2 (Figure 1), as well as *S. aureus* phage 80alpha, phage 85 and phiNM2 (Table
220 S3). In phage phiNM2, gene AVT76_GP14 encodes a protein with 43% identity to PacK, but
221 no homologous proteins exist in phage 80alpha, phage 85 or CNP_x. To identify how these
222 phages activate Stk2, we isolated mutants of phiNM2 and phage 85 capable of infecting *S.*
223 *aureus* cells expressing *stk2*. Sequencing of these mutants revealed a H230T mutation in gene
224 AVT76_GP14 of phage phiNM2 and a K97G mutation in gene ST85ORF023 of phage 85. To
225 confirm that these phage genes encode activators of Stk2, we cloned them under the control of a
226 Ptet promoter on plasmid pE194, producing plasmids pFD16 and pFD20, respectively (Table
227 S5). After induction with anhydrotetracycline, cells were killed in the presence of *stk2* but not
228 in its absence (Figure 4C). The mutations identified in these genes were also confirmed to
229 abolish Stk2-mediated cell death (data not shown). These results show that, in addition to
230 PacK, two other phage proteins can activate Stk2.

231
232 **Identification of Stk2 phosphorylation targets.** Our results suggest that Stk2 triggers an Abi
233 phenotype through phosphorylation of one or several host proteins. To identify the
234 phosphorylation target(s) of Stk2, we first characterized *S. aureus* colonies that survive the
235 induction of *pacK* in the presence of Stk2 with the goal of identifying mutants of the target
236 proteins. Unfortunately, all of the 36 analyzed colonies carried mutations either in the *stk2* or
237 *pacK* genes, but no other mutant could be identified (data not shown). This result suggests
238 that several mutations might be required to survive Stk2 activation; these would occur at a
239 lower frequency than point mutations in *stk2* or *pacK*.

240
241 We then performed a phosphoproteome analysis of cells expressing *pacK* in either the
242 presence or absence of *stk2*. Expression of PacK was induced from plasmid pAS10 (Ptet-
243 *pacK*) in exponentially growing cells. After 30 min of induction, proteins were precipitated
244 and digested followed by titanium dioxide-based phosphopeptide enrichment (Larsen et al.,
245 2005). To confidently differentiate basal and Stk2-induced phosphorylation events, we
246 labeled the different proteomes with mass spectrometry-differentiable stable isotopes of
247 dimethyl (Boersema et al., 2009). We identified 32 phosphopeptides that could only be found
248 in the presence of Stk2 (Table 1). These include several proteins related to translation,
249 including elongation factors Tu and P, 50S ribosomal proteins L6, L5, L31 and the MetG

250 methionine-tRNA ligase. This extensive phosphorylation of the translation machinery likely
251 indicates that translation is modified after Stk2 activation. In addition, we identified proteins
252 involved in global transcription control, cell cycle control, stress response, DNA topology,
253 DNA repair and central metabolism. This suggests a coordinated response influencing many
254 aspects of the cellular machinery, and a general shift towards stress response and growth
255 arrest. Phosphopeptides corresponding to Stk2 itself could also be identified. Three residues
256 are phosphorylated in the activation loop (S272, T275, T278), as well as a serine S163
257 between the P-loop and the catalytic site. These residues were also identified in the *in*
258 *vitro* autophosphorylation assay (Figure 2). Phosphorylation of the trigger PacK protein could
259 also be identified at residue S176, suggesting that Stk2 interacts with PacK directly. Mutation
260 of the S176 residue to alanine did not have any impact on the Abi phenotype (Figure S3).

