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Chromosomal localization of PemIK toxin-antitoxin system results in the loss of toxicity – Characterization of *pemIK$_{Sa1}$-Sp* from *Staphylococcus pseudintermedius*

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ABSTRACT

Toxin-antitoxin (TA) systems are ubiquitous in bacteria and on numerous occasions have been postulated to play a role in virulence of pathogens. Some *Staphylococcus aureus* strains carry a plasmid, which encodes the highly toxic PemIKSa TA system involved in maintenance of the plasmid but also implicated in modulation of gene expression. Here we showed that *pemIKSa1-Sp* TA system, homologous to the plasmid-encoded PemIKSa, is present in virtually each chromosome of *S. pseudintermedius* strain, however exhibits sequence heterogeneity. This results in two length variants of the PemKSa1-Sp toxin. The shorter (96 aa), C-terminally truncated toxin is enzymatically inactive, whereas the full length (112 aa) variant is an RNase, though nontoxic to the host cells. The lack of toxicity of the active PemKSa-Sp2 toxin is explained by increased substrate specificity. The *pemISa1-Sp* antitoxin gene seems pseudogenized, however, the whole *pemIKSa1-Sp* system is transcriptionally active. When production of N-terminally truncated antitoxins using alternative start codons is assumed, there are five possible length variants. Here we showed that even substantially truncated antitoxins are able to interact with PemKSa-Sp2 toxin and inhibit its RNase activity. Moreover, the antitoxins can rescue bacterial cells from toxic effects of overexpression of plasmid-encoded PemKSa toxin. Collectively, our data indicates that, contrary to the toxic plasmid-encoded PemIKSa TA system, location of *pemIKSa1-Sp* in the chromosome of *S. pseudintermedius* results in the loss of its toxicity. Interestingly, the retained RNase activity of PemKSa1-Sp2 toxin and functionality of the putative, N-terminally truncated antitoxins suggest the existence of evolutionary pressure for alleviation/mitigation of the toxin's toxicity and retention of the inhibitory activity of the antitoxin, respectively.

1. Introduction

Toxin-antitoxin (TA) systems are predominantly associated with maintenance of genetic material, stress response and persister phenotype (Harms et al., 2018; Song and Wood, 2018). TA systems are widespread in bacteria and found both on plasmids and chromosomes. Regardless the genomic localisation of TA operons, the antitoxin inhibits the deleterious effect of its cognate toxin – either by direct protein-protein interaction or by blocking toxin synthesis through interference with its mRNA (Kang et al., 2018). A wide variety of stress stimuli, such as high temperature, changes of environmental conditions, oxidative damage, nutritional deficiency, bacteriophage infection or antibiotic treatment can perturb the balance the toxin and antitoxin level. The resulting activation of the toxin leads to various physiological effects most often resulting in inhibition of cell growth and division (Song and Wood, 2020). For the cell, the severity of the consequences resulting from toxin activation and the reversibility of the effect itself largely depend on TA operon localisation. For plasmid-encoded TA systems, the loss of mobile genetic element's (MGE) encoding at cell division eliminates daughter cells from the population.

Abbreviations: IPTG, isopropylβ-D-thiogalactoside; ORF, open reading frame; MGE, mobile genetic element; SEC, size exclusion chromatography; TA, toxinantitoxin

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Cytoplasmatically inherited, inactive TA complex eventually releases the active toxin which poisons the daughter cell devoid of the TA-encoding plasmid. The toxin's action is irreversible and when prolonged, leads to dormancy and eventually death. Only cells that inherited the TA-encoding plasmid are able to grow effectively, which ensures stable propagation of TA dependent MGEs (Gerdes et al., 1986; Jaffé et al., 1985). In contrast, chromosome-encoded TA systems cannot be easily lost at cell division. It has been suggested that chromosomal TA systems are simply selfish genes (Ramisetty and Santhosh, 2017), but accumulating evidence suggests that such systems have a regulatory function on the overall cell metabolism (Harms et al., 2018). A fine-tuned mechanism of reversible switching between active and inactive state of a TA system and directed action of a toxin on selected cell components would constitute prerequisites for such a role. In fact, regulation of expression of TA system components is facilitated by the genetic organisation of TA operons, where the antitoxin gene precedes the gene encoding the toxin, which ensures priority in the production of the former. More significantly, some antitoxin proteins bind the respective TA operon promoters and the interaction is modulated by the toxin. The phenomenon, known as conditional cooperativity, regulates expression of some TA systems (Overgaard et al., 2008). Regarding the postulated global regulatory effects of toxins on cell metabolism – the activity of many toxins is directed against gene transcripts. It is widely debated if the experimentally demonstrated targeting of only limited number of cellular transcripts is a random effect or rather reflects a global evolution of cellular transcripts. In either case, the fact is that the overall protein synthesis is not entirely switched off at the toxin activation and the activated TA system is able to return to the "dormant" state, which allows the argument for its regulatory role (Bukowski et al., 2013).

TA systems are currently classified into seven types, according to the antitoxin nature and the mechanism of action of particular systems (Song and Wood, 2018). Type II, the most ubiquitous type of TA systems is of particular relevance to this study. In the type II TA systems both the toxin and the antitoxin are proteins and the majority of toxins act as RNases: endoribonuclease (DinJ-YafQ), ribosome-dependent (YefM-YoeB) or independent mRNase (MqsAR) or mRNA interferase (MazEF, RelBE, YafNO) (Deter et al., 2017). Type II toxins characterized to date selectively recognize and target 3–7 bp long RNA sequences (Masuda and Inouye, 2017). However, within type II TA systems are also toxins targeting DNA gyrase and cell wall synthesis, CcdB and PezT, respectively (Mutschler et al., 2011; Van Melderen, 2001).

Staphylococcus pseudintermedius together with *S. intermedius* and *S. delphini* are Gram-positive, coagulase-positive bacteria commonly classified as *Staphylococcus intermedius*-group (SIG) (Sasaki et al., 2007). SIG bacteria are part of normal skin flora, but have also been associated with variety of infections and diseases. SIG are typically animal pathogens, most commonly isolated from dogs and cats (Pires dos Santos et al., 2016), however, cases of human infection have also been reported (Magleby et al., 2019; Somayaji et al., 2016; Van Hoovels et al., 2006). Of further relevance, many *S. pseudintermedius* strains have acquired multi-drug resistance over the last ten years and are hard to treat. More importantly, it is postulated that they serve as transfer vectors of resistance genes to human infecting species. This involves methicillin-resistant *S. pseudintermedius* (MRSP), responsible for infections, some of which are impossible to treat with the currently available antibiotics (Pires dos Santos et al., 2016).

So far, only a few TA systems have been experimentally characterized in *Staphylococci*. These include *yefM/yoeB* (Wen et al., 2018; Yoshizumi et al., 2009), *mazEF* (Schuster et al., 2013; Zhu et al., 2009) and related *pemIK* (Bukowski et al., 2013). An analysis of available staphylococcal genomes revealed that the *mazEF* system is present in practically every examined strain, while *pemIK* systems are sparse (~3% of tested strains) and often located in mobile genetic elements. Among staphylococcal *pemIK* systems, one third belonged to *pemIK-Sa1* homology group. Contrary to its plasmid-encoded character in other staphylococci, *pemIK-Sa1* was found on chromosomes of SIG species (Bukowski et al., 2017). The rationale for *pemIK-Sa1* system shift onto the chromosome of SIG species remains unclear.

