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A regulatory role for *Staphylococcus aureus* toxin-antitoxin system PemK_{Sa}

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Toxin-antitoxin systems were shown to be involved in plasmid maintenance when they were initially discovered, but other roles have been demonstrated since. Here we identify and characterize a novel toxin-antitoxin system (*pemK_{Sa}*) located on *Staphylococcus aureus* plasmid pCH91. The toxin (PemK_{Sa}) is a sequence-specific endoribonuclease recognizing the tetrad sequence U↓AUU, and the antitoxin (PemI_{Sa}) inhibits toxin activity by physical interaction. Although the toxin-antitoxin system is responsible for stable plasmid maintenance our data suggest the participation of *pemK_{Sa}* in global regulation of staphylococcal virulence by alteration of the translation of large pools of genes. We propose a common mechanism of reversible activation of toxin-antitoxin systems based on antitoxin transcript resistance to toxin cleavage. Elucidation of this mechanism is particularly interesting because reversible activation is a prerequisite for the proposed general regulatory role of toxin-antitoxin systems.

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Toxin–antitoxin (TA) systems were initially characterized as components of low copy number plasmids, which ensured their stable maintenance^{1,2}. It was established that daughter cells that lose the plasmid are selectively eliminated by the deleterious activity of the long half-life toxin, as the pool of labile antitoxin rapidly diminishes in the absence of synthesis of new molecules. However, the later discovery of TA systems located on bacterial chromosomes initiated a still largely unsettled debate concerning the broader biological role of TA systems^{1,3–6}.

It is currently thought that TA systems have a general role in regulation of the stress response. Recent studies have demonstrated great diversity among TA systems. Different systems sense diverse stimuli, and act as global regulators of gene expression. Under favourable growth conditions genes of most TA systems are coexpressed and the deleterious activity of the toxin is prevented by the antitoxin⁷. However, various stress stimuli alter the toxin/antitoxin balance, usually by inducing increased proteolytic degradation of the labile antitoxin^{8–10}. The activated toxin induces a dormant state or other adaptations that enable the bacteria to survive environmentally unfavourable conditions^{11–13}. However, it is unclear how the bacteria recover from this state, although it is evident that the ability to return to equilibrium is a prerequisite in an efficient regulatory system. This important issue was addressed in our study.

Class II TA systems include toxins characterized by ribonucleolytic activity. Nonspecific ribonuclease toxins degrade total cellular RNA; their potential regulatory role is limited to cell growth inhibition (dormancy), with prolonged activation of these toxins leading to cell death (plasmid maintenance). However, many class II toxins are sequence-specific ribonucleases. For example, the MazF toxin from *Escherichia coli* hydrolyses mRNA at the A↓CA sequence¹⁴, where the arrow denotes the site of hydrolysis; the MazF homologues MazF-mt3, MazF-mt7, MazF_{sa} and MazF-cd from *Mycobacterium tuberculosis*, *S. aureus* and *Clostridium difficile* recognize pentad target sequences (UU↓CCU or CU↓CCU, U↓CGCU, U↓ACAU and U↓A↓C↓AU, respectively)^{15–17}; whereas MazF-hw from *Haloquadrada walsbyi* recognizes seven consecutive nucleotides (UU↓ACUCA)¹⁸. The activity of highly sequence-specific toxins leads to selective degradation and, as a result, inhibition of translation from only a limited pool of transcripts; this process is termed RNA interference¹⁹, and probably constitutes an efficient means of global regulation of gene expression.

TA-driven RNA interference has been implicated in the regulation of virulence and antibiotic resistance in numerous pathogens including *M. tuberculosis*¹⁶, *S. aureus*^{15,20}, *Yersinia pestis*²¹, *Helicobacter pylori*²², *Streptococcus mutans*²³ and *C. difficile*¹⁷, and also among enterococci²⁴. However, the occurrence of this mechanism remains largely speculative, and the very attractive concept of global regulation of virulence factor expression by TA systems in pathogenic bacteria still awaits thorough experimental characterization.

Here we describe a novel TA system PemIK_{sa} in *S. aureus* CH91 and demonstrate that the toxin PemK_{sa} is a sequence-specific ribonuclease while the antitoxin PemI_{sa} inhibits the toxin by physical interaction. We show that the system efficiently maintains plasmids, but argue that nonrandom distribution of toxin target sequences in the staphylococcal transcriptome supports the concept of TA system regulation of gene expression and provide experimental examples of such regulation. Moreover, we propose a possible mechanism of reversible activation of TA systems, which is indispensable for the postulated regulatory role of PemIK_{sa} and other similar systems.