261
262 **Role of Stk1 in the Stk2-mediated immunity.** The question of whether the phosphopeptides
263 identified are directly phosphorylated by Stk2 remains to be investigated. Indeed, it is possible
264 that the activation of Stk2 results in the activation of Stk1 which would lead to secondary
265 phosphorylation events. For instance, the elongation factor P identified in our analysis was
266 previously reported as a target of Stk1 (Lomas-Lopez et al., 2007). In further support of this
267 possible role of Stk1 in the phage defense phenotype, we detected the phosphorylation of Stk1
268 at two threonines in the activation loop (T164 and T166). The phosphopeptide carrying these
269 residues was 1.5-fold more abundant in the presence of Stk2 than in its absence (Table S7).
270 To investigate whether Stk1 could play a role in Stk2-mediated phage defense, plasmid
271 pDB31 carrying *stk2* was introduced by electrotransformation in *S. aureus* strain NCTC8325-
272 4 and in the corresponding *stk1* deletion mutant (Debarbouille et al., 2009). The EOP of phage
273 phiNM1 on cells carrying both *stk1* and *stk2* is 4.4×10^{-6} , but when only *stk2* is present the
274 EOP jumps to 2×10^{-2} (Figure 5A). Thus, in the absence of *stk1*, we can still observe some
275 protective effect of *stk2*, but ultimately *stk1* is required for efficient immunity. We also
276 investigated whether *stk2* could trigger cell death in the absence of *stk1*. *S. aureus* NCTC8325-
277 4 and the *stk1* mutant were electrotransformed with both pDB31 (*stk2*) and pAS10 (Ptet-
278 *pacK*) plasmids. Upon induction of PacK expression, cells were killed with identical
279 efficiencies regardless of the presence of *stk1* (Figure 5B). This demonstrates that while *stk1*
280 is necessary for efficient antiviral immunity, it is not required for Stk2-mediated cell death.

281 282 **Discussion**

283
284 Recent bioinformatics analyses have led to the discovery that bacteriophage defense systems
285 frequently cluster together in bacterial genomes (Makarova et al., 2011). Here we report the
286 discovery of a novel defense system in close proximity to the type III CRISPR and type I RM
287 system of *S. epidermidis* RP62A. SERP2479, or Stk2, is responsible for abortive infection and
288 cell death upon phage infection. Stk2 belongs to the family of eukaryotic-like serine/threonine
289 kinases but differs from previously described eSTKs in its lack of transmembrane or PASTA
290 domains. We were able to confirm the kinase activity of Stk2 *in vitro* and identified nine auto-
291 phosphorylated residues. Four of these residues were corroborated *in vivo*, including three in
292 the activation loop (S272, T275, T278), as well as a serine (S163) close to the ATP-binding
293 region (Figure 2A). All three residues of the activation loop are essential for *in*
294 *vitro* autophosphorylation, while an S163A mutation actually increased the kinase activity,
295 suggesting a regulatory role. The phosphorylation of several residues in the activation loop of
296 eSTKs has been reported before and seems to be a common feature of these kinases (Young et
297 al., 2003).

298

299 Our data suggest that while Stk2 is able to autophosphorylate *in vitro*, it is only activated in the
300 presence of a phage protein *in vivo*. We identified three such phage proteins by analyzing the
301 genomes of mutant phages able to propagate on cells carrying *stk2*: Gene SAPPV1_GP14
302 (*pacK*) from phage phiNM1, geneAVT76_GP14 from phage phiNM2, a distant homologue of
303 *pacK* with 43% protein identity, and gene ST85ORF023 from phage 85, which shows no
304 identity to PacK. The PacK protein carries a P-loop NTPase domain and leads to a defect in
305 phage DNA packaging when mutated. This defect could either be due to problems in DNA
306 replication leading to DNA molecules in a bad conformation for packaging, or to a defect in
307 packaging itself. It is currently identified in databases as the chromosomal replication initiator
308 DnaA. However, we believe this to be a simple case of incorrect annotation, as no significant
309 homologies can be found between PacK and DnaA proteins. The function of ST85ORF023 is
310 not known, and no protein domain of known function can be identified. These activator
311 genes are located within the phage lytic operon, which likely explains why Stk2-induced cell
312 death is not triggered when the phage enters lysogeny (Figure 3D). However, the induction of
313 a lysogenic phage in cells carrying *stk2* also leads to cell death. The ability of Stk2 to
314 tolerate prophages while maintaining an active defense against the phage lytic cycle is
315 reminiscent of the similar capacity of type III CRISPR systems to tolerate lysogenic phages
316 (Goldberg et al., 2014).

