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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.

Introduction

The arms race between bacteria and phages has led to the evolution of many bacterial defense systems that can act at every stage of the phage life cycle, blocking phage adsorption, DNA injection, degrading phage DNA, and interfering with phage replication or the production of phage proteins (Labrie et al., 2010). These defense systems are mechanistically diverse and can vary considerably among bacterial species or even among different isolates of a particular species. At a glance, bacterial defense against phages has little in common with eukaryotic antiviral systems. In plants, defense is primarily conducted via RNA-mediated interference (RNAi), while in vertebrates, pattern-recognition receptors (PRR) recognize nucleic acids and proteins from pathogens and activate the interferon, proinflamatory and adaptive immune responses (Kanneganti, 2010; Palm and Medzhitov, 2009; Pumplin and Voinnet, 2013; Sadler and Williams, 2008). Serine/Threonine kinases (STKs) play a critical role at different stages of the antiviral response in both plants and vertebrates. They behave as switches that are activated by phosphorylation of one or several residues in an activation loop (Huse and Kuriyan, 2002). Some STKs, such as the interferon-induced, double-stranded RNA-activated protein kinase (PKR) in mammals, can also directly sense and interfere with viruses (Yan and Chen, 2012). Upon activation by dsRNA, PKR phosphorylates the translation initiation factor eIF2α, blocking translation and viral protein synthesis. A similar mechanism was also recently described in plants. The NIK1 STK of *Arabidopsis* was shown to phosphorylate the ribosomal protein L10 and globally suppress translation as an antiviral immunity strategy (Zorzatto et al., 2015).

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

Here we report the discovery of an eSTK involved in phage defense in *Staphylococci*. The Stk2 protein is activated when a specific phage protein, PacK, is present in the cell. The activation ofStk2results in cell death through phosphorylation of proteins involved in essential cellular processes, including translation, transcription, control of cell cycle and others. Infected cells die before releasing infectious phages thereby protecting neighboring bacteria. This altruistic defense strategyis known as abortive infection (Abi) and can be performed by mechanically diverse systems(Chopin et al., 2005).*Staphylococci* carry another STK known as Stk1 or PknB, which is important for cell-wall structure, antimicrobial resistance, and virulence(Beltramini et al., 2009; Debarbouille et al., 2009; Donat et al., 2009; Truong-Bolduc et al., 2008). The existence of a second STK, known as Stk2, present in only some isolates of *S. aureus*was noted in a few studies but its function remained mysterious(Debarbouille et al., 2009; Didier et al., 2010). We now demonstrate that Stk2 provides immunity against bacteriophages through an Abi mechanism. Interestingly, the Stk1 kinase is also involved in this defense pathway, suggesting a phosphorylation cascade reminiscent of Eukaryotic viral defense pathways.

Results

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

SERP2479 contains a serine/threonine kinase domain that is easily identified by prediction algorithm such as hhmer or CD-search (Figure 2A)(Finn et al., 2011; Marchler-Bauer and Bryant, 2004). Proteins identical to SERP2479 are found in several strains of *Staphylococcus aureus* (Figure 1C). In particular, protein SA0077whose sequence is 100% identical to SERP2479 was previously described in *S. aureus* strain N315.It was named Stk2 and shown to phosphorylate the virulence regulatorSarA*in vitro*, but could not be assigned a physiological role(Didier et al., 2010). Closely related proteins are also present in more distantly related Firmicutes, including some *Bacilli* and *Streptococci* (Table S2). In addition to the kinase domain, a distinctive feature of these proteins is the presence of large N-term and C-term domains of unidentified fold or function.