Results

S. aureus plasmid pCH91 encodes a toxin–antitoxin system.

We identified a putative TA system encoded on *S. aureus* plasmid pCH91. The putative toxin (PemK_{sa}; 112 amino acids) and the putative antitoxin (PemI_{sa}; 89 amino acids) were encoded by the SACH_a18 and SACH_a19 loci, respectively. To verify the predicted function of those proteins, the respective coding sequences (CDSs) were cloned into the expression plasmids pETDuet-pemK_{sa} and pETDuet-pemI_{sa}. Expression plasmid pACYCDuet-pemI_{sa}, which is compatible with pETDuet, was also constructed. Furthermore, plasmid pETDuet-pemIK_{sa} was prepared where pemK_{sa} and pemI_{sa} were cloned under the control of two independent T7 promoters. Upon induction with isopropyl-β-D-thiogalactoside (IPTG), *E. coli* BL21(DE3) carrying pETDuet-pemI_{sa} expressed large quantities of His₆-PemI_{sa}, as evidenced by SDS–polyacrylamide gel electrophoresis (PAGE) (Fig. 1a and Supplementary Fig. S1). Protein expression was not observed from pETDuet-pemK_{sa} under comparable experimental conditions. Rather, IPTG induction resulted in growth inhibition (Supplementary Fig. S2) while coexpression of PemI_{sa} from pACYCDuet-pemI_{sa} restored the production of PemK_{sa}. A similar effect occurred when both proteins were coexpressed from pETDuet-pemIK_{sa}, which indicates that PemI_{sa} eliminated the toxicity of PemK_{sa} to the host; thus, PemIK_{sa} exhibits characteristics of a typical TA system.

PemK_{sa} and PemI_{sa} physically interacted inside the cell, as PemI_{sa} was copurified from the cell lysate using His₆-PemK_{sa} (Fig. 1b and Supplementary Fig. S1). Indeed, purified PemI_{sa} forms a heterodimer with purified PemK_{sa}, as evidenced by gel filtration (Fig. 1c,d and Supplementary Figs S3 and S4).

PemK_{sa}-induced growth inhibition was partially reversed by a R84A mutation. Growth inhibition was still evident following induction of mutant production, but was not as pronounced as in the case of wild-type PemK_{sa} (Supplementary Fig. S2). Moreover, mutant production was detectable in cell lysates using SDS–PAGE, whereas accumulation of the wild-type was not detected under similar conditions (Fig. 1a). This suggests that the deleterious effect of PemK_{sa}R84A was reduced but not completely eliminated.

PemIK_{sa} system allows stable plasmid maintenance. To investigate the stability of pCH91 in *S. aureus* strain CH91 the bacterium was subjected to cultivation under conditions known to cure bacteria of plasmids (4 °C, 42 °C, ultraviolet radiation). The presence of pCH91 was monitored using the reporter activity of staphopain C, a cysteine protease naturally overproduced from pCH91 (ref. 25). Under no tested conditions was the reporter activity reduced during the course of experiment (14 days), which indirectly suggests strong internal selective pressure for pCH91 maintenance.

To determine whether PemIK_{sa} mediates plasmid stability, *E. coli* cells were cotransformed with pETDuet-pemK_{sa} and pACYCDuet-pemI_{sa}, and the stability of pACYCDuet-pemI_{sa} was monitored during cultivation in the presence of selective antibiotic pressure towards pETDuet-pemK_{sa} but not pACYCDuet-pemI_{sa} (Supplementary Fig. S5). We assumed that if PemI_{sa} is essential to overcoming the deleterious activity of PemK_{sa}, then pACYCDuet-pemI_{sa} would stably propagate despite the absence of external selective antibiotic pressure. Indeed, pACYCDuet-pemI_{sa} was maintained throughout the experiment (23 days), but was largely lost on day 23 when pETDuet-pemK_{sa} was substituted with an empty pETDuet control plasmid (Fig. 2a).

To verify the plasmid maintenance function of the PemIK_{sa} system in a homologous host, we introduced the pemIK_{sa} cassette under control of its native promoter into the pALCP2G reporter

Figure 1 | Basic properties of the components of the PemK_{Sa} toxin-antitoxin system. (a) Coexpression of PemI_{Sa} restored the accumulation of PemK_{Sa}. SDS-PAGE of total cell lysates of *E. coli* BL21(DE3) carrying plasmids driving the expression of His₆-PemK_{Sa} (toxin), His₆-PemK_{Sa}R84A mutant, His₆-PemI_{Sa} (antitoxin), and coexpression of PemI_{Sa} and His₆-PemK_{Sa}. Recombinant protein production was induced with IPTG as indicated. Bands corresponding to PemI_{Sa} and PemK_{Sa} (or PemK_{Sa}R84A) are marked with a single and a double arrowhead, respectively. (b) PemK_{Sa} and PemI_{Sa} form complexes *in vivo*. His₆-tagged PemK_{Sa} was coexpressed with native PemI_{Sa}. Toxin was recovered from cell lysate by affinity binding and copurified proteins were analysed by SDS-PAGE. Arrowheads as in panel a. (c) *In vitro* demonstration of PemK_{Sa}-PemI_{Sa} complex formation. Purified components of the TA system were subjected to gel filtration separately or after mixing (using approximately a 1.5 molar excess of the antitoxin). (d) Analysis of fractions from gel filtration. SDS-PAGE of selected fractions (top), and agarose gel analysis of the effect of particular fractions on MS2 phage RNA (bottom). Only PemK_{Sa} (double arrowhead) but not PemI_{Sa} (single arrowhead; the bands below that corresponding to PemI_{Sa} represent degradation products of labile antitoxin) or the PemK_{Sa}-PemI_{Sa} complex exhibited ribonucleolytic activity against MS2 RNA.

plasmid constitutively expressing green fluorescent protein (GFP). *S. aureus* strain RN4220 containing the resulting pALCP2G-pemIK_{Sa} was cultured in liquid media without selective antibiotic. Empty pALCP2G was used as a control. The presence of the plasmid was monitored on subsequent days using GFP fluorescence, and on the last day of the experiment by comparative quantitative plating on agar media containing and deprived of a selective antibiotic. Under these conditions, pALCP2G-pemIK_{Sa} was stably maintained throughout the experiment (6 days), whereas control plasmid was largely eliminated (Fig. 2b). These data directly demonstrate the plasmid stabilizing role of the PemIK_{Sa} system in *S. aureus*.