317
318 An analysis of Staphylococcal phages in the RefSeq database shows that 23% carry
319 homologues of PacK, while 13% carry homologues of ST85ORF023 (Table S4). Phage CNP_x,
320 which was used in this study to first identify Stk2 in *S. epidermidis* carries a protein with 85%
321 identity to ST85ORF023. Interestingly, a blast analysis only identified activators of Stk2 in
322 Siphoviridae; none were found in other phage families. The ability of Stk2 to sense multiple
323 phage proteins is fascinating, and leads to deeper questions about the regulation of the kinase
324 activity.

325
326 We hypothesize that the activation of Stk2 by PacK is the result of a direct interaction
327 between the two proteins, as PacK itself phosphorylated during the response. After sensing
328 the presence of the infecting phage, the activation of Stk2 results in the phosphorylation of a
329 large number of proteins involved in several core functions of the cell including translation,
330 transcription and cell division (Table 1). The modulation of the activity of these proteins
331 through phosphorylation is likely responsible for cell death, preventing phage propagation
332 concurrently. It is worthwhile to note that while the SarA protein was previously reported as a
333 phosphorylation target of Stk2, we did not identify it in our analysis (Didier et al., 2010). This
334 could be explained by the fact that a serine-rich segment of SarA was not covered by our
335 mass-spectrometry analysis (data not shown).

336
337 Another STK known as Stk1 (sometimes also named PknB or PrkC), present in all
338 Staphylococci, is also involved in this antiviral defense pathway. Deletion of *stk1* strongly
339 impairs the ability of Stk2 to protect *S. aureus* against phages (Figure 5A). Nonetheless, the
340 activation of Stk2 leads to cell death even in the absence of Stk1 (Figure 5B). Thus, the role
341 of Stk1 in this defense pathway is likely to ensure that phage particles are not produced before
342 cells are killed by Stk2. It remains to be investigated whether this occurs by accelerating cell
343 death, slowing down the phage, or some other mechanism. The phosphoproteome analysis
344 performed here does not allow for differentiation of direct targets of Stk2 from targets
345 phosphorylated by Stk1 as a result of Stk2 activation. Future work will focus on elucidating
346 the molecular interaction between Stk2 and PacK, for which we only provide circumstantial
347 evidence, as well as deciphering the exact phosphorylation cascade occurring during the
348 response.

349
350 In sum, we provide strong evidence for a bacterial antiviral defense pathway involving a
351 complex phosphorylation cascade and resulting in cell death through the modification of
352 several essential cellular pathways. Other bacterial eSTKs have been shown to target different
353 components of translation (EF-Tu, EF-P), transcription (various sigma and anti-sigma
354 factors), cell division machinery (FtsZ) and central metabolism (Pereira et al., 2011), but none
355 so far have been linked to phage defense. On the contrary, some phages have been described
356 as using STKs in order to manipulate the host translation machinery for their own benefit
357 (Robertson and Nicholson, 1992). It is also interesting to note that STKs play critical roles in
358 the antiviral defense of eukaryotes. In particular, there are striking similarities between Stk2,
359 the mammalian PKR and the plant NIK1. All three STKs are activated by viral infection and
360 target the translation machinery. Moreover, PKR not only inhibits the initiation of translation
361 through phosphorylation of eIF-2 α (Meurs et al., 1990), can also trigger cell death through
362 apoptosis (Dai et al., 2012; Hsu et al., 2004; Stark et al., 1998). Viral defense strategies which
363 involve the Serine/Threonine phosphorylation of essential cellular pathways thus exist in both
364 eukaryotes and bacteria. eSTKs have also recently been identified in archaea (Kennelly,
365 2014). In particular the Ph0512p kinase from *Pyrococcus horikoshii* OT3 was shown to
366 phosphorylate the archaeal homolog of eIF2 α (aIF2 α) *in vitro* (Tahara et al., 2004). It is
367 tempting to hypothesize that Ph0512p and other archaeal kinases could also be involved in
368 viral defense, making this a universal strategy conserved across all domains of life.