Here, we analyse the *pemIK-Sa1* TA system prevalence in *S. pseudintermedius* and examine its functionality. Our results prove that the system is present in virtually all *S. pseudintermedius* strains, but displays significant sequence heterogeneity. Despite being an active RNase, the toxin does not significantly affect the viability of bacterial cells, which may be explained by its restricted substrate specificity. *PemI-Sa1* antitoxin encoding genes often seem pseudogenized, however the existence of N-terminally truncated variants expressed from alternative start codons cannot be excluded, especially for the fact we demonstrate in this study, which is that such variants retain their ability to bind and inhibit the toxin when recombinantly expressed.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. coli strains TOP 10 and BL21(DE3) were used for DNA cloning and recombinant protein production. *S. pseudintermedius* strains used in the study are listed in Supplementary table 3. Strains 235, 244, 21007, LMG22220 and LMG22221 were used for the analysis of the $pemI_{Sa1}$-*Sp* and $pemK_{Sa1}$-*Sp* transcription level. *E. coli* were cultured in Luria Broth (LB) while *S. pseudintermedius* in Tryptic Soy Broth (TSB). Bacteria were incubated at 37 °C with thorough aeration (180 rpm) for 16 h. For production of recombinant proteins, the temperature was lowered to 18 °C upon induction with 1 mM IPTG (isopropyl-β-D-thiogalactoside). For *E. coli* carrying plasmid constructs derived from pETDuet-1 (Novagen), pBAD/His B (Thermo Fisher Scientific) and pACYCDuet-1 (Novagen) media were supplemented respectively with ampicillin (100 μg ml^{-1}) or chloramphenicol (34 μg ml^{-1}).

2.2. TA genes detection and construction of recombinant plasmids

The putative loci of the toxin-antitoxin operon $pemIK_{Sa1}$-*Sp* were amplified from the bacterial chromosome of *S. pseudintermedius* strains 9, 200, 221, 223, 229, 235, 244, 258kot, 265, 1025, 21007, IKB402, IKB7616, IKB7824, IKB8470, IKB8512, IKB8686, LMG22219, LMG22220, LMG22221, using the pemIKpseudUpA-F and pemIKpseudDown_Rev primers. Amplicons were separated on agarose gel, purified and sequenced using the above primers. Components of the toxin-antitoxin system $pemIK_{Sa1}$-*Sp* ($pemI_{Sa1}$-*Sp* antitoxin; $pemK_{Sa1}$-*Sp* toxin) coding for different lengths of the respective proteins were amplified from chromosomal DNA of *S. pseudintermedius*: $pemI_{Sa1}$-*Sp1* and $pemK_{Sa1}$-*Sp2* from strain LMG22220; $pemI_{Sa1}$-*Sp2* from strain 235, $pemI_{Sa1}$-*Sp3* from strain 21007; $pemI_{Sa1}$-*Sp4* from strain LMG22221; $pemI_{Sa1}$-*Sp5* and $pemK_{Sa1}$-*Sp1* from strain 244. Primers used in the study and obtained plasmid constructs are listed in the Table S1 and Table S2, respectively. Amplicons were ligated into pTZ-57R/T vector (Thermo Fisher Scientific) and sequenced. The correctly cloned genes were excised and inserted into expression vectors: pETDuet™-1, pACYCDuet™-1, pBAD/His B or pETDuet-AF (Bukowski et al., 2013). QuickChange site-directed mutagenesis (Stratagene) was used to obtain LY12ED mutant of pETDuet-$PemK_{Sa1}$-Sp4.

2.3. In vivo co- and cross-interactions of $PemIK_{Sa1}$-*Sp* system components

In the co- and cross-interaction tests, the co-transformants of *E. coli* BL21 (DE3), containing recombinant plasmids pBAD_$PemK_{Sa1}$-Sp2, pBAD_$pemK_{Sa1}$ (Bukowski et al., 2013) encoding arabinose-induced toxins and pACYCDuet-$PemI_{Sa1\text{-}Sp}$, pACYCDuet-$pemI_{Sa}$ (Bukowski et al., 2013) coding for IPTG-induced antitoxins were used. Empty pACYCDuet-1 and pBAD/His B were used as a control. The overnight cultures of co-transformants were diluted 1:100 in LB and grown to OD_{600} of 0.1. Then, the bacteria were ten-fold serial diluted (10°, 10^1, 10^2, 10^3, 10^4 and 10^5) and plated in a form of dots (2 μL) on 1.5 % agar

solidified LB medium supplemented with respective antibiotics and inducer(s): 0.2 % (w/v) L(+)-arabinose (induction of toxin production), 0.05 mM IPTG (induction of antitoxin production) or both and incubated at 37 °C, for 16 h.

2.4. Production and purification of recombinant proteins

Target proteins were overproduced in *E. coli* BL21(DE3) grown in TSB medium at 18 °C for 16 h with thorough aeration (180 rpm). Expression was induced by IPTG (final concentration 1 mM). All analysed proteins, except of the $PemK_{Sa1}$-Sp1 were isolated from bacteria by sonication and purified in native conditions by immobilized metal affinity chromatography (IMAC) on the Ni-NTA resin (Qiagen) and by Size Exclusion Chromatography (SEC) on the Superdex 75 HR column (GE Healthcare). $PemK_{Sa1}$-Sp1 toxin was isolated from bacteria by incubation of pellets in a buffer containing 6 M GuHCl and purified in denaturing conditions by IMAC on Ni-NTA resin. Refolding of this toxin was optimized using QuickFold™ Protein Refolding Kit (Molecular Dimensions Ltd.). All purified proteins were homogenous, as determined using SDS-PAGE (Schägger and von Jagow, 1987).

2.5. RNA cleavage by PemK toxins

The analysis was performed according to the modified protocol described in Zhu et al., 2006, with the exception that the RNA chaperone CspA was not included. Briefly, the 0.5 μg of purified PemK toxin (PemK-Sa1 or $PemK_{Sa1}$-Sp2) was incubated with 1 μg of MS2 RNA (Sigma Aldrich) in 10 mM Tris pH 8.0 containing 10 mM EGTA, for 30 min, at 37 °C. The cleavage products were analysed by agarose gel electrophoresis in TAE buffer.

2.6. Analysis of sites of RNA hydrolysis by PemK toxins by primer extension method

Cleaved MS2 RNA was subjected to primer extension analysis according to the modified protocol described in (Nadratowska-Wesołowska et al., 2010). 0.2 μg of processed RNA was mixed with 1 μL of ^{32}P-labelled primer (listed in the Table S3) and incubated for 20 min. at 70 °C. Then slowly cooled to 40 °C and transferred to ice. Next, reverse transcriptase buffer (M-MuLV Buffer; Sigma Aldrich), deoxyribonucleotide triphosphates (dNTPs), reverse transcriptase M-MulV (Sigma Aldrich) and RNA inhibitors were added. The reaction was carried out for 60 min at 42 °C and stopped by incubation of the probes for 5 min at 95 °C. The reaction products were separated by electrophoresis in a 7 % polyacrylamide gel, containing 8 M urea. The bands were autoradiographically visualized and analysed using Quantity One software (Bio-Rad).