PemK_{Sa} is a sequence-specific RNase. We investigated the mechanism of PemK_{Sa} toxicity against the host. Incubation of phage MS2 RNA in the presence of purified PemK_{Sa} resulted in RNA degradation, suggesting PemK_{Sa} has ribonucleolytic activity. The degradation pattern was distinct (not smeared), suggesting that the ribonuclease activity of PemK_{Sa} is sequence specific or secondary structure specific (Fig. 1d). We identified the sites of hydrolysis of MS2 RNA using a modified primer extension method¹⁶. The identified cleavage sites (Supplementary Table S1 and Supplementary Fig. S6) defined a perfect consensus (U↓AUU) recognized and hydrolysed by PemK_{Sa}. No other sites of hydrolysis were detected. Although there are a total of 10

Figure 2 | PemK_{sa} system maintains plasmid stability in *E. coli* and *S. aureus*. (a) *E. coli* was cotransformed either with the PemK_{sa} expressing and chloramphenicol resistance (cam) providing pACYCDuet-pemK_{sa} plasmid, and the PemK_{sa} expressing and ampicillin resistance (amp) providing pETDuet-pemK_{sa} plasmid (filled circles), or pACYCDuet-pemK_{sa} and empty pETDuet (empty circles). The cells were cultured in the presence of ampicillin, and the stability of pACYCDuet (chloramphenicol resistance) was monitored. Only in the presence of PemK_{sa} was the PemK_{sa}-encoding plasmid stably maintained. (b) *S. aureus* was transformed with pALCP2G-pemK_{sa} (filled circles), a GFP-expressing reporter plasmid containing pemK_{sa} under the control of its native promoter, or empty pALCP2G (empty circles). The cells were cultured in the absence of selective antibiotic, and plasmid stability was monitored by virtue of GFP reporter activity. Only the pemK_{sa}-containing but not the empty plasmid was stably maintained. Both panels: representative results of two independent experiments.

UAUU sites in MS2 RNA (RefSeq accession no. NC_001417), only seven were hydrolysed by PemK_{sa} in our experiments, most probably because of steric occlusion of the remaining three sites (Supplementary Table S1).

We also purified the PemK_{sa}R84A mutant and assayed its ability to degrade MS2 RNA. The activity of PemK_{sa}R84A was estimated to be ~10% of that of the wild-type (a 10-fold excess of mutant was required to obtain digestion comparable to that obtained with wild-type PemK_{sa}). Using primer extension experiments, we demonstrated that R84A mutation affects only the rate of hydrolysis, and not its specificity.

Using an *in vitro* MS2 RNA digestion assay and purified proteins, we demonstrated that PemK_{sa} stoichiometrically inhibited the ribonuclease activity of PemK_{sa} at a 1:1 molar ratio. The activity of the PemK_{sa}R84A mutant was also stoichiometrically inhibited by PemK_{sa}, demonstrating that the mutation did not affect the TA interface.

Nonrandom distribution of UAUU in *S. aureus* transcriptome.

Analysis of available genomic data demonstrated that, unlike pemK_{sa} of strain CH91, homologous pemK systems are almost exclusively chromosomal (Supplementary Fig. S7). Therefore, we investigated whether plasmid maintenance was the only function of pemK_{sa}. A nonspecific RNase would clearly suffice for a process of elimination of daughter cells not inheriting the plasmid. On the other hand, a sequence-specific RNase, such as PemK_{sa} could exert a global regulatory effect by degrading only a selected pool of transcripts, provided that the recognized sequences are organized in the transcriptome in a nonrandom fashion. To evaluate whether the latter was the case, we calculated the expected distribution of the UAUU sequence in mRNA, and compared this with the observed distribution in the transcriptome of *S. aureus* strain ED98. This strain was chosen for analysis because its genomic sequence is known (in contrast to strain CH91), and it carries a pCH91 related pAvX plasmid²⁶ coding a TA system identical to PemK_{sa}. Statistically, the PemK_{sa} target sequence should appear every 77 bases, and thus should occur at least once in every gene. Among 2,689 open reading frames (ORFs) encoded in the genome of *S. aureus* ED98 we identified 121 ORFs in which the PemK_{sa} target sequence was either absent (56 ORFs) or present, but statistically underrepresented (65 ORFs; $P' \leq 0.01$). Interestingly, this group of ORFs was composed of nonrandom transcripts, and contained a substantial number of transcripts encoding proteins implicated in virulence (Supplementary Fig. S8). These included a transcription regulator Fur²⁷; delta-hemolysin²⁸; extracellular enzymes comprising metalloprotease, lipase and coagulase²⁹; and host protein-binding proteins including clumping factor A, von Willebrand factor-binding protein, fibronectin-binding protein, and serine-aspartate repeat proteins³⁰ (for a full list see Supplementary Data 1). We also identified 214 ORFs in which the PemK_{sa} target sequence was statistically overrepresented ($P' \geq 0.99$). The majority of transcripts in this group encoded transmembrane transporters (Supplementary Fig. S8 and Supplementary Data 1). The nonrandom distribution of the PemK_{sa} target sequence in the staphylococcal transcriptome argues in favour of the hypothesis of a regulatory role for PemK_{sa}.