369

370 **Experimental Procedures**

371 **Bacterial strains and growth conditions**

372 *S. epidermidis* LM1680 (Hatoum-Aslan et al., 2014), *S. aureus* RN4220 (Nair et al., 2011) and
373 derivative strains were grown in TSB media at 37°C with shaking at 200 rpm. *S. epidermidis*
374 LM1680 and *S. aureus* RN4220 were used as hosts for recombinant plasmids.
375 Chloramphenicol (10 μ g/mL), erythromycin (10 μ g/ml), and ampicillin (100 μ g/ml) were
376 added to the medium to prevent loss of plasmids derived from pC194, pE194 (Horinouchi and
377 Weisblum, 1982a, b), and pET15b (Novagen), respectively. *E. coli* BL21 (DE3) AD494
378 (Novagen) was used for expression of recombinant proteins, and grown in LB medium
379 supplemented with 100 μ g/mL ampicillin at 37°C.

380

381 **Isolation of phage CNP_x**

382 Phage CNP_x (GenBank accession KU598975) was isolated as a plaque on a soft-agar lawn of
383 *S. epidermidis* LM1680 that was infected with phage CNPH82 (Daniel et al., 2007). LM1680
384 is resistant to phage CNPH82 and the isolation of the CNP_x was a single fortuitous event that
385 might have occurred via contamination with an environmental phage and recombination with
386 CNPH82. Indeed CNP_x shares close to 100% homology with CNPH82 over 65% of its
387 genome length, but carries a divergent segment of ~13 kb in the region of the genome
388 containing the lysogenic operon and the early lytic genes.

389

390 **Introduction of plasmids in Staphylococci**

391 Plasmid constructions are detailed in the supplemental experimental procedures. Lists of
392 plasmids and oligonucleotides used in this study are provided in supplementary Tables S5 and
393 S6. After DNA assembly all plasmids were first electroporated in *S. aureus* strain RN4220.
394 Briefly, cells were grown to an optical density (600nm) of 0.8 and washed three times in cold
395 water and concentrated 100x in 10% glycerol. Electroporation of dialyzed DNA was
396 performed in 2mm cuvettes using the following settings: 100 Ω , 2.5kV, 25 μ F. In order to
397 introduce plasmids in other Staphylococci strains, plasmids were extracted from RN4220

398 using the NucleoSpin Plasmid kit (Macherey Nagel) with the following modification: 4ug of
399 lysostaphin (Ambi) was added to the A1 buffer, and cells were incubated 1H at 37°C in this
400 buffer before resuming the protocol as described. Plasmids extracted from RN4220 can then
401 be introduced in other Staphylococci through electroporation following the same protocol.

402

403 **Overproduction and purification of Stk2 and derivatives**

404 Plasmids pET15Ω*stk2(sa0077)* and derivative mutants were introduced into *E. coli*
405 BL21λDE3/AD494. The transformants were grown in 1 liter of LB medium with shaking at
406 25°C until OD₆₀₀ = 0.5, IPTG (0.5 mM) was added to induce protein production, and
407 incubation was pursued for 6 h at 25°C. Cells were then harvested by centrifugation at 3000g
408 for 10 min. Since Stk2 and its mutants were not soluble and retained in inclusion bodies, a
409 step of denaturation/renaturation using guanidine chloride according to London(London et al.,
410 1974)and Goldberg (Goldberg et al., 1996)was performed before the purification on a Ni-
411 NTA column.