2.7. Assessment of functionality of $PemI_{Sa1}$-Sp antitoxins

The *in vivo* functionality of the $PemI_{Sa1}$-Sp antitoxins was analysed, assessing the ability to rescue the production of PemK-Sa1 recombinant toxin during a co-expression assay (Bukowski et al., 2013). *E. coli* BL21(DE3) were transformed with the pETDuetAF-$PemI_{Sa1}$-Sp plasmids coding for PemK-Sa1 toxin and five analysed variants of $PemI_{Sa1}$-Sp antitoxins (Table S2). The overnight culture was diluted 1:100 in LB and incubated at an OD_{600} in the range of 0.6 − 0.8. IPTG (1 mM) was added and the culture was continued for 3 h. 1 mL samples were collected every hour and the presence of the target protein(s) was determined by SDS-PAGE and Western Blot, using the Anti-His-tag antibodies.

2.8. In vitro $PemIK_{Sa1}$-Sp complex formation

240 μg of the purified toxin $PemK_{Sa1}$-Sp2, 500 μg of tested antitoxin $PemI_{Sa1}$-Sp or the mixture of both proteins were loaded on the Superdex 75 HR column equilibrated with SEC Buffer (50 mM NaH_2PO_4 pH 8.0, 100 mM NaCl, 20 % glycerol (*v/v*)) and resolved with the flow rate of 0.7 mL/min. The peak fractions were analysed by SDS-PAGE and by cleavage test on RNA MS2.

2.9. Analysis of transcript levels of $pemIK_{Sa1}$-Sp system components

S. pseudintermedius strains LMG22220, 235, 244, 21007, LMG22221 and 244 were cultured in liquid medium TSB. At OD_{600} of 1 samples (1 mL) were collected by centrifugation and bacterial pellet was suspended in 1 mL of TRI Reagent (Sigma Aldrich). Bacteria were mechanically disintegrated using glass beads and Precellys equipment (Bertin Instruments). Then, 200 μL of chloroform was added to the obtained cell lysate and the samples were vigorously shaken for 20 s, and subsequently centrifuged (15,000 rcf, 10 min, 22 °C). The upper aqueous phase was collected and further processed using GeneJet Purification Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Residual DNA was removed with On-Column DNase I Digestion Set (Sigma Aldrich). Complementary DNA was obtained for isolated RNA in the reverse transcription reaction using the Maxima H minus reverse transcriptase (Thermo Fisher Scientific) and random hexamers. cDNA and primers specific for analysed genes (Table S1) were used in Real-Time PCR reaction. The level of genes' transcription was determined relative to expression of DNA gyrase subunit B.

3. Results

3.1. Chromosomal toxin-antitoxin system $pemIK_{Sa1}$-Sp is widespread among strains of S. pseudintermedius and widely heterogeneous

Our earlier report on the discovery of PemIKSa TA systems encoded on *S. aureus* plasmids argued their regulatory role, despite the location on mobile genetic elements (Bukowski et al., 2013). Using bioinformatic analysis of a limited number of staphylococcal genomes available at the time, we identified the presence of homologous systems located on bacterial chromosomes of staphylococcal species other than *S. aureus*. We found variants of the PemIK system in all four analysed genomes of *S. pseudintermedius*, however the limited genomic data at the time did not allow us to conclude the system prevalence, functionality or heterogeneity. In this report we screened our collection of twenty *S. pseudintermedius* strains for *pemIK* operons. Using pemIKpseudUpA-F and pemIKpseudDown_Rev primers (Table S1) we amplified putative *loci*. The amplicons were detected by DNA electrophoresis for all analysed strains, however differed slightly in size. Thus, we sequenced the amplified DNA and analysed the obtained sequences for the presence of open reading frames (ORF) homologous to *pemIKSa* genes. Quite surprisingly, we detected significant heterogeneity within amplicon sequences involving 2 length variants of $PemK_{Sa1}$-Sp toxin and 5 length variants of $PemI_{Sa1}$-Sp antitoxin (Figs. 1, S1 and S2). The short (96 aa) toxin variant resulted from two-nucleotide insertion in the vicinity of 3′ end of *pemK* gene, generating a frame shift and consequently a premature stop codon. The long variant of the toxin ($PemK_{Sa1}$-Sp2; 112 aa) corresponds to the prototype PemK-Sa1 toxin from *S. aureus*. The extent of sequence similarity of PemK toxins form *S. pseudintermedius* and *S. aureus* is variable through the protein and lowest in the C-terminal region. The accumulation of single-nucleotide insertions/deletions and substitutions in antitoxin encoding genes produces a more complicated picture with frame shifts and stop codons located at the very beginning of the putative PemI coding sequences suggesting pseudogenisation. However, pseudogenisation of the antitoxin gene in the presence of a functional toxin gene would imply constitutive toxin activity. Thus, we hypothesized that alternative start codons may be used to express N-terminally truncated antitoxins. In this scenario, five putative antitoxin variants, $PemI_{Sa1}$-Sp1-5, consisting of 26, 30, 31, 40 and 59 amino acids, respectively, could be expressed in different strains (Fig. 1A).