The presence of the PemK_{sa} target sequence in an RNA molecule does not necessarily imply its hydrolysis, as the process may be sterically hindered by secondary structures, RNA-binding proteins and other factors. Therefore, we experimentally verified *in vitro* and *in vivo* the anticipated differential vulnerability of representative transcripts from the groups described above to PemK_{sa} degradation. *In vitro*, the stability of transcripts exposed to PemK_{sa} was monitored using electrophoresis. Expectedly, the more recognition sites were present in a particular mRNA, the more efficiently it was degraded by PemK_{sa} (Fig. 3a and Supplementary Fig. S9). To further verify the correlation, we have synthesized ScpB encoding gene (*scpBalt*) in which all eight PemK_{sa} target sequences were replaced with sequences encoding identical amino acids, but using different codons. Such transcript was not affected by PemK_{sa} *in vitro* contrary to *scpB* transcript, which was efficiently degraded (Fig. 3a). Moreover, we have synthesized NrdR encoding gene (*nrdRalt*) in which we introduced nine UAUU sequences in a way, which did not affect the amino acid sequence of encoded protein. Such transcript was efficiently degraded by PemK_{sa} *in vitro* contrary to *nrdR* transcript, which was resistant to PemK_{sa}-mediated hydrolysis (Fig. 3a).

To verify the ability of PemK_{sa} to differentially target transcripts *in vivo*, we have produced selected staphylococcal mRNAs in *E. coli* and evaluated their stability upon ectopic expression of PemK_{sa}. The stability of tested transcripts inversely

Figure 3 | PemK_{Sa} modulates gene expression by differentially targeting mRNA transcripts. (a) mRNA transcripts were incubated with PemK_{Sa} for the indicated time and analysed using agarose gel electrophoresis. The number of PemK_{Sa} target sites (UAUU) in each transcript is denoted in superscript. Representative transcripts belonging to the groups where the PemK_{Sa} target sequence is: underrepresented (*agA* and *nrdR*), overrepresented (*adhC* and *ftsW*) or follows random distribution (*scpB* and *argR*) were chosen. *scpBalt* and *nrdRalt* are versions of *scpB* and *nrdR* genes where PemK_{Sa} target sequences were removed and introduced, respectively. (b) Transcripts encoding PemI_{Sa}, PemK_{Sa} and the whole, functionally deficient *pemIK_{Sa}** transcript were treated with PemK_{Sa} *in vitro*. Despite the presence of the PemK_{Sa} target sequence in each transcript, no hydrolysis was observed in *pemI_{Sa}*, whereas the toxin encoding part, as well as the whole *pemIK_{Sa}** transcript were only inefficiently hydrolysed. (c) SDS-PAGE of total cell lysates of *E. coli* BL21(DE3) cotransformed with arabinose inducible pBAD-pemK_{Sa} plasmid, and the respective IPTG inducible pACYCDuet-based plasmids. Single arrowheads indicate proteins expressed from pACYCDuet. Note that transcripts resistant to hydrolysis by PemK_{Sa} (a) drove expression of respective proteins in the presence of PemK_{Sa} *in vivo*, while no expression occurred from transcripts susceptible to PemK_{Sa}. The timescale and major events are indicated at the top of the panel. (d) Experiment identical to that in c save that production of antitoxin was monitored (single arrowhead; toxin—double arrowhead). (e) Influence of PemK_{Sa} on the accumulation of transcripts expressed from pACYCDuet. At each indicated time point total cellular RNA was isolated, reverse transcribed and the amount of particular transcripts was analysed by PCR.

correlated with the number of PemK_{Sa} target sites and correlated with the stability determined in the *in vitro* experiments (Fig. 3e and Supplementary Fig. S10).

To confirm the above findings in the native host and further assess the possible effects of cellular factors, we have introduced PemK_{Sa}-encoding gene under the control of inducible promoter

(pCN51-pemK_{Sa}) into *S. aureus* RN4220. Induction of the PemK_{Sa} production resulted in growth inhibition, both in liquid and on solid media, in comparison with the bacteria carrying empty plasmid (Fig. 4a,b). Total RNA was isolated at various time points after induction of the toxin expression and the relative amount of selected transcripts was assessed using reverse transcriptase PCR. Again, transcript stability inversely correlated with the number of PemK_{Sa} target sequences. In case of a single transcript (*argR*) protection against PemK_{Sa} mediated degradation was observed in *S. aureus* compared with *E. coli* model and the results obtained *in vitro* (Fig. 4c and Supplementary Fig. S11).