412

413 **In vitro phosphorylation assay**

414 Phosphotransfer to Stk2 and its derivatives purified was performed in a buffer containing 25
415 mM Tris-HCl, pH7.5, 1 mM DTT, 2.5 mM MnCl₂, 10 mM ATP and 5 μCi of [γ-³²P]-ATP
416 and incubated at 37°C 10 min to 30 min following the substrate. The reaction was stopped by
417 the addition of 20% Laemmli 5X (Sigma), followed by electrophoresis on SDS-PAGE and
418 autoradiography.

419

420 **Phosphoamino acid analysis**

421 The method used to detect acid-stable phosphoamino acids was described previously (Duclos et
422 al., 1991).

423

424 **Phage production**

425 Phages were mixed with *S. aureus* RN4220 in soft TSA agar supplemented with CaCl₂
426 (5 mM) and then poured on top of TSA agar plate supplemented with CaCl₂ (5 mM). The
427 plates were incubated overnight at 37°C. Soft TSA agar lawns were then resuspended in PBS
428 solution (1X), centrifuged and the lysate containing the phage was filtered on a 0.22 μm filter.
429 To measure phage titers, serial dilutions were spotted on a soft agar lawn of RN4220 in TSA
430 agar supplemented with CaCl₂ (5 mM) and PFUs were quantified after incubation overnight
431 at 37°C.

432

433 **Efficiency of plaquing assays**

434 Phage lysates containing ~10⁷ PFU/μl of CNPX, phage 80alpha, phage 85, phiNM1, phiNM2,
435 phiNM4 or Staph1N were serially diluted and spotted on soft TSA agar lawns supplemented
436 with 5 mM of CaCl₂ and containing either *S. epidermidis* LM1680 or *S. aureus* RN4220, TB4,
437 8325-4, or 8325-4(Δ*stk1*) cells containing the indicated plasmids. PFUs were quantified after
438 incubation overnight at 37°C.

439

440 **Efficiency of center of infection (ECOI)**

441 RN4220 cells carrying plasmid pC194 or pDB31 were grown to an optical density of 0.6 and
442 incubated 10 min at 37°C with phage phiNM1 at an MOI of 5. Cells were then washed twice
443 in fresh TSB to remove unbound phages and plated on a lawn of RN4220 cells. ECOI was
444 obtained by dividing the number of plaques (or center of infections) obtained after infecting
445 cells carrying pDB31 by the number of plaques obtained with cells carrying pC194.

446

447 **Phage DNA isolation and sequencing**

448 Samples of phage lysates were treated with DNase and RNase to a final volume of 200 μ l for
449 30 min at 37°C followed by treatment with EDTA (pH 8.0, 5 mM) and proteinase K (0,5
450 mg/ml) for 30 min at 37°C. Phage DNA was then purified using a PCR purification kit
451 (Macherey Nagel). Phage DNA was sequenced using the Nextera library preparation kit from
452 Illumina and sequenced on a MiSeq device.

453

454 **Adsorption assay**

455 Recipient RN4220 cells were grown to an OD of 0.6 and incubated with phage phiNM1 at an
456 MOI of 1 for 10min. Cells were then centrifuged and the number of phages remaining in the
457 lysates was quantified (n_{ad}). Adsorption efficiency is computed as $(1 - n_{ad}/n_{tot})$ where n_{tot} is the
458 total number of phages added to the sample and reported as percentages.

