The Axe-Txe Complex of Enterococcus faecium Presents a Multilayered Mode of Toxin-Antitoxin Gene Expression Regulation

Lidia Boss¹, Łukasz Labudda¹, Grzegorz Węgrzyn¹, Finbarr Hayes², Barbara Kędzierska*¹

1 Department of Molecular Biology, University of Gdańsk, Gdańsk, Poland, 2 Faculty of Life Sciences and Manchester Institute of Biotechnology, the University of Manchester, Manchester, United Kingdom

Abstract

Multidrug-resistant variants of human pathogens from the genus Enterococcus represent a significant health threat as leading agents of nosocomial infections. The easy acquisition of plasmid-borne genes is intimately involved in the spread of antibiotic resistance in enterococci. Toxin-antitoxin (TA) systems play a major role in both maintenance of mobile genetic elements that specify antibiotic resistance, and in bacterial persistence and virulence. Expression of toxin and antitoxin genes must be in balance as inappropriate levels of toxin can be dangerous to the host. The controlled production of toxin and antitoxin is usually achieved by transcriptional autoregulation of TA operons. One of the most prevalent TA modules in enterococcal species is axe-txe which is detected in a majority of clinical isolates. Here, we demonstrate that the axe-txe cassette presents a complex pattern of gene expression regulation. Axe-Txe cooperatively autorepress expression from a major promoter upstream of the cassette. However, an internal promoter that drives the production of a newly discovered transcript from within axe gene combined with a possible modulation in mRNA stability play important roles in the modulation of Axe:Txe ratio to ensure controlled release of the toxin.

Introduction

Recent analyses of the dynamics of invasive infections causing bacteraemia in European countries showed the fastest increase in the number of infections caused by Enterococcus sp. relative to other tested pathogens [1]. The treatment of infections caused by these bacteria is particularly difficult because of their intrinsic resistance to certain groups of antibiotics including penicillins, cephalosporins, and aminoglycosides. Moreover, the tendency of enterococci to acquire and exchange a wide variety of resistance determinants through horizontal transfer of mobile genetic elements such as plasmids and transposons further reduces the antibiotics available to treat certain enterococcal infections [2,3].

Molecular mechanisms responsible for the spread and stable maintenance of antibiotic resistance genes located on plasmids are well documented for model bacteria such as Escherichia coli. One of the stabilisation mechanisms that assures effective propagation of low copy number bacterial plasmids is their active segregation to daughter cells during cell division. Additionally, plasmids encode toxin-antitoxin (TA) systems that act in postsegregational killing of cells that have failed to acquire a plasmid at division [4]. In these daughter cells devoid of a plasmid, the degradation of antitoxin and the lack of its gene expression leads to the release of the toxin which interacts with its intracellular target, leading to cell death or inhibition of metabolic processes. Thus, as progeny die if the plasmid is lost, bacteria become “addicted” to TA modules located on plasmids. TA complexes are also widely encoded by chromosomes of prokaryotes. Here, the toxin is activated in response to diverse stress and nutritional stimuli that result in downregulation of metabolism and/or programmed cell death. Chromosomal TAs are also implicated in antibiotic persistence, biofilm formation, and bacteriophage resistance [5].