The above data collectively suggest that PemK_{Sa} is capable of selectively targeting particular transcripts in the cellular pool of mRNA, possibly in a dose-dependent manner. As such, PemK_{Sa} may function as a global regulator of *S. aureus* gene expression, downregulating the housekeeping metabolism and transmembrane transport while preserving the expression of selected virulence related genes.

Return to equilibrium following temporary system activation.

If PemK_{Sa} has a regulatory function, mechanisms must exist to return the system to equilibrium following temporary activation. It is widely accepted that TA systems are induced by degradation of labile antitoxin, but there is no explanation for how, or whether, these systems return to equilibrium. We hypothesized that restoration of the equilibrium of temporarily activated PemK_{Sa} system is a self-perpetuating process dependent on the resistance of the antitoxin transcript to the ribonucleolytic activity of the toxin.

PemI_{Sa}- and PemK_{Sa}-encoding transcripts contain one and two PemK_{Sa} target sequences, respectively. However, when we treated

the transcripts encoding the TA system components, PemI_{Sa} and PemK_{Sa}, or the whole *pemIK*_{Sa} transcript, with purified toxin, the transcripts remained largely unaffected (Fig. 3b and Supplementary Fig. S9) under conditions where susceptible transcripts (such as those of *AdhC* or *FstW*) were completely degraded (Fig. 3a). Significantly, the part of *pemIK*_{Sa} transcript encoding the antitoxin was fully stable while that encoding the toxin was partially hydrolysed, but only inefficiently (Fig. 3b). Concurrently, computational modelling of the secondary structures of *pemIK*_{Sa} mRNA demonstrated that all putative cleavage sites are located in double-stranded regions (Supplementary Fig. S12), and are, therefore, inaccessible to PemK_{Sa}. As ribosomes and RNA-binding proteins may alter higher-order mRNA structures, we have verified the stability of *pemIK*_{Sa} transcript *in vivo*. To exactly define the time point of PemK_{Sa} activation, its transcript was provided *in trans* while the analysed *pemIK* transcript (*pemIK*_{Sa}*) contained frameshifts in both PemI_{Sa} and PemK_{Sa}-encoding fragments, both resulting in premature termination of translation. When the *pemIK*_{Sa}* transcript was challenged with ectopically expressed PemK_{Sa}, only negligible decrease in the amount of the transcript was observed compared with other susceptible transcripts evaluated in the same conditions (Fig. 3e). This suggests that PemK_{Sa} has no or only little effect on antitoxin translation, and in the absence of tertiary effects, the system should spontaneously return to equilibrium as soon as the stimulus that initially triggered the TA system imbalance is removed.

To assess whether the antitoxin is continuously synthesized, or if the overall burden associated with toxin activation arrests cellular protein synthesis, sequential expression of PemK_{Sa} and PemI_{Sa} was attempted. *E. coli* was transformed with

Figure 4 | PemK_{Sa} induces growth inhibition and selective transcript degradation in *S. aureus*. (a) Induction (CdCl₂) of ectopic production of PemK_{Sa} in the cell, results in growth inhibition of *S. aureus* RN4220 in liquid medium. Representative results of two independent experiments. (b) Same as in a, but on solid media. The bacteria were diluted as indicated and plated on inducing (CdCl₂) and non-inducing solid media. (c) Transcripts are differentially susceptible to PemK_{Sa} in *S. aureus*. PemK_{Sa} expression was induced and total mRNA was isolated at indicated time points. Level of indicated transcripts was assessed by reverse transcriptase PCR and electrophoretic analysis of reaction products. *adhC* was transcriptionally silent, which did not allow to assess its *in vivo* stability (compare with Fig. 3). All panels: Expression of PemK_{Sa} was driven from pCN51-pemK_{Sa} while the control contained empty pCN51 plasmid only.

pBAD-pemK_{sa} and pACYCDuet-pemI_{sa}, the expression of recombinant toxin and antitoxin was sequentially induced, and the accumulation of both proteins was monitored. Consistent with other experiments in this study (Supplementary Fig. S2), induction of toxin expression in the absence of antitoxin expression resulted in growth inhibition, and no toxin accumulation was detected. Induction of antitoxin production alone resulted in efficient expression. Simultaneous induction of PemI_{sa} and PemK_{sa} resulted in detectable expression of both proteins. Significantly, sequential induction of toxin followed by induction of antitoxin, even when separated by a period of 3 h, resulted in efficient translation of antitoxin (Fig. 3d and Supplementary Fig. S13). This indicates that the protein synthesis apparatus remains functional long after toxin activity is induced. Further supporting this interpretation, recombinant NrdR (a transcript not affected by PemK_{sa}) was expressed despite prior toxin induction, whereas no production of recombinant ArgR or ScpB (both transcripts are susceptible to PemK_{sa}) was observed after induction of the toxin (Fig. 3a,c).

To verify that inhibition of ScpB production upon PemK_{sa} induction is a direct effect of *scpB* transcript degradation by PemK_{sa} and not other tertiary effects we evaluated if *scpBalt* is capable to drive efficient expression of ScpB in the presence of PemK_{sa}. *In vivo*, ScpB protein was efficiently produced from *scpBalt* gene despite ectopic expression of PemK_{sa} (Fig. 3c). Conversely, no accumulation of NrdR protein was observed when *nrdRalt* was used instead of *nrdR* (Fig. 3c).