459

460 **Growth curves**

461 *S. aureus* strains (RN4220, RN4220/pC194, RN4220/pDB31, RN4220/pDB275,
462 RN4220/pFD6) were grown in triplicate overnight at 37°C and diluted 1:100 in 200 μ l of TSB
463 broth in a 96-well microplate that was incubated at 37°C with shaking in an Infinite M200
464 PRO reader (TECAN). Absorbance was measured at 600 nm every 10 min. For RN4220,
465 RN4220/pC194, and RN4220/pDB31, after 1h of growth ($OD_{600} \approx 0.2$), 10 μ l of phiNM1
466 ($4 \cdot 10^7$ PFU/ μ l) phage was added. For RN4220, RN4220/pDB275, and RN4220/pFD6, when
467 OD_{600} reached 0.2, the cultures were induced by anhydrotetracycline (aTc) during 1h and
468 then phage 80alpha ($5 \cdot 10^7$ PFU/ μ l) was added.

469

470 **Prophage induction**

471 *S. aureus* strains (RN4220, RN4220::phiNM1, RN4220::phiNM1/pC194,
472 RN4220::phiNM1/pDB31) were grown in triplicate overnight at 37°C, diluted 1:100 in TSB
473 broth and incubated at 37°C with shaking. When cultures reached $OD_{600} \approx 0.4$, mitomycin
474 C was added at a final concentration of 2 μ g/ml. After 3h of incubation in the presence or in
475 the absence of mitomycin C, the samples were serially diluted and plated to quantify the
476 number of surviving bacteria. Samples were also centrifuged to recover the supernatant and
477 measure the phage titer.

478

479 **Induction of candidate activators of Stk2**

480 *S. aureus* strains were grown in triplicate overnight at 37°C, diluted 1:100 in TSB broth and
481 incubated at 37°C with shaking. When cultures reached $OD_{600} \approx 0.2$, aTc was added at a final
482 concentration of 0.5 μ g/ml. All the strains were grown in parallel without aTc as a control.
483 After 1h30 of incubation in the presence or in the absence of aTc, the samples were serially
484 diluted and 5 μ l was spotted in TSA with appropriate antibiotics to count viable bacteria.

485

486 **Mass Spectrometry**

487 Mass spectrometry methods are detailed in the supplemental experimental procedures.

488

489

490

491 **Author Contributions**

492 F.D., J.-P.D, A.S. and D.B. performed the experiments. B.D., F.D. and D.B. wrote the
493 manuscript. H.M. performed the mass spectrometry experiments and analyzed the data. A.B.
494 performed the phylogenetic analysis.

495

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Figure Legends

617

618 **Figure 1. Discovery of a novel phage defense system in *S. epidermidis* strain RP62A.** A) Strains with various deletions in the region of the CRISPR locus (numbered as in Jiang et al.,
619 2013) were screened for sensitivity to bacteriophage CNP_x. ORFs in the genomic region are
620 represented in green. Each horizontal line represents a strain and the line is discontinued in
621 the deleted region. Blue lines indicate strains sensitive to phage CNP_x while red lines indicate
622 resistance. The region that is sufficient to provide resistance is highlighted in purple. The
623 exact position of the candidate region is 2535598-2551561 and the ORFs it contains are
624 described in Table S1. B) ORFs contained in the candidate region were cloned either alone or
625 two at a time on plasmid pC194 to give plasmids pDB31 (*serp2479, stk2*), pDB32 (*serp2480*),
626 pDB33 (*serp2481+serp2482*) and pDB34 (*serp2483+serp2484*)(see Table S1). Efficiency of
627 plaquing (EOP) of phage CNP_x is reported on *S. epidermidis* strain LM1680 containing these
628 different plasmids (mean+sd, n=3). No plaques were recovered in bacteria carrying gene
629 *Serp2479 (stk2)* (EOP detection limit of 10⁻⁵). C) A tree of Staphylococci was constructed
630 based on the assembled complete genomes available in GenBank. Red indicates the presence
631 of proteins with a minimum of 90% identity to Stk2 (SERP2479) over 100% of the sequence
632 length. More distantly related proteins were also identified in Staphylococci that are not
633 present in this tree and are reported in Table S2.