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

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

Characterization of the Stk2 kinase activity. The *stk2* gene was cloned with a 6x His N-terminal tag inplasmid pET15b and introduced in*E. coli* BL21 (DE3). Uponinduction with IPTG, efficient overproduction of His6-Stk2 fusion protein was obtained,though in the form of inclusion bodies. The His6-Stk2 product was then purified by adenaturation/renaturation method using guanidinium chloride, followed by a step of purification on anaffinity column. Finally, the linked His6 was removed through proteolysis by thrombin (Figure 2B).Autophosphorylation activity was tested in the presence of various divalent cations: Mn2+,Mg2+, Ca2+, Zn2+ and Co2+ (Figure 2C). It was observed that purified Stk2 was significantly labelled *in vitro*in the presence of [γ-32P] ATP and Mn2+ (Figure 2C, lane 4). The ability of Stk2 to autophosphorylate in these conditions indicates that it displaysintrinsic kinase activity. To exclude the possibility of contamination by an exogenous kinasethat mightinterfere with the phosphorylation of Stk2, the invariant lysine152 involved in the binding of the ATP phosphoryl donor, was mutated to isoleucine. As expectedStk2-K152Icould no longer autophosphorylate(Figure 2B, lane 5).

The phosphoamino acid content of the labelled protein was determined after acidhydrolysis and two-dimensional analysis (Duclos et al., 1991). Both phosphoserine and phosphothreoninewere revealed on the corresponding autoradiogram (Figure 2D), indicating that Stk2was modified exclusively on these two types of residues. NanoLC/nano-spray/tandem mass spectrometry was then used for the identification of phosphorylated peptides and for the localization of the phosphorylation sites in Stk2(Molle et al., 2006). Nine phosphorylation sites could be identified including three sites in the activation loop of Stk2 (S272, T275 and T278) (Figure 2A).Various mutated proteins were produced and purified, and the effect of substitution of the different residues to alanine on kinase activity was analyzed by measuring autophosphorylation activity(Figure 2E). Kinase activity was completely abolished when the substitution was on T147, S272, T275, and T278. In contrast, substitution of S246 had not effect on the activity of Stk2, andreplacement of S163, S337 and S360 unexpectedly increased Stk2 activity. Altogether these results show that Stk2 is indeed an active STK.

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

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

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

It is worth nothing that the number of bacteria that survive phiNM1 infection is similar in the presence and absence of *stk2* (Figure 3C). Wild-type RN4220 cells can survive phiNM1 infection when the phage enters lysogeny and integrates in the genome. Interestingly, analysis of cells that survive phiNM1 infection in the presence of *stk2* revealed that some (3/8) had lysogenized phage phiNM1, whilethe remainder (5/8) most likely did not receive a phiNM1 phage particle, eliminated the phage without dying or mutated its receptor (Figure S1A). These results suggest that *stk2* kills staphylococci only if the phage enters its lytic cycle. To confirm this, we sought to induce the lytic cycle of phage phiNM1 lysogenized in cells carrying *stk2* or a control plasmid (Figure 3D). Prophages were induced with mitomycin C. In the presence of *stk2*, the culture stopped growing but did not lyse. In agreement with this observation, $3*10^4$-fold fewer phage particles were recovered after induction of cells carrying *stk2*compared to cells carrying the control plasmid(Figure 3D). Note that in the absence of mitomycin C, phage phiNM1 is spontaneously induced at a lower rate. Under these conditions, the presence of *stk2* also limits the number of phages released. Altogether, these experiments demonstrate that presence of phage DNA is not recognized by Stk2; instead,the Abi phenotype of Stk2 is only triggered during the lytic cycle of the phage.

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

To understand what might activateStk2, we isolated phage mutants capable of infecting*S. aureus* strain RN4220 carrying pDB31. Theefficiency of plaquing(EOP) of phage phiNM1 on cells carrying *stk2* is only $5*10^{-7}$ (Table S3). Nonetheless, some plaques can be recovered and propagated on cells expressing *stk2*(Figure S2A). Phages isolated in this way retain the ability to infect cells carrying *stk2* even after being passaged on cells lacking *stk2*, suggesting that the new phenotype is the result of mutation and not epigenetic variation. These phiNM1 mutants form small plaques and are harder to propagate than the wild-type phage (data not shown).Two mutant phageswere sequenced (phiNM1k1 and phiNM1k2), and both carried independent miss-sense mutations (V200M and K30E) in the same gene, SAPPV1_GP14. These results indicate that this protein likely activates Stk2. SAPPV1_GP14 contains a P-loop NTPasedomain frequently found in proteins involved in molecular motion. An interesting observation enabled us to link the function of this gene to either DNA replication or packaging. When sequencing phage phiNM1k1 and phiNM1k2 we observed that coverage was highest shortly after the packaging site of the phage and then slowly dropped over the rest of the sequence (Figure 4A). This contrasts sharply with the wild-type phage which shows almost uniform coverage throughout the sequence.A possible explanation for this skewed coverage is that the capsids from which DNA was purified do not all contain the full phage genome; instead, most capsids only contain the part of the phage genome that is packaged first. Phage particles which contain only part of the genome would likely not be