To date, five different TA types based on the nature and mode of action of the antitoxin have been proposed [6]. Our current study focuses on type II TA systems, in which both the...
toxin and the antitoxin are proteins. In this group, TA modules generally have similar organizations and modes of expression regulation [5,7–9]. The cassettes usually consist of a pair of genes forming an operon. The first gene encodes a more labile antitoxin which is a target for Clp or Lon proteases, whereas the second gene specifies a stable toxin. Strong and specific interactions between toxin and antitoxin proteins, as well as precise transcriptional regulation of their expression, are characteristic feature of TA complexes. Expression of the two genes must be in balance as inappropriate levels of toxin can be dangerous to the host. The controlled production of toxin and antitoxin is achieved by transcriptional regulation of TA operons. Usually, type II TA operons are negatively autoregulated at the transcriptional level, but the detailed molecular mechanisms that underpin this process are still poorly understood for most TA modules. Nevertheless, a common pattern involves binding of the antitoxin to palindromic sequences in the promoter region by its N-terminal domain, making the antitoxin the principal factor for transcriptional repression. The C-terminal domain of the antitoxin generally binds to the toxin which acts as a co-repressor by increasing the affinity and stability of the regulatory complex. This canonical pattern of transcriptional autoregulation characterizes the best described type II TA cassettes, including YefM-YoeB, RelBE, MazEF, CcdAB and Kis-Kid [10–14]. Additionally, cooperative binding of certain TA complexes to operator DNA occurs only when toxins and antitoxins are in proper stoichiometric relationships. Excess toxin stimulates operon transcription by releasing the TA complex from the operator site which prevents uncontrolled toxin activation [15,16]. Nevertheless, some exceptions to this general pattern of type II TA regulation are known. Binding of the antitoxin alone is sufficient for full repression of the parDE TA operon in low copy number plasmid RK2 [17]. Additional genes are involved in repression of the paaR-paaA-parE and ε-ζ-ω TA systems. In the case of the PaaA antitoxin-ParE toxin complex in E. coli O157:H7, it autorepresses the main promoter only partially, but the PaaR protein is needed for full down-regulation of transcription [18]. On the other hand, in the case of the ε-ζ-ω system of plasmid pSM19035, the ω toxin and ε antitoxin have no roles in transcriptional control. Instead, transcription of the operon is efficiently repressed solely by the ω protein [19]. Unlike its E. coli homologues, the chromosomal type II mazEF operon of Staphylococcus aureus is not autoregulated. Instead, the global transcriptional regulator SarA activates the cassette, whereas the alternative sigma factor σ6 represses its transcription, probably indirectly [20].

As TAs are key for both maintenance of mobile genetic elements that specify antibiotic resistance and in bacterial persistence and virulence, dissection of these systems in pathogenic bacteria, including enterococci, is crucial [21]. Par and Axe-Txe encoded by plasmids of Enterococcus faecalis and E. faecium, respectively, were among the first TA systems identified in enterococci [22–24]. The par locus specifies two small RNA molecules, RNA I and RNA II. The former is translated into a 33 amino acid toxic peptide whose expression is regulated posttranscriptionally by RNA II [25]. Differential decay patterns of RNA I and RNA II elicit translation of the former in plasmid-free cells. The toxin disrupts cell membrane function by an as yet unknown mechanism [26].

The type II axe-txe module was first identified on the multidrug resistant pRUM plasmid from a clinical isolate of E. faecium. Axe-Txe is a plasmid maintenance complex not only in enterococci, but also in evolutionary diverged species, including Bacillus sp. and E. coli. Axe-Txe is homologous to the YefM-YoeB complex of E. coli [24]. Txe (85 amino acids) is a positively charged toxin that is neutralized by Axe (89 amino acids), a negatively charged antidote. When liberated from the complex, Txe acts as an endoribonuclease that cleaves cellular mRNA downstream of AUG start codons [27]. Txe thereby inhibits bacterial growth and cell division [24]. Axe-Txe and certain other TA modules are found widely in antibiotic resistant enterococci, including vancymycin resistant isolates [28–30].

In this study, we investigated mechanisms underpinning regulation and expression control of the axe-txe module. Our studies show that the expression of axe-txe genes is different than in other described TA systems. Notably, an internal promoter that drives the production of a novel transcript was detected within the axe gene. This message, together with mRNA stability control, may be a part of a complex regulatory circuit that tunes the ratio of Axe antitoxin to Txe toxin.

Materials and Methods

Strains

E. coli DH5α was used for plasmid construction and Rosetta(DE3) for crude extract preparation with Axe and Axe-Txe overproduction from pET22axe and pET22at_axe-txe, respectively. Strain SC301467 [31] was used for DNA and RNA isolation and for luminescence assays, and C600polA1 was used in plasmid stability assays. Bacteria were grown in Luria-Bertani (LB) medium at 37°C. Ampicillin and chloramphenicol were added to final concentrations of 100 and 34 or 10 µg/ml, respectively, when required.