635 **Figure 2. Stk2 is an active Serine/Threonine kinase.** A) Schematic presentation of the
636 position of phosphoresidues in protein Stk2 and location of different domains. The kinase
637 domain, the activation loop, the cystein-rich region, and the position of the residues K152 and
638 D243 are shown. The position of phosphoresidues is indicated by dotted bars. Residues in red
639 were also shown to be phosphorylated *in vivo* (see Table S7). B) SDS-PAGE analysis of
640 purified Stk2 (lane 2) and Stk2 mutant K152I (lane 3) after staining with Coomassie blue.
641 Molecular mass standards are shown on the left (lane 1). Autophosphorylation of Stk2 (lane
642 4) and mutant K152I (lane 5) in the presence of radioactive [γ -³²P] ATP is shown.
643 Radioactive molecules were detected by autoradiography. C) Effect of cations on
644 Stk2 autophosphorylation activity *in vitro*. D) 2D-analysis of phosphorylated amino acids in
645 Stk2. The acid-stable phosphoamino acids from [γ -³²P]-labelled Stk2 were separated by
646 electrophoresis in the first dimension (1D) followed by ascending chromatography in the
647 second dimension (2D). (P-Tyr) phosphotyrosine, (P-Ser) phosphoserine, and (P-Thr)
648 phosphothreonine were located by ninhydrin staining. Phosphorylated molecules were
649 revealed by autoradiography. E) Effect mutations on the kinase activity of Stk2. Purified wild-
650 type and mutants of Stk2 were incubated in the presence of radioactive [γ -³²P] ATP, protein
651 were separated by SDS-PAGE, and radioactive molecules were detected by autoradiography.

652 **Figure 3. The Stk2 kinase triggers cells death during the phage lytic cycle.** A) EOP of
653 phage phiNM1 on RN4220 *S. aureus* cells in the presence of the WT Stk2 protein (pDB31) or
654 various mutants: K152Q (pDB81), D243A (pDB82) and T275A (pDB83). B) Growth curve
655 of *S. aureus* RN4220 carrying control plasmid pC194 or pDB31 (pC194 Δ *stk2*). Phage
656 phiNM1 was added after 1H of growth at a multiplicity of infection(MOI) of 10. C) PFU and
657 CFU recovered after infection of RN4220 cells carrying *stk2* or not. Cells were grown to OD

658 = 0.2 and incubated with phiNM1 for 2H. Cells were then plated on TSA to measure CFUs,
659 and the filtered supernatant was spotted on a top-agar lawn of RN4220 cells to measure PFUs.
660 Upon infection with phiNM1 and in the presence of *stk2*, cells are killed but the phage is not
661 amplified. D) PFU and CFU recovered after induction with mitomycin C (MMC) of growing
662 RN4220 carrying a phiNM1 lysogenic phage or not, in the presence or absence of *stk2*. Upon
663 induction, cells carrying a lysogenic phage are killed regardless of the presence of *stk2*, but
664 the production of phage is inhibited in the presence of *stk2*. Note that PFU are recovered even
665 in the absence of MMC due to the spontaneous induction of the phage. See also Figure S1.

666 **Figure 4. A phage protein triggers Stk2-mediated cell death.** A) Two mutants of phage
667 phiNM1 able to infect RN4220 cells carrying *stk2* were isolated and sequenced. Coverage
668 along the phage genome is plotted and mutations are highlighted. Both phages carry a
669 mutation in gene *pacK*(SAPPV1_GP14), V200M and K30E for phages phiNM1K1 and
670 phiNM1K2 respectively. B) To test whether PacK is sufficient to activate Stk2, the *pacK* gene
671 was cloned under the control of a Ptet promoter on a pE194 vector giving pAS10, and
672 introduced in cells carrying plasmid pDB31. C) RN4220 cells carrying genes
673 *pacK*(SAPPV1_GP14) from phage phiNM1 (pAS10), AVT76_gp14 from phage phiNM2
674 (pFD16) or ST85ORF023 from phage 85 (pFD20) under the control of a Ptet promoter,
675 together with plasmids pC194 or pDB31 were grown to OD~0.2 and induced with
676 anhydrotetracycline. After 1H of induction cells were plated and colonies quantified. See also
677 Figure S2.