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

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

It is interesting to note that Stk2 can provide resistance to phages which do not carry PacK (see Tables S3 and S4), suggesting that it can be activated by other phage proteins. In particular, this is true for*S. epidermidis* phage CNPx which was used in this study to first identify Stk2 (Figure 1), as well as *S. aureus*phage 80alpha, phage 85 and phiNM2 (Table S3). In phage phiNM2, geneAVT76_GP14encodes a protein with 43% identity to PacK, but no homologous proteins exist in phage 80alpha, phage85 or CNPx. To identify how these phages activate Stk2, we isolated mutants of phiNM2 and phage 85 capable of infecting*S. aureus* cells expressing *stk2*. Sequencing of these mutantsrevealed a H230T mutation in gene AVT76_GP14of phage phiNM2 and a K97G mutation in gene ST85ORF023of phage 85.To confirm that these phage genesencode activators of Stk2,we cloned themunder the control of a Ptet promoter on plasmid pE194,producing plasmids pFD16 and pFD20, respectively (Table S5). After induction with anhydrotetracycline, cellswere killed in the presence of *stk2* but not in its absence (Figure 4C). The mutations identified in these genes were also confirmed to abolish Stk2-mediated cell death (data not shown). These results show that, in addition to PacK, two other phage proteins can activate Stk2.

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

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

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

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

Discussion

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

Our data suggest that while Stk2 is able to autophosphorylate*in vitro*, it is only activated in the presence of a phage protein*in vivo*. We identified three such phage proteins by analyzing the genomes of mutant phages able to propagate on cells carrying stk2: Gene SAPPV1_GP14 (*pacK*) from phage phiNM1, geneAVT76_GP14from phage phiNM2, a distant homologue of *pacK* with 43% protein identity, and gene ST85ORF023 from phage 85, which shows no identity to PacK. The PacK protein carries a P-loop NTPase domain and leads to a defect in phage DNA packaging when mutated. This defect could either be due to problems in DNA replication leading to DNA molecules in a bad conformation for packaging, or to a defect in packaging itself. It is currently identified in databases as the chromosomal replication initiator DnaA. However, we believe this to be a simple case of incorrect annotation, as no significant homologies can be found between PacK and DnaA proteins. The function of ST85ORF023 is not known, and no protein domain of known function can be identified. Theseactivator genesare located within the phage lytic operon, which likely explains why Stk2-induced cell death is not triggered when the phage enters lysogeny (Figure 3D). However, the induction of a lysogenic phage in cells carrying *stk2*also leads to cell death. The ability of Stk2 to tolerateprophages while maintaining an active defense against the phage lytic cycleis reminiscent of the similar capacity of type III CRISPR systems to tolerate lysogenic phages (Goldberg et al., 2014).

An analysis of Staphylococcal phages in the RefSeq database shows that 23% carry homologues of PacK, while 13% carry homologues of ST85ORF023(Table S4). Phage CNPx, which was used in this study to first identify Stk2in *S. epidermidis* carries a protein with 85% identity to ST85ORF23. Interestingly,a blast analysis only identified activators of Stk2 in Siphoviridae; none were found in other phage families. The ability of Stk2 to sense multiple phage proteins is fascinating, and leads to deeper questions about the regulation of the kinase activity.