Overall, the susceptibility of mRNA to the toxin determines the fate of particular protein synthesis upon TA system induction, whereas the protein translation apparatus remains functional. These results strongly support our hypothesis of self-perpetuating restoration of TA system equilibrium.

Discussion

The plasmid maintenance function of TA systems has been well established^{1,2}, but increasing number of recent studies suggest a regulatory role of TA systems in broadly defined stress response^{7,31}. Our current understanding of the latter function of TA systems is largely speculative, but if confirmed, TA systems are likely to emerge as one of the most versatile global regulatory systems in bacteria. Multiple chromosome-encoded TA systems have been identified³², and many of these contain site-specific ribonucleases. The low-specificity mycobacterial toxins MazF-mt1 (U↓AC) and MazF-mt6 (cleaves preferentially in U-rich regions) cause growth inhibition when heterologously expressed in *E. coli*, whereas MazF-mt3 (UU↓CCU, CU↓CCU) and MazF-mt7 (U↓CGCU) show almost no effect^{16,33}. PemK_{sa} (U↓AUU) induces an intermediate effect when expressed in *E. coli*, inhibiting growth in liquid, but not on solid media. Although the above differential influence may be modulated by other factors (such as for example the level of overexpression), it seems reasonable to hypothesize that the longer the recognition sequence the greater the potential to selectively target only parts of the transcriptome. Importantly, although *pemIKsa* was identified on a plasmid in strain CH91, almost identical systems are present throughout the *Staphylococcus* genus, and with only one exception are localized on the bacterial chromosome (Supplementary Fig. S7). This suggests that the system may be transmitted between the chromosome and plasmids, but also that its primary role is distinct from plasmid maintenance.

Several studies have demonstrated that recognition sequences of particular interferases are distributed in a nonrandom fashion in the transcriptome of host species. This suggested that bacterial genomes have coevolved with interferases to distinguish susceptible and resistant gene pools, and thus facilitate global regulation.

Only simple models, based on frequencies of particular nucleotides in all CDSs, have previously been used to predict the expected random distribution of interferase target sequences in the transcriptome^{14,16,34}. Comparison with the observed distribution enabled groups of transcripts with significantly underrepresented or overrepresented interferase recognition sequences to be distinguished. Here we proposed a more biologically relevant model based on frequencies of coding triplets. Our model provides predictions that better fit the observed distribution, and limits the number of sequences outside the assumed confidence interval, which enables better prediction of the pools of transcripts likely to be differentially regulated by the interferase (Supplementary Fig. S14). It is of note that for the tested interferases these pools of transcripts are surprisingly functionally consistent. For example, the MazF-mt3 or MazF-mt7 recognition sequence is underrepresented in a subset of genes of the PE and PPE families, which are important in the immunopathogenicity of *M. tuberculosis*¹⁶. Further, the recognition sequence for MazF-cd is underrepresented in *tox*B and *cwpV* genes, encoding factors implicated in virulence¹⁷. We argue that PemIK_{sa} is involved in virulence modulation in *S. aureus*, based on the fact that the PemK_{sa} recognition sequence is underrepresented in a range of genes encoding virulence factors. As the MazF_{sa} toxin has also been implicated in regulation of staphylococcal virulence¹⁵, interferase-mediated modulation of virulence factors emerges as a common trait. Another large group of transcripts potentially regulated by PemK_{sa} includes those encoding transmembrane transporters. This is consistent with other studies arguing that not only groups of genes related to virulence are potentially susceptible to TA-mediated regulation (although some transmembrane transporters are involved in pathogenesis). For example, in the soil bacterium *Bacillus subtilis* a group of transcripts encoding proteins involved in antibiotic synthesis has been predicted to be resistant to MazF_{Bs}, based on statistical analysis³⁵. Although the specificities of MazF_{Bs}, MazF_{sa} and MazF-cd are identical, the range of affected transcripts differs. This clearly indicates adaptive evolution of the genomes to differentially exploit the regulatory potential of similar interferases among different species.

Although computational analyses consistently demonstrate functionally related pools of transcripts as being potentially resistant/susceptible to interferase action, the regulatory function of interferases remains a tempting hypothesis rather than a proven fact. The experimental evidence concerning the presumed regulatory role of TA interferases is almost nonexistent. Most significantly, the differential susceptibility of four different transcripts to MazF_{sa} has been demonstrated. mRNA encoding pathogenic adhesive factor SraP was rapidly degraded by MazF_{sa} (ref. 15), whereas transcripts of housekeeping genes (including *recA*, *gyrB* and *sarA*) were resistant³⁴. This is the only previous direct experimental demonstration of TA-mediated regulation of transcript stability we are aware of. Thus, our comprehensive demonstration of a direct correlation between the predicted and observed susceptibility of different transcripts to interferase *in vitro*, and the observation that resistant, but not susceptible transcripts are able to drive the expression of their respective proteins *in vivo* despite activation of the TA system, strongly support the proposed regulatory function of TA systems.