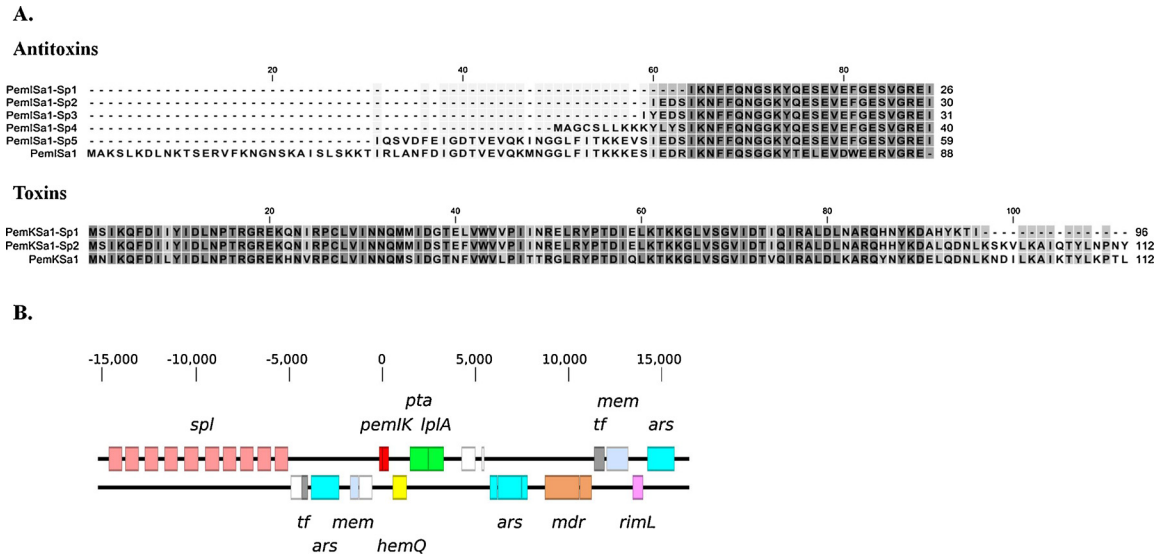

Fig. 1. Sequence variants of PemIK$_{Sa1}$-Sp toxin-antitoxin system components identified in the analysed pool of *S. pseudintermedius* chromosomes. **(A)** Comparison of the amino acid sequences of antitoxins (PemI$_{Sa1}$-Sp; panel A) and toxins (PemK$_{Sa1}$-Sp; panel B), with reference to prototypical plasmid-encoded PemIK$_{Sa}$, reveals two and five length variants of the toxin and antitoxin, respectively. PemI$_{Sa1}$-Sp antitoxins and PemK$_{Sa1}$-Sp1 toxin are respectively N- and C-terminally truncated compared to *S. aureus* prototypes. **(B)** Localization of *pemIK$_{Sa1}$-Sp* toxin-antitoxin system within chromosome of *S. pseudintermedius* (based on strain HKU10-03). *spl,* serine protease like endopeptidases; *tf,* putative transcription factor; *ars,* genes likely involved in arsenic resistance; *mem,* putative membrane protein; *pemIK,* toxin-antitoxin system *pemIK$_{Sa1}$-Sp; hemQ,* hemoprotein HemQ; *pta,* phosphotransacetylase; *lplA,* lipoate-protein ligase A; *mdr,* multidrug resistance efflux pump; *rimL,* acetyltransferase.

To verify the conclusions of our amplicon analysis we searched the whole genome sequencing data available for *S. pseudintermedius*. Indeed, PemIK$_{Sa1}$-Sp operon was identified in 222 out of 223 analysed genomes demonstrating its wide penetrance. In each case, the TA system was located in the same genetic context between genes coding for putative membrane protein and hemoprotein HemQ. Moreover, in a vicinity of *pemIK$_{Sa1}$-Sp* we did not identified transposons or known insertion elements (Fig. 1B). Again, two length variants of the PemK$_{Sa1}$-Sp toxin were observed, with the long variant PemK$_{Sa1}$-Sp2 (112 aa) being by far more prevalent (214 hits). The shorter variant (PemK$_{Sa1}$-Sp1, 96 aa) was present only in less than 4% (8 hits) of analysed genomes. Among antitoxins, the most prevalent variants were PemI$_{Sa1}$-Sp4 and PemI$_{Sa1}$-Sp5 (151 and 44 hits, respectively). The "full length" antitoxin consisting of 89 aa (similar to prototype PemISa from *S. aureus* plasmid) was identified only in 5 *S. pseudintermedius* genomes (Suppl. data set 1).

3.2. The *pemIK$_{Sa1}$-SpTA* system is transcriptionally active

TA systems often autoregulate their own expression through antitoxin and/or toxin-antitoxin complex interaction with the promoter (Garcia-Pino et al., 2010). Degeneration of *pemIK$_{Sa1}$-Sp* operons and possible pseudogenisation of antitoxin genes put into question the transcriptional activity and functionality of the whole system. To evaluate this, we isolated RNA from *S. pseudintermedius* strains (LMG22220, 235, 244, 21007, LMG22221 and 244), exemplifying each of the identified variants of *pemI$_{Sa1}$-Sp* and *pemK$_{Sa1}$-Sp* genes, and analysed the level of respective transcripts by qRT-PCR. Transcripts encoding each tested variant were detected in relevant strains (Fig. S3), which potentially allows for their expression at the protein level. The level of transcription of all *pemI$_{Sa1}$-Sp* antitoxins was higher than their corresponding *pemK$_{Sa1}$-Sp* toxins despite expression of both genes from a single promoter (one operon). It likely suggests premature transcription termination, possibly induced by hairpin structures in toxin encoding fragment (Fig. S1). Strain 235 was characterized by an overall lower relative level of expression of *pemIK* components, probably as a result of a point mutation in the putative promoter region (Fig. S1).

3.3. The PemK$_{Sa1}$-Sp1 toxin is non-toxic and its natural C-terminal truncation abolishes RNase activity

Frameshifts and truncations within *pemIK$_{Sa1}$-Sp* operon (Fig. S1) may indicate its translational incompetence (pseudogenization) despite the transcriptional activity. Lack of relevant antibodies has prevented us from direct verification of system component expression. However, the results of a set of indirect experiments presented below argue the functionality of major identified variants of PemK$_{Sa1}$-Sp toxins and PemI$_{Sa1}$-Sp antitoxins *in vivo*.

Coding sequences of *pemK$_{Sa1}$-Sp1* (96 aa) from strain 244 and *pemK$_{Sa1}$-Sp2* (112 aa) from strain LMG22220 were cloned into an arabinose-inducible expression vector pBAD/His B. *E. coli* were transformed with recombinant plasmids and plated in serial dilutions as dots on a solid medium containing L(+)-arabinose. Expectedly, the positive control bacteria transformed with pBAD-PemK$_{Sa1}$ plasmid encoding PemK toxin from *S. aureus* CH91 were significantly inhibited in the presence of the inducer compared to the negative control bacteria carrying an empty plasmid. Surprisingly, we did not observe growth inhibition of *E. coli* carrying pBAD-PemK$_{Sa1}$-Sp1 or pBAD-PemK$_{Sa1}$-Sp2 plasmids (Fig. 2D). This result indicates that despite the high level of sequence identity (82.2 % for PemK$_{Sa1}$-Sp1 and 80.9 % for PemK$_{Sa1}$-Sp2) to PemK$_{Sa1}$ toxin from *S. aureus*, the *S. pseudintermedius* derived toxins are not toxic to *E. coli*.

E. coli is routinely used to verify the functionality of putative TA systems where the toxin overexpression in the absence of relevant antitoxin results in an observable growth defect which is rescued by concomitant overexpression of the antitoxin (Kato et al., 2019). However, this model fails in case of PemK$_{Sa1}$-Sp2 which shows no toxic effect even in the absence of antitoxin – likely because of very limited substrate specificity. It may not be excluded, nonetheless, that PemK$_{Sa1}$-Sp2 has a different effect in host cells, which are phylogenetically distant from *E. coli*. To test such possibility, a cadmium-inducible pCN51-PemK$_{Sa1}$-Sp2 plasmid expressing enzymatically active PemK$_{Sa1}$-Sp2 was transformed to *S. pseudintermedius* strain LMG22221. Growth inhibition was not observed upon stimulation of the transgene production with cadmium when compared to an empty plasmid control (Fig. S5). This demonstrates that PemK$_{Sa1}$-Sp2 is non-toxic to the native host even in