We hypothesize that the activation of Stk2 by PacK is the result of a direct interaction between the two proteins, as PacKisitself phosphorylated during the response.After sensing the presence of the infecting phage, the activation of Stk2results in the phosphorylation of a large number of proteins involved in several core functions of the cell including translation, transcription and cell division (Table 1). The modulation of the activity of these proteins through phosphorylation is likely responsible for cell death, preventing phage propagation concurrently. It is worthwhile to note that while the SarA protein was previously reported as a phosphorylation target of Stk2, we did not identify it in our analysis(Didier et al., 2010). This could be explained by the fact that a serine-rich segment of SarA was not covered by our mass-spectrometry analysis (data not shown).

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

In sum, we provide strong evidence for a bacterial antiviral defense pathway involving a complex phosphorylation cascade and resulting in cell death through the modification of several essential cellular pathways. Other bacterial eSTKs have been shown to target different components of translation (EF-Tu, EF-P), transcription (various sigma and anti-sigma factors), cell divisionmachinery (FtsZ) and central metabolism (Pereira et al., 2011),but none so far have been linked to phage defense. On the contrary, some phages have been described as using STKs in order to manipulate the host translation machinery for their own benefit (Robertson and Nicholson, 1992). It is also interesting to note that STKs play critical roles in the antiviral defense of eukaryotes. In particular, there are striking similarities between Stk2, the mammalian PKR and the plant NIK1. All three STKs are activated by viral infection and target the translation machinery. Moreover, PKR not only inhibits the initiation of translation through phosphorylation of eIF-2α (Meurs et al., 1990), can also trigger cell death through apoptosis (Dai et al., 2012; Hsu et al., 2004; Stark et al., 1998). Viral defense strategies which involve the Serine/Threonine phosphorylationof essential cellular pathwaysthus exist in both eukaryotes and bacteria. eSTKs have also recently been identified in archaea (Kennelly, 2014). In particular the Ph0512p kinase from *Pyrococcushorikoshii OT3* was shown to phosphorylate the archaeal homolog of eIF2α (aIF2α) *in vitro* (Tahara et al., 2004). It is tempting to hypothesize that Ph0512p and other archaeal kinases could also be involved in viral defense, making this a universal strategy conserved across all domains of life.

Experimental Procedures

Bacterial strains and growth conditions

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

Isolation of phage CNPx

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

Introduction of plasmids in Staphylococci

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

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

Overproduction and purification of Stk2 and derivatives

Plasmids pET15Ω*stk2*(*sa0077*) and derivative mutantswere introduced into *E. coli* BL21λDE3/AD494. The transformants were grown in 1 liter of LB medium with shaking at 25°C until OD_{600} = 0.5, IPTG (0.5 mM) was added to induce protein production, and incubation was pursued for 6 h at 25°C. Cells were then harvested by centrifugation at 3000g for 10 min. Since Stk2 and its mutants were not soluble and retained in inclusion bodies, a step of denaturation/renaturation using guanidine chloride according to London(London et al., 1974)and Goldberg (Goldberg et al., 1996)was performed before the purification on a Ni-NTA column.

***In vitro* phosphorylation assay**

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

Phosphoamino acid analysis

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

Phage production

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

Efficiency of plaquing assays

Phage lysates containing ~10^7 PFU/µl of CNPX, phage 80alpha, phage 85, phiNM1, phiNM2, phiNM4 or Staph1Nwere serially diluted and spotted on soft TSA agar lawns supplemented with 5 mM of CaCl2 and containing either *S. epidermidis* LM1680 or *S. aureus*RN4220, TB4, 8325-4, or 8325-4(Δ*stk1*) cells containing the indicated plasmids. PFUs were quantified after incubation overnight at 37°C.