Given the recent speculation about the regulatory role of TA systems, it is surprising that no consistent model of their reversible activation has been provided. It is generally accepted that TA systems are activated in response to external stimuli. However, regulatory function requires both an activating mechanism and an efficient means of switching between the active and inactive states. Although obvious, this requirement has never previously been comprehensively investigated with respect

We experimentally demonstrated that all the requirements necessary for the proposed mechanism to function were fulfilled. First, we showed that the *pemIK_{Sa}* transcript was relatively resistant to PemK_{Sa} compared with other, susceptible genes. Second, we demonstrated that PemK_{Sa}-associated RNA interference did not directly affect the cell translation machinery, which remained functional despite toxin activation. Third, we showed that PemI_{Sa} was expressed even in the presence of active toxin. A simple mechanism such as that described here could potentially regulate all TA systems where the toxin is a sequence-specific RNase. Indeed, the pentad sequences recognized by MazF-mt3, MazF-mt7, MazF_{Sa}, MazF_{Bs} and MazF-cd are absent in the transcripts of their respective antitoxins^{15-17,35}. Moreover, toxins other than PemK_{Sa} do not interfere with cell translation machinery^{17,36}. Currently, the most important question remaining is whether global analyses of interferase effects on transcriptomes and proteomes will confirm the assumptions concerning their universal regulatory role in bacteria.

Bacterial strains and culture conditions. *E. coli* strains DH5 α and BL21(DE3) were cultured in Luria broth (LB). *S. aureus* strains CH91 and RN4220 were grown in tryptic soy broth. All cultures were incubated at 37°C with thorough aeration. For plasmid propagation, the media were supplemented with ampicillin (amp) (100 $\mu\text{g ml}^{-1}$) or chloramphenicol (cam) (34 $\mu\text{g ml}^{-1}$ and 10 $\mu\text{g ml}^{-1}$ for *E. coli* and *S. aureus*, respectively).

Protein expression and purification. Recombinant proteins were produced in *E. coli* BL21(DE3). Expression was induced with 1 mM IPTG. Six histidine tagged antitoxin PemI_{Sa} was expressed from pETDuet-pemI_{Sa}. A similarly tagged toxin mutant was expressed from pETDuet-pemK_{Sa}R84A. Both proteins were recovered using affinity chromatography under native conditions. His₆-tagged PemK_{Sa} was coexpressed with native PemI_{Sa} using pETDuet-pemIK_{Sa}. PemK_{Sa} was purified from the complex with PemI_{Sa} using affinity chromatography in denaturing conditions, and refolded by dialysis against 50 mM sodium phosphate pH 8.0 containing 300 mM NaCl and 20% glycerol. For each protein, the final purification step consisted of gel filtration on Superdex 75 (GE Healthcare) in 50 mM sodium phosphate, pH 8.0 containing 100 mM NaCl (buffer GF). All preparations obtained were homogenous, as determined using SDS-PAGE⁴⁰.

Plasmid maintenance tests. *E. coli* BL21(DE3) were double-transformed with pETDuet-pemK_{Sa} and pACYCDuet-pemI_{Sa} or pETDuet and pACYCDuet-pemI_{Sa}, inoculated into LB supplemented with amp, and cultured overnight (ON). Each

following day a new culture was set up by 1,000 times dilution of the ON culture into fresh LB with amp. Simultaneously, the aliquot of bacteria was diluted and plated on LB agar with amp. Colonies were counted and transferred on LB agar with amp and cam using replica plate method⁴¹. The percentage of cam resistant colonies relative to amp resistant colonies was determined. *S. aureus* RN4220 was transformed either with pALCP2G-pemK_{sa} or empty pALCP2G, inoculated to tryptic soy broth with no selective antibiotic, and incubated ON. Subsequent cultivation was performed as described above, save that bacteria were plated on tryptic soy broth agar and the percentage of colonies that retained the ability to express GFP was determined. On the last day of the experiment, the percentage of colonies resistant to cam relative to all colonies was determined by replica plate method.

Assessment of RNA hydrolysis sites by primer extension. The total RNA of phage MS2 was reacted with PemK_{sa} according to the protocol of Zhu *et al.*¹⁶, with the exception that the RNA chaperone CspA was not included. Briefly, 1 µg of MS2 RNA and 10 pmol of PemK_{sa} were incubated at 37 °C in 10 mM Tris-HCl (pH 8.0) containing 10 mM EGTA in a total volume of 10 µl for 30 min. Primer extension was performed as described by Nadratowska-Wesolowska *et al.*⁴² with slight modifications. Briefly, toxin-digested MS2 RNA was mixed with 0.5 pmol of ³²P-labelled primer (Supplementary Table S5), incubated for 20 min at 70 °C, then slowly cooled to 40 °C and transferred to an ice bath. M-MuLV buffer, deoxyribonucleotide triphosphates, RevertAid M-MuLV Reverse Transcriptase and RNase inhibitor (Fermentas) were added. The reaction was continued for 60 min at 42 °C, and terminated by addition of denaturing solution (95% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue) and incubation at 70 °C for 5 min. The reaction products were separated by electrophoresis in 8% polyacrylamide gel containing 8 M urea, and the bands were visualized on X-ray film.