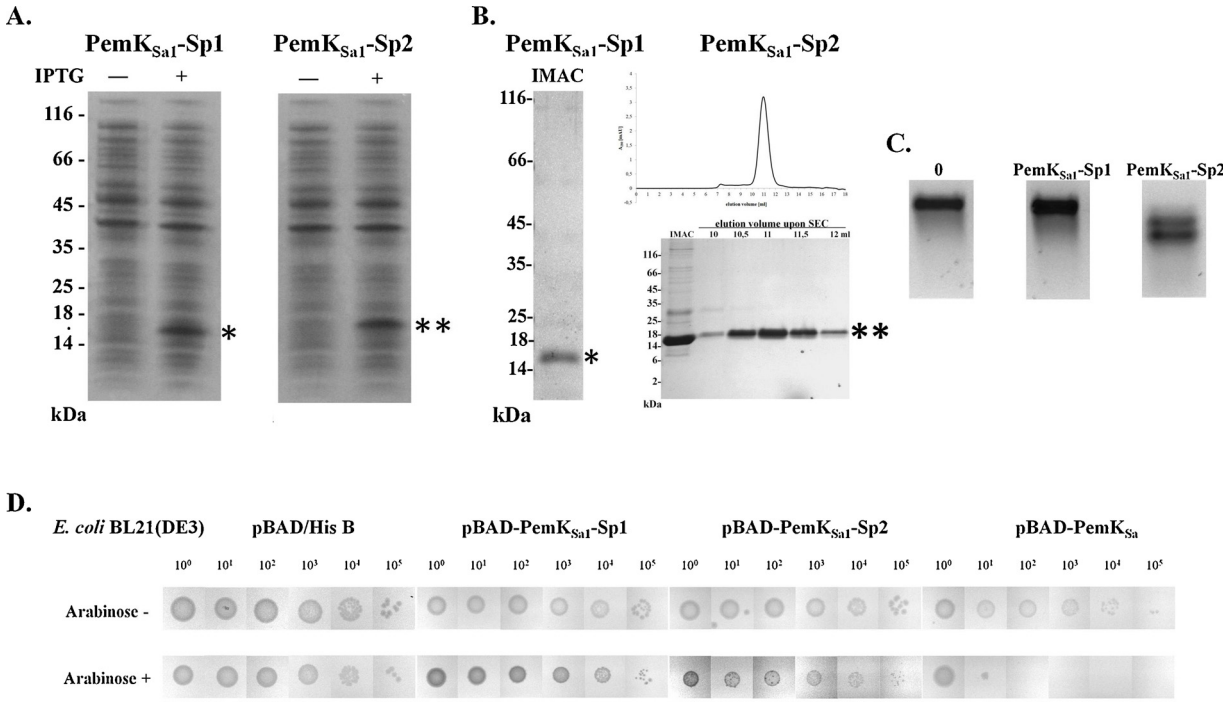

Fig. 2. PemK$_{Sa1}$-Sp toxins and their activity *in vitro* and *in vivo*. (A.) Production of 6xHis-tagged PemK$_{Sa1}$-Sp toxins in *E. coli* BL21(DE3). Expression of tested toxin was induced by IPTG from the following vectors: pETDuet-PemK$_{Sa1}$-Sp1-His (for PemK$_{Sa1}$-Sp1;*) and pETDuet-PemK$_{Sa1}$-Sp2-His (for PemK$_{Sa1}$-Sp2;**; Table S2); and was monitored by SDS-PAGE. **(B.) The purification of the PemK$_{Sa1}$-Sp toxins.** PemK$_{Sa1}$-Sp1 was purified by Ni-NTA affinity chromatography (IMAC) in denaturing conditions and subjected to refolding, whereas the PemK$_{Sa1}$-Sp2 by IMAC, in native conditions, and Size Exclusion Chromatography (SEC). Purity of the obtained preparations were verified by SDS-PAGE. **(C.) Endoribonucleolytic activity of tested PemK$_{Sa1}$-Sp toxins towards RNA from MS2 phage.** The PemK$_{Sa1}$-Sp2 toxin shows endoribonucleolytic activity, while the C-terminally truncated PemK$_{Sa1}$-Sp1 is inactive. 0, the negative control. **(D.) Analysis of toxicity of PemK$_{Sa1}$-Sp toxins toward *E. coli* cells.** Serially diluted (10°-10^{5}) *E. coli* BL21(DE3) cells (at OD_{600} equal 0.2), transformed with and pBAD/His B (empty), pBADPemK$_{Sa1}$-Sp1 or pBAD-PemK$_{Sa1}$-Sp2 vectors were spotted on solid medium LBA (upper panel) or LBA supplemented with inducer (0.2 % L(+)-arabinose; lower panel). Contrary to *S. aureus* PemK-Sa, the *S. pseudintermedius* PemK$_{Sa1}$-Sp toxins were not toxic for *E. coli*, as evidenced by the lack of inhibition of bacterial growth on the medium supplemented with the inducer.

the absence of the antitoxin.

We hypothesized that the difference in toxicity between PemK toxins derived from *S. aureus* and *S. pseudintermedius* resulted from the lack of RNase activity of the latter toxins. To verify this assumption, we recombinantly expressed N-terminally His-tagged *S. pseudintermedius* toxin variants obtaining efficient overproduction in *E. coli* BL21(DE3). Recombinant proteins were purified using chromatographic techniques (Fig. 2A and B). PemK$_{Sa1}$-Sp1 was obtained from the insoluble fraction and refolded. PemK$_{Sa1}$-Sp2 was recovered from the soluble fraction. Toxins were contacted with MS2 RNA and the substrate integrity was subsequently analysed by agarose gel electrophoresis. Degradation products were not observed upon incubation of MS2 RNA with PemK$_{Sa1}$-Sp1, suggesting the protein does not exhibit RNase activity. On contrary, PemK$_{Sa1}$-Sp2 exhibited RNase activity while the banded pattern of the product indicated a certain degree of substrate specificity (Fig. 2C). Seven sites of hydrolysis were identified by primer extension method (Nadratowska-Wesołowska et al., 2010) and the sites were identical to those previously identified for PemK-Sa1 from *S. aureus* (Bukowski et al., 2013). However, contrary to PemK-Sa1 where all the U'AUU sites are utilized with equal efficiency, PemK$_{Sa1}$-Sp2 strongly preferred a single site (CU'AUUU), recognized three additional sites with relatively high efficiency, while it almost completely excluded three remaining sites (Fig. S4). Such result suggests that the specificity of PemK$_{Sa1}$-Sp2 encompasses the four-nucleotide specificity determined for PemK-Sa1, but also extends beyond this sequence which was not the case for *S. aureus* toxin.