Efficiency of center of infection (ECOI)

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

Phage DNA isolation and sequencing

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

Adsorption assay

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

Growth curves

S. aureus strains (RN4220, RN4220/pC194, RN4220/pDB31, RN4220/pDB275, RN4220/pFD6) were grown in triplicate overnight at 37°C and diluted 1:100 in 200 µl of TSB broth in a 96-well microplate that was incubated at 37°C with shaking in an Infinite M200 PRO reader (TECAN). Absorbance was measured at 600 nm every 10 min. For RN4220, RN4220/pC194, and RN4220/pDB31, after 1h of growth (OD600≈0.2), 10 µl of phiNM1 (4.10^7 PFU/µl) phage was added. For RN4220, RN4220/pDB275, and RN4220/pFD6, when OD600 reached 0.2, the cultures were induced by anhydrotetracycline (aTc) during 1h and then phage 80alpha (5.10^7 PFU/µl) was added.

Prophage induction

S. aureus strains (RN4220, RN4220::phiNM1, RN4220::phiNM1/pC194, RN4220::phiNM1/pDB31) were grown in triplicate overnight at 37°C, diluted 1:100 in TSB broth and incubated at 37°C with shaking. When cultures reached OD600≈0.4,mitomycin Cwas added at a final concentration of 2 µg/ml. After 3h of incubation in the presence or in the absence of mitomycin C, the samples were serially diluted and plated to quantify the number of surviving bacteria. Samples were also centrifuged to recover the supernatant and measure the phage titer.

Induction of candidate activators of Stk2

S. aureus strains were grown in triplicate overnight at 37°C, diluted 1:100 in TSB broth and incubated at 37°C with shaking. When cultures reached OD600≈0.2, aTc was added at a final concentration of 0.5 µg/ml. All the strains were grown in parallel without aTc as a control. After 1h30 of incubation in the presence or in the absence of aTc, the samples were serially diluted and 5 µl was spotted in TSA with appropriate antibiotics to count viable bacteria.

Mass Spectrometry

Mass spectrometry methods are detailed in the supplemental experimental procedures.

Author Contributions

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

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Figure Legends

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., 2013) were screened for sensitivity to bacteriophage CNPx. ORFs in the genomic region are represented in green. Each horizontal line represents a strain and the line is discontinued in the deleted region. Blue lines indicate strains sensitive to phage CNPx while red lines indicate resistance. The region that is sufficient to provide resistance is highlighted in purple. The exact position of the candidate region is 2535598-2551561 and the ORFs it contains are described in Table S1. B) ORFs contained in the candidate region were cloned either alone or two at a time on plasmid pC194 to give plasmids pDB31 (*serp2479*, *stk2*), pDB32 (*serp2480*), pDB33 (*serp2481+serp2482*) and pDB34 (*serp2483+serp2484*)(see Table S1). Efficiency of plaquing (EOP) of phage CNPx is reported on *S. epidermidis*strain LM1680 containing these different plasmids (mean+sd, n=3). No plaques were recovered in bacteria carrying gene *Serp2479* (*stk2*) (EOP detection limit of 10^{-5}). C) A tree of Staphylococci was constructed based on the assembled complete genomes available in GenBank. Red indicates the presence of proteins with a minimum of 90% identity to Stk2 (SERP2479) over 100% of the sequence length. More distantly related proteins were also identified in Staphylococci that are not present in this tree and are reported in Table S2.

Figure 2. Stk2 is an active Serine/Threnonine kinase. A) Schematic presentation of the position of phosphoresidues in protein Stk2 and location of different domains. The kinase domain, the activation loop, the cystein-rich region, and the position of the residues K152 and D243 areshown. The position of phosphoresidues is indicated by dotted bars. Residues in red were also shown to be phosphorylated *in vivo* (see Table S7). B) SDS-PAGE analysis of purified Stk2 (lane 2) and Stk2 mutant K152I (lane 3) after staining with Coomassie blue. Molecular mass standards are shown on the left (lane 1). Autophosphorylation of Stk2 (lane 4) and mutant K152I (lane 5) in the presence of radioactive [γ-32P] ATP is shown. Radioactive molecules were detected by autoradiography. C) Effect of cations on Stk2autophosphorylationactivity *in vitro*.D) 2D-analysis of phosphorylated amino acids in Stk2. The acid-stable phosphoamino acids from [γ-32P]-labelled Stk2 were separated by electrophoresis in the first dimension (1D) followed by ascending chromatography in the second dimension (2D). (P-Tyr) phosphotyrosine, (P-Ser) phosphoserine, and (P-Thr) phosphothreonine were located by ninhydrin staining. Phosphorylated molecules were revealed by autoradiography. E) Effect mutations on the kinase activity of Stk2. Purified wild-type and mutants of Stk2 were incubated in the presence of radioactive [γ-32P] ATP, protein were separated by SDS-PAGE, and radioactive molecules were detected by autoradiography.