678 **Figure 5. Stk1 is required for efficient Stk2-mediated immunity.** A) EOP of phage
679 phiNM1 is reported against *S. aureus* strain NCTC8325-4 harboring *stk1* or not (Δ *stk1*) in the
680 presence of *stk2* (pDB31) or a control plasmid (pC194). B) NCTC8325-4 cells harboring *stk1*
681 or not (Δ *stk1*) in the presence of *stk2* (pDB31) or a control plasmid (pC194) were
682 transformed with plasmid pAS10 expressing *pacK* under the control of Ptet promoter. Cells
683 were grown to OD \approx 0.2 and induced with anhydrotetracycline. After 2H of induction, cells
684 were plated and colonies quantified. Induction of *pacK* expression triggers cell death in the
685 presence of *stk2* regardless of the presence or absence of *stk1*.

Table 1. List of proteins phosphorylated upon expression of PacK and in the presence of Stk2 and which are never found phosphorylated in the absence of Stk2. A detailed list of all phosphopeptides identified with match metrics and measured ratios are provided in Table S7.

	Gene Name	Protein Descriptions	Accession #
Transcription	greA	Transcription elongation factor GreA	A6QHF1
	sigA	RNA polymerase sigma factor SigA	P0A0J0
	nusA	Transcription termination/antitermination protein NusA	Q2G2D2
Translation	metG	Methionine--tRNA ligase	A6QEE3
	tuf	Elongation factor Tu	A6QEK0
	efp	Elongation factor P	A6QH73
	rpmE2	50S ribosomal protein L31 type B	A6QJW4
	rplF	50S ribosomal protein L6	A6QJ77
	rplE	50S ribosomal protein L5	A6QJ80
Cell cycle	ftsZ	Cell division protein FtsZ	A6QG86
	sepF	Cell division protein SepF	Q2FZ86
	gpsB	Cell cycle protein GpsB	Q2FYI5
Stress response	clpX	ATP-dependent Clp protease ATP-binding subunit ClpX	Q2FXQ7
	clpB	Chaperone protein ClpB	Q2FZS8
	ydaG / yzzA	General stress protein 26	Q2FVN7
	AQ00_RS06590	alkaline shock protein (Asp23) / Stress response regulator gls24 homolog	Q2FZ59
	tela	Tellurite/ Toxin anion resistance protein	Q2FYM7
DNA topology and repair	ssb	Single-stranded DNA-binding protein	A6QE48
	parE	DNA topoisomerase 4 subunit B	A6QGQ7
	mutS	DNA mismatch repair protein MutS	Q2FYZ9
Central metabolism and Biosynthesis	hemL1	Glutamate-1-semialdehyde 2,1-aminomutase 1 (protoporphyrin-IX biosynthesis)	A6QHK1
	pgk	Phosphoglycerate kinase (Glycolysis)	A6QF82
	pgi	Glucose-6-phosphate isomerase (Glycolysis pathway)	Q2FZU0
	dltA	D-alanine--poly(phosphoribitol) ligase subunit 1 (LTA biosynthesis)	Q2FZW6
Others	pacK / SAPPV1_GP14	pacK (Stk2 activation protein)	A6QDW1
	stk2	SERP2479	Q5HK71
	ylaL	Uncharacterized protein	A6QFW6
	esxA	Virulence factor EsxA (ESAT-6-like protein)	Q2G189
	obg	GTPase	Q2FXT1
	phoP	Alkaline phosphatase synthesis two-component response regulator	Q2FXN6
	AQ00_RS00105	short-chain dehydrogenase	Q2FV41