Coexpression assay. Bacteria were grown in LB containing selection antibiotics to optical density at 600 nm (OD₆₀₀) ~0.5 before induction of recombinant proteins expression. In case of pETDuet-pemK_{sa} and pACYCDuet-pemI_{sa} cotransformants, the expression from both plasmids was induced simultaneously with IPTG. In case of pBAD-pemK_{sa} carrying cotransformants, PemK_{sa} expression was induced with arabinose and partner coexpression was induced with IPTG at time points indicated on Fig. 3c. Expression of recombinant proteins were analysed by SDS-PAGE.

In vitro PemK_{sa} complex formation. Samples containing 30 nmol of PemK_{sa}, 45 nmol of PemI_{sa} or mixture of above were analysed by gel filtration using the Superdex 75 HR column (GE Healthcare) in buffer GF. Fractions were analysed by SDS-PAGE and MS2 RNA degradation assay.

Bioinformatic analysis. The probability (*P*) of finding *K* interbase recognition sequence(s) in a CDS of length *L* can be calculated using the binominal distribution according to equation 1:

$$P = p_{\text{tot}}^K (1 - p_{\text{tot}})^{n-K} \binom{n}{K} \quad (1)$$

where *n* is the number of sites where a super-motif of length *l'* (containing an interbase recognition sequence of length *l*) can be placed in the CDS, according to equations 2 and 3:

$$n = \frac{L - l'}{3} + 1 \quad (2)$$

$$l' = 3 \left\lceil \frac{l+2}{3} \right\rceil \quad (3)$$

note that biologically meaningful *L* values are multiples of three. The super-motif is defined as the smallest number of sequential codons necessary to accommodate the interbase recognition sequence in all three possible positions with respect to an open reading frame (Supplementary Fig. S14). The total probability (*p*_{tot}) of finding the interbase recognition sequence along the super-motif is related to the frequency of particular codons (*π*_i). The frequency of codons in mRNA of *S. aureus* strain ED98 was determined based on all CDSs in GenBank records CP001781, CP001782, CP001783 and CP001784. To avoid bias, the initial and stop codons were omitted from the calculations.

CDS of *S. aureus* ED98 were separated into three groups according to the distribution of interbase recognition sequences. Group I included transcripts where the interbase recognition sequence was strongly underrepresented (*P*' < 0.01; equation 4) or absent. Group II contained transcripts that best fitted the probability distribution (0.01 < *P*' < 0.99). Group III contained transcripts where the recognition sequence was strongly overrepresented (*P*' > 0.99).

$$P' = \sum_{k=0}^K p_{\text{tot}}^k (1 - p_{\text{tot}})^{n-k} \binom{n}{k} \quad (4)$$

The fitness of the resulting model was assessed by comparing the adjusted coefficient of determination based on sequence length standardized deviations with

a similar coefficient calculated for the model based on nucleotide frequencies (the latter approach has been used by others to computationally assess the likely impact of mRNA interferences on the bacterial transcriptome^{14,16,34}). The internet tool implementing our model for analysis of user provided sequences is available at <http://mol058.mol.uj.edu.pl/pemmetstat>.

All analysed genes were classified into three groups: 'factors related to pathogenesis', 'transmembrane transporters' and the remaining genes. Factors related to pathogenesis were annotated according to NCBI BioSystems database (<http://www.ncbi.nlm.nih.gov/biosystems>). This annotation was completed manually by referring to the literature. 'Transmembrane transporters' were assigned according to the annotation of *S. aureus* ED98 genome. Classification and annotation of genes are presented in Supplementary Data 1.

Susceptibility of staphylococcal mRNA to PemK_{sa}. The mRNA of PemI_{sa}, PemK_{sa} and reporter proteins was obtained from the respective PCR amplified genomic sequences using TranscriptAid T7 High Yield Transcription Kit (Fermentas). In each case, 1 µg of mRNA was reacted with 10 pmol of PemK_{sa} at 37 °C for various time periods in 20 µl reaction mixture containing 10 mM Tris-HCl (pH 8.0) and 10 mM EGTA. The degree of hydrolysis was analysed using agarose gel electrophoresis.

In vivo analysis of transcript levels. Bacteria were collected at different time points from liquid culture by centrifugation. *E. coli* cells were resuspended in TRI Reagent. *S. aureus* cells were mechanically disintegrated in TRI using glass beads. Subsequent steps were performed using GeneJET RNA Purification Kit (Thermo Scientific). DNA contamination was removed with On-Column DNase I Digestion Set (Sigma-Aldrich). Complementary DNA was obtained using RevertAid Premium Reverse Transcriptase (Thermo Scientific) and random hexamers. In each case, 200 ng of total cellular RNA was used. PCR was carried out using the obtained complementary DNA and primers specific for each characterized gene (Supplementary Table S3). The products were analysed by agarose gel electrophoresis in TAE buffer.

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Author contributions

B.W. discovered pCH91 plasmid and identified the TA system; M.B., G.D. and B.W. planned research and designed experiments; M.B., R.L., W.M.H., E.B. and B.W. performed the experiments; M.B. designed and performed computational studies; M.B., A.S.P., G.D., G.W. A.D. and B.W. analysed data; M.B. G.D. and B.W. wrote the paper. M.B. and B.W. contributed equally to this study.

Additional information

Supplementary Information accompanies this paper at <http://www.nature.com/naturecommunications>

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