Figure 3. The Stk2 kinase triggers cells death during the phage lytic cycle. A) EOP of phage phiNM1 on RN4220 *S. aureus* cells in the presence of the WT Stk2 protein (pDB31) or various mutants: K152Q (pDB81), D243A (pDB82) and T275A (pDB83). B) Growth curve of *S. aureus* RN4220 carrying control plasmid pC194 or pDB31 (pC194Ω*stk2*). Phage phiNM1 was added after 1H of growth at a multiplicity of infection(MOI) of 10. C) PFU and CFU recovered after infection of RN4220 cells carrying *stk2* or not. Cells were grown to OD

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

Figure 4. A phage protein triggers Stk2-mediated cell death. A) Two mutants of phage phiNM1 able to infect RN4220 cells carrying *stk2* were isolated and sequenced. Coverage along the phage genome is plotted and mutations are highlighted. Both phages carry a mutation in gene *pacK*(SAPPV1_GP14), V200M and K30E for phages phiNM1K1 and phiNM1K2 respectively. B) To test whether PacKis sufficient to activate Stk2, the *pacK* gene was cloned under the control of a Ptetpromoter on a pE194 vector giving pAS10, and introduced in cells carrying plasmid pDB31. C) RN4220 cells carrying genes *pacK*(SAPPV1_GP14) from phage phiNM1 (pAS10), AVT76_gp14from phage phiNM2 (pFD16) or ST85ORF023from phage 85 (pFD20) under the control of a Ptet promoter, together with plasmids pC194 or pDB31 were grown to OD~0.2 and induced with anhydrotetracycline. After 1H of induction cells were plated and colonies quantified. See also Figure S2.

Figure 5. Stk1 is required for efficient Stk2-mediated immunity. A) EOP of phage phiNM1 is reported against *S. aureus* strain NCTC8325-4 harboring *stk1* or not (Δ*stk1*) in the presence of *stk2* (pDB31) or a control plasmid (pC194). B) NCTC8325-4 cells harboring *stk1* or not (Δ*stk1*) in the presence of *stk2* (pDB31) or a control plasmid (pC194)were transformedwith plasmid pAS10 expressing *pacK* under the control of Ptet promoter. Cells were grown to OD ≈ 0.2 and induced with anhydrotetracycline. After 2H of induction, cells were platedand colonies quantified. Induction of *pacK* expression triggers cell death in the 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/antiterminationproteinNusA	Q2G2D2
Translation	metG	Methionine--tRNA ligase	A6QEE3
	tuf	Elongation factor Tu	A6QEK0
	efp	Elongation factor P	A6QH73
	rpmE2	50S ribosomal protein L31 type B	A6QIW4
	rplF	50S ribosomal protein L6	A6QJ77
	rplE	50S ribosomal protein L5	A6QJ80
Cell cycle	ftsZ	Cell division proteinFtsZ	A6QG86
	sepF	Cell division proteinSepF	Q2FZ86
	gpsB	Cell cycle proteinGpsB	Q2FYI5
Stress response	clpX	ATP-dependent Clp protease ATP-binding subunit ClpX	Q2FXQ7
	clpB	ChaperoneproteinClpB	Q2FZS8
	ydaG / yzzA	General stress protein 26	Q2FVN7
	AQ00_RS06590	alkaline shock protein (Asp23) / Stress response regulator gls24 homolog	Q2FZ59
	telA	Tellurite/ Toxin anion resistanceprotein	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	Uncharacterizedprotein	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-chaindehydrogenase	Q2FV41