3.4. Natural N-terminally truncated PemI$_{Sa1}$-Sp antitoxin variants retain inhibitory activity

All 5 variants of PemI antitoxins in *S. pseudintermedius* identified in this study contain substantial N-terminal truncations relative to the canonical PemISa (88 aa) from *S. aureus*. To verify if these antitoxins are still capable of toxin inhibition, a classical coexpression assay (Kato et al., 2019) was utilized. The coding sequences of *pemI$_{Sa1}$-Sp* antitoxin variants (*Sp1-5*, respectively form strains LMG22220, 235, 21007, LMG22221 and 244) were cloned into expression vector pACYCDuet-1 under control of IPTG-inducible T7 promoter. *E. coli* BL21(DE3) were co-transformed with plasmids pACYCDuet-PemI$_{Sa1}$-Sp1-5 and arabinose inducible pBAD-pemK$_{Sa1}$ plasmid expressing PemK-Sa1 (Bukowski et al., 2013), and plated on solid medium containing proper inducers. PemK-Sa1 toxin from *S. aureus* CH91 was used instead of PemK$_{Sa1}$-Sp for the lack of observable toxic effect of the latter (see above). Expectedly, the growth of all transformants was inhibited exclusively on plates containing arabinose while no effect was seen on plates containing only IPTG. PemI$_{Sa1}$-Sp2, PemI$_{Sa1}$-Sp3 and PemI$_{Sa1}$-Sp5 sustained bacterial growth in the presence of both inducers indicating functional inhibition of PemK-Sa1 toxin *in vivo*. PemI$_{Sa1}$-Sp1 and PemI$_{Sa1}$-Sp4 were ineffective in alleviating the toxic effects of PemK-Sa1 (Fig. 3B).

Another model, which allows for the evaluation of antitoxin functionality, relies on the observation that efficient overexpression of PemK-Sa1 toxin in *E. coli* is possible only when the antitoxin is concomitantly supplied (Bukowski et al., 2013). To further evaluate the activity of Sp1-5 antitoxin variants, a set of recombinant plasmids was prepared based on pETDuet-AF expressing a functional PemIK-Sa1 TA system (Bukowski et al., 2013), in which the PemI-Sa1 antitoxin coding sequence was replaced with sequences encoding the tested *S.*

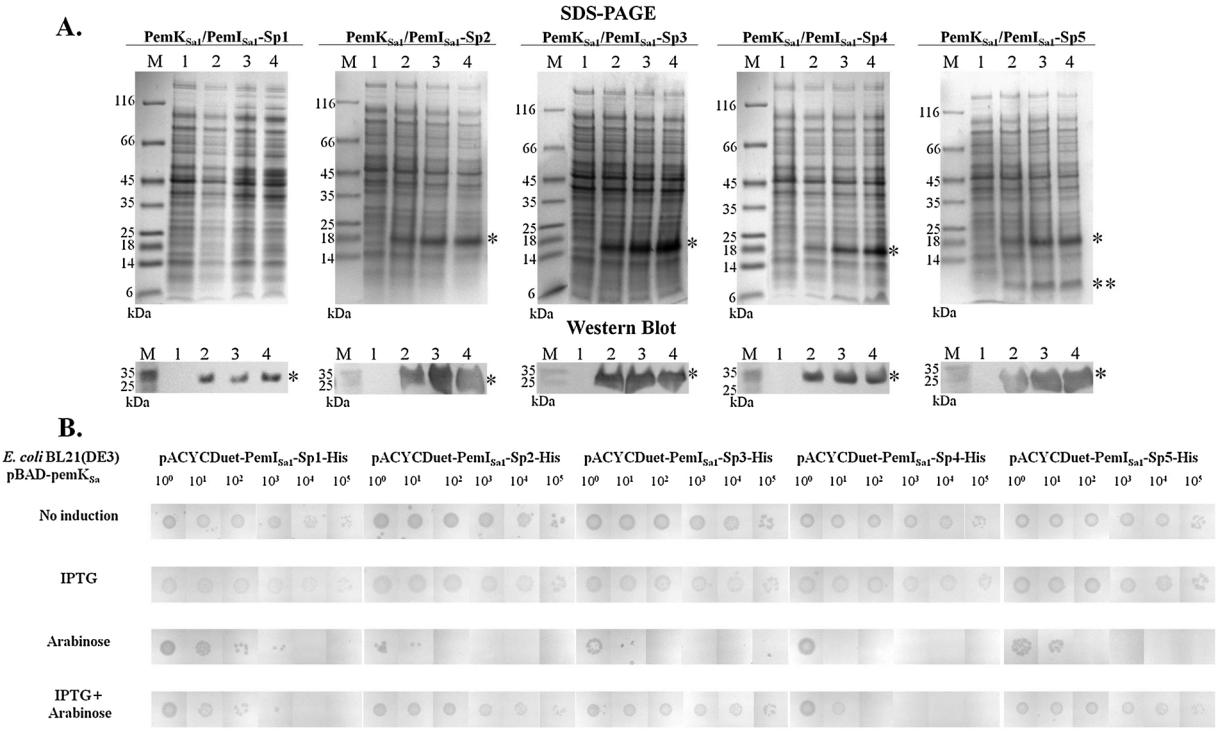

Fig. 3. Functionality of the PemI$_{Sa1}$-Sp antitoxins. (A) Co-production test. PemK$_{Sa1}$ toxin was co-expressed with tested variants of PemI$_{Sa1}$-Sp antitoxins in *E. coli*. Lysates before (lane 1) and three subsequent hours after induction with IPTG (lanes 2-4) were separated by SDS-PAGE (upper panel) and the 6xHis-tagged PemK$_{Sa1}$ toxin was identified by Western Blot (lower panel) using anti-His-tag antibodies. PemI$_{Sa1}$-Sp2, PemI$_{Sa1}$-Sp3, PemI$_{Sa1}$-Sp4 and PemI$_{Sa1}$-Sp5 rescued the production of PemK$_{Sa1}$ toxin (*) as observed by SDS-PAGE. PemI$_{Sa1}$-Sp1 had little protective effect and PemK$_{Sa1}$ toxin production was discernible only on Western Blot. M-molecular mass standards. **(B) Toxicity protection assay.** The serial dilutions of *E. coli* co-transformed with pBAD-PemK$_{Sa1}$ and one of the following plasmids: pACYCDuet™-1 (empty), pACYCDuet-PemI$_{Sa1}$-Sp1-His, pACYCDuet-PemI$_{Sa1}$-Sp2-His, pACYCDuet-PemI$_{Sa1}$-Sp3-His, pACYCDuet-PemI$_{Sa1}$-Sp4-His or pACYCDuet-PemI$_{Sa1}$-Sp5-His, were spotted on solid medium supplemented with the inducers: IPTG and L(+)-arabinose for induction of antitoxins and toxin expression, respectively. Growth inhibitory effect of PemK$_{Sa1}$ was abolished only by co-expression of PemI$_{Sa1}$-Sp2, PemI$_{Sa1}$-Sp3, PemI$_{Sa1}$-Sp5 antitoxins, but not PemI$_{Sa1}$-Sp1 and PemI$_{Sa1}$-Sp4 variants.

pseudintermedius antitoxin variants. *E. coli* were transformed with the resulting plasmids and the presence of histidine-tagged PemK-Sa1 was monitored by SDS-PAGE and Western Blot upon IPTG induction of co-expression (Fig. 3A). Efficient expression of PemK-Sa1 toxin was supported only by Sp2, Sp3 and Sp5 variants of PemI-Sp antitoxin, which corresponds with the results of *in vivo* protection assay, indicating the functionality of Sp2, Sp3 and Sp5 variants. Expression of PemK-Sa1 was significantly compromised when Sp4 was used and detectable only by Western blot with Sp1 variant. Additionally, the growth rate of bacteria carrying the plasmids encoding PemK$_{Sa1}$/PemI$_{Sa1}$-Sp1 and PemK$_{Sa1}$/PemI$_{Sa1}$-Sp4 was compromised compared to the other tested variants, either in the presence or absence of IPTG (the latter likely reflecting promoter leakage). These results indicate only limited functionality of Sp1 and Sp4 variants.

The above antitoxin functionality tests relied on inhibition of *S. aureus* toxin by tested *S. pseudintermedius* antitoxins. To test the presumed activity of Sp2, Sp3 and Sp5 against the cognate toxin, we evaluated the capability of complex formation between PemK$_{Sa1}$-Sp2 toxin and corresponding PemI$_{Sa1}$-Sp antitoxin variants by gel filtration. In parallel, the ability of tested antitoxin variants to inhibit the RNase activity of the toxin in *in vitro* assays was evaluated. Gel filtration experiments followed by SDS-PAGE inspection of the elution fractions demonstrated complex formation between His-tagged PemK$_{Sa1}$-Sp2 toxin and His-tagged PemI$_{Sa1}$-Sp antitoxins variants 2, 3 and 5 (Fig. 4, panels A and B). Moreover, variants 2, 3 and 5 protected MS2 RNA from degradation by PemK$_{Sa1}$-Sp2 (Fig. 4, panels C). These results indicate that antitoxin variants Sp2, Sp3 and Sp5 are, indeed, capable of inhibiting the cognate toxin.

Our data demonstrate that the activity of the PemI-Sp antitoxin variants is unrelated to the extent of N-terminal truncation. Highly truncated variants Sp2 and Sp3 retained their activity, whereas longer variant Sp4 was inactive. The detailed sequence analysis provided hints into the differences in antitoxin variant activity. Sp4 sequence differs from the other antitoxin variants at the N-terminus (contains LY in place of ED; Fig. 1A). Introduction of LY62ED substitution into pET-Duet-AF-PemI$_{Sa1}$-Sp4 plasmid rescued the coexpression of PemK$_{Sa1}$ toxin to a certain extent (Fig. S6), demonstrating that indeed the substitution of acidic residues (ED) with uncharged ones (LY) in Sp4 is responsible for highly reduced activity of Sp4.

4. Discussion

Toxin-antitoxin systems are intriguingly widespread in bacterial genomes. TA systems were identified both in the chromosomal DNA and mobile genetic elements such as plasmids (Bukowski et al., 2017, 2013; Zhu et al., 2006). A rapidly growing number of genomic sequences revealed that particular TA systems may be found in phylogenetically distant bacteria and in different regions of the genome in related species, which suggests their mobile character (Bukowski et al., 2017). The driving force of such mobility and its potential consequences for physiology of bacteria remain elusive. Another poorly explained aspect concerns the prevalence of TA systems, which ranges from a single strain to a wide occurrence of a particular system among diverse strains and species. MazEF system is located exclusively on the chromosome of all staphylococcal strains tested and co-evolves with the bacteria. Such co-evolution argues for an important physiological role in staphylococci (Bukowski et al., 2017). Indeed, the system is clustered in an operon with *sigB* locus. *SigB* encodes the alternative sigma factor

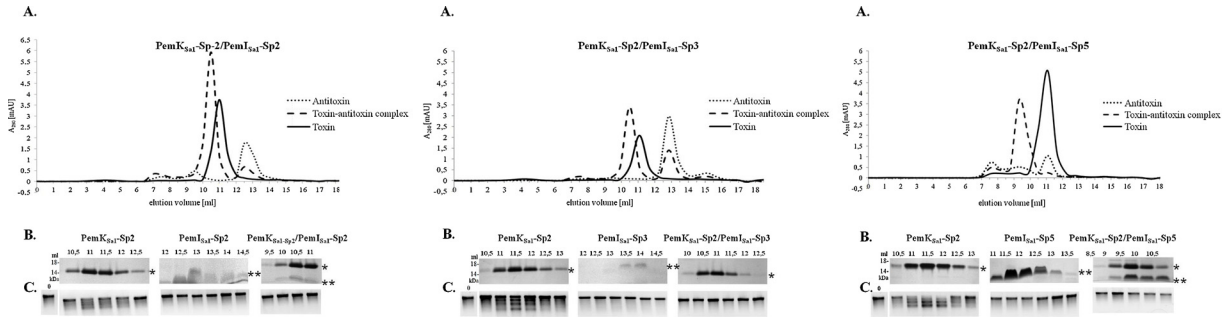

Fig. 4. Complex formation between PemK$_{Sa1}$-Sp2 toxin and PemI$_{Sa1}$-Sp2, PemI$_{Sa1}$-Sp3 and PemI$_{Sa1}$-Sp5 antitoxins. (A.) Size Exclusion Chromatography (SEC) of PemK$_{Sa1}$-Sp2 toxin, different PemI$_{Sa1}$-Sp antitoxin variants and mixtures. (B.) SDS-PAGE and (C.) agarose gel electrophoresis from MS2 RNA cleavage test of fractions obtained from SEC. On SDS-PAGE gels, bands corresponding to PemK$_{Sa1}$-Sp2 toxin (*) and PemI$_{Sa1}$-Sp antitoxin (**) are marked.

and stress regulator σ^B of significant importance in staphylococcal physiology. Importantly, *mazEF* promoter is needed for full activity of σ^B (Donegan and Cheung, 2009). Moreover, in *S. aureus* MazF toxin was shown to be able to affect transcripts of virulence factors and thus modulate the pathogenic potential (Schuster et al., 2015; Zhu et al., 2009). Interestingly, in non-pathogenic *S. equorum,* MazF toxin targets important metabolic and regulatory gene transcripts (Schuster et al., 2013) despite having exactly the same substrate specificity as *S. aureus* homologue. The above facts suggest that it is the genomes that co-evolve with the toxin and not the other way round.

Prior data suggested that the prevalence/penetrance of PemIK-Sa1 system in staphylococcal genomes was low (up to 1%). The system was found mostly on plasmids. Among rare cases of the chromosomal PemIK-Sa1, its localization within the genome varied considerably (Bukowski et al., 2017). In this work we show that PemIK$_{Sa1}$-Sp has high penetrance in *S. pseudintermedius* chromosomes while being characterized by significant sequence heterogeneity. This heterogeneity and especially truncations resulted in prior underestimation of the system penetrance in analyses relying on full-length PemI-Sa1 queries (Bukowski et al., 2017). In all examined *S. pseudintermedius* strains, *pemIK$_{Sa1}$-Sp* is found in the same genetic context, which suggests that the system emerged (or was acquired) in one genetic event, presumably in the distant past, however we did not identified any genetic hallmarks explaining mechanism of its emergence.

Both components of type II TA systems are proteins. It follows that a functional system requires transcription and translation of respective genes. Using qRT-PCR we have demonstrated that *pemIK$_{Sa1}$-Sp* is transcriptionally active. This seemingly contrasted with the finding of significant truncations in antitoxin genes, which had suggested pseudogenization. Additionally, recent findings in other systems demonstrated toxin inactivation by promoter mutations when antitoxins were non-functional or absent (Fernandez-Garcia et al., 2019). Pseudogenization of the antitoxin would result in the production of only the toxin that should poison the cell, or at least arrest its growth, neither of which did we observe in *S. pseudintermedius*. Possible explanations are that either the toxin is not toxic itself or truncated antitoxins are still functional. Here, we provided evidence that both the above assumptions are true. Using *E. coli* we demonstrated that both length variants of the PemK$_{Sa1}$-Sp toxin are non-toxic. The shorter version (PemK$_{Sa1}$-Sp1) has likely lost the RNase activity, which explains the lack of toxicity. The structure for PemK toxin is not available, however the crystal structure of homologous MazF toxin demonstrates that the last 20 aa form an alpha helix which is involved in the toxin dimerization (Kamada et al., 2003; Zorzini et al., 2016). It was demonstrated that only a dimer effectively binds substrate RNA (Li et al., 2006). Inability to form a dimer by C-terminally truncated PemK$_{Sa1}$-Sp1 would explain its lack of activity and structural instability.

Surprisingly, the full-length version (PemK$_{Sa1}$-Sp2) retained RNase activity despite the lack of toxicity. We explain this by increased substrate specificity compared to *S. aureus* PemK-Sa1, which is significantly toxic to *E. coli* and to the native host (Bukowski et al., 2013). The average number of transcripts accessible to sequence-specific RNases correlates inversely with the length of the target sequence. The sequence recognized by PemK-Sa1 (UAUU) appears every 77 bases in random transcripts. Such sequence is thus expected at least once in every single gene. Indeed, among 2,689 open reading frames (ORFs) within the genome of *S. aureus* ED98 (carrying *pemIK-Sa1* system on pAvX plasmid) the target sequence is absent only in 56 ORFs (2%). Penta-nucleotide sequence (UACAU) recognized by MazF$_{Sa}$, is expected on average every 578 bases. Here the number of transcripts devoid of the target sequence reaches 967 genes – 36 % of all transcripts. The CUAUUU sequence effectively hydrolyzed by PemK$_{Sa1}$-Sp2 is absent in 1666 ORFs (out of 2452 ORFs found in *S. pseudintermedius* HKU10-03) which renders 68 % of all genes resistant to PemK$_{Sa1}$-Sp2. Only 48 transcripts contain 3 or more (max. 5) target sites. These statistical considerations may explain the lack of toxicity of PemK$_{Sa1}$-Sp2. However, we should bear in mind that the toxicity may as well result from a small number of cleavages in crucial genes. It has been demonstrated that hydrolysis of only a single essential gene transcript in *E. coli* by MazF-hw toxin (targeting a seven-nucleotide sequence UUACUCA) is enough to abolish the growth of bacteria (Yamaguchi et al., 2012).

Interestingly, despite the fact that both natural variants of PemK$_{Sa1}$-Sp toxin are non-toxic and the antitoxins are significantly truncated compared to their *S. aureus* homologues, the antitoxins still retain the inhibitory activity against their cognate toxins, the significance of which is unclear. The shortest functional antitoxin (PemI$_{Sa1}$-Sp2) consists of 30 aa, which is only one-third of the full-length prototype PemI-Sa1 (89 aa). This is, however, consistent with prior data. Antitoxins within type II TA systems consist of two domains. The N-terminal domain interacts with DNA, often with the TA system promoter. The C-terminal domain binds the toxin, inhibiting its activity (Kamada et al., 2003). Thus, in retrospect, it is not surprising that the truncated antitoxins of *S. pseudintermedius* retain inhibitory activity.

The plasmid-encoded TA systems clearly play a role in the maintenance of these mobile genetic elements by eliminating bacteria that have lost the plasmid during the cell division process. This requires a deleterious and irreversible mechanism. When the MGE is lost, the labile antitoxin is quickly depleted while the stable toxin hinders the growth of the daughter cells that have lost the plasmid. But, the role of plasmid-located TA systems is not limited to stable maintenance of MGEs, as exemplified by PemIK-Sa1 (Bukowski et al., 2013). PemIK-Sa1 and certain other plasmid-encoded systems additionally act as regulators of gene expression. Here, the toxin activation is likely reversible. In turn, it seems that chromosomally encoded systems act by multilevel mechanisms simultaneously affecting a large number of targets. Such control would imply fine-tuning by co-evolution of genomes and toxin specificities. Additionally, reversibility would be another requirement. However, the functions of chromosomally encoded TA systems are less well defined and the understanding of their role is the subject of an ongoing investigation.

Further complexity is introduced by cross-interactions and competition between homologues (paralogues) of TA system (Yang et al., 2010; Zhu et al., 2010). Competition between plasmid-encoded TA systems (referred to as "addiction modules") and those located at the chromosome (or other plasmids), where the latter provide "anti-addiction" antitoxins, leads to a complicated arms-race between chromosomally- and plasmid-encoded TA systems, which may result in elimination/exclusivity of certain combinations (Goeders and Van Melderen, 2014). Cross-reactivity of *S. pseudintermedius* antitoxins with *S. aureus* toxin described in this study may suggest an exclusion mechanism based on anti-addiction, preventing stable acquisition of homologous PemIK systems by horizontal gene transfer. This is, however, highly speculative at this moment.

Concluding, the chromosomal localization of $pemIK_{Sa1}$-Sp resulted in the loss of toxicity of the $PemK_{Sa1}$-Sp toxin through increased sequence specificity. In such a case, the $PemI_{Sa1}$-Sp antitoxin should be released from two important functions, (i) inhibitory and (ii) the regulatory, which control expression of the whole system. Nonetheless, our study demonstrates that even substantial N-terminal truncation of the antitoxin does not deprive it of the inhibitory activity. This indicates potential evolutionary pressure to maintain antitoxin functionality. When the absence of toxicity of the toxin is taken into account, the existence of such pressure and its relevance seem perplexing and definitely require further investigation.

Author contributions

MJ, MB, and BW identified the TA systems and designed the experiments. MJ, RŁ, KH, and MH, performed the experiments. MB designed and performed computational analysis. MJ, MB, RŁ, KH, MH, ASP, GW, PG, GD and BW analysed the data. MJ, KH and BW wrote the paper.

CRediT authorship contribution statement

Monika Janczak: Conceptualization, Funding acquisition, Investigation, Project administration, Visualization, Writing - original draft, Writing - review & editing. **Karolina Hyz:** Investigation, Visualization, Writing - original draft. **Michal Bukowski:** Conceptualization, Investigation, Software, Writing - review & editing. **Robert Lyzen:** Investigation. **Marcin Hydzik:** Investigation, Visualization. **Grzegorz Wegrzyn:** Resources, Supervision. **Agnieszka Szalewska-Palasz:** Resources, Supervision. **Przemyslaw Grudnik:** Investigation, Resources. **Grzegorz Dubin:** Resources, Writing - review & editing. **Benedykt Wladyka:** Conceptualization, Investigation, Project administration, Supervision, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2020.126529.

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