Plasmids and oligonucleotides

Oligonucleotides and plasmids used in this study are listed in Tables 1 and 2, respectively.

Crude extract preparation

Bacteria were grown at 37°C in 10 ml of LB medium with appropriate antibiotic until OD600 ~0.5. Expression of axe (pET22axe) or axe-txe (pET22at_axe-txe) was induced with 1 mM IPTG and incubation continued for 3 hours. Cells were harvested at 1600 g for 10 min. The pellet was resuspended in 1 ml of buffer comprising 20 mM Tris–HCl pH 7.5 and 50 mM NaCl. The cells were sonicated and then centrifuged for 30 min at 15500 g at 4°C. Supernatant was dialysed against the same buffer containing 10% glycerol. The samples were aliquoted and stored at −20°C.

Promoter fusion studies and bioluminescence assays

Strain SC301467 harbouring derivatives of pBBrux-amp with the lux operon under transcriptional control of fragments containing different elements of axe-txe operon were used.
Primer extension analysis

The promoters in the axe-txe cassette region were mapped with a \( ^{32}P \)-labeled primer (primer 15) that anneals to the lux gene downstream from the region of interest. Total cellular RNA from strain SC301467 harbouring pBBRlux–based plasmids possessing transcriptional fusions of \( p_{\mu} \) or \( p_{\text{axemut}} \) promoter-operator regions to the lux operon (pluxat or pluxaxe) were combined with the labeled primer. Primer extension reactions were done in total volumes of 10 µl containing 10 µg RNA, 0.6 pmol of labeled primer, RevertAid H Minus Reverse Transcriptase buffer (50 mM Tris-HCl pH 8.3, 50 mM KCl, 4 mM MgCl\(_2\), 10 mM DTT), 1 mM of each dNTPs, 10 U RiboLock RNase Inhibitor. Samples were denatured at 99°C for 2 min, and then incubated at 50°C for 1 hour. Next, 0.5 µl of 200 U/µl RevertAid H Minus Reverse Transcriptase (Fermentas) were added and samples were incubated at 42°C for 30 min. 5 µl of loading dye (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol) were added and samples were denatured for 10 min at 99°C prior loading on a 6% sequencing gel along with sequencing reactions performed with the same labeled primer and appropriate plasmid DNA (SequiTherm EXCEL™ II DNA Sequencing Kit, Epicenter) according to the protocol.

Electrophoretic mobility shift assays (EMSA)

5'-bionylated, double-stranded PCR fragments that included the \( p_{\mu} \) (primers 19/20) and \( p_{\text{axemut}} \) (primers 21/22) regulatory regions were used in EMSA. Reactions containing 0.1 nM of biotin–labeled DNA and bacterial crude extract at concentrations of 0, 1.25, 2.5, 5, 10, 12.5 and 25 µg/ml total protein were assembled in binding buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM DTT, 5 mM MgCl\(_2\), 10 mM DTT), 1 mM of each dNTPs, 0.015 mM of UTP and 0.8 µCi \( ^{32}P \) labeled primer (primer 15) that anneals to the lux operon (pluxat or pluxaxe) were combined with the labeled primer. Primer extension reactions were done in total volumes of 10 µl containing 10 µg RNA, 0.6 pmol of labeled primer, RevertAid H Minus Reverse Transcriptase buffer (50 mM Tris-HCl pH 8.3, 50 mM KCl, 4 mM MgCl\(_2\), 10 mM DTT), 1 mM of each dNTPs, 10 U RiboLock RNase Inhibitor. Samples were denatured at 99°C for 2 min, and then incubated at 50°C for 1 hour. Next, 0.5 µl of 200 U/µl RevertAid H Minus Reverse Transcriptase (Fermentas) were added and samples were incubated at 42°C for 30 min. 5 µl of loading dye (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol) were added and samples were denatured for 10 min at 99°C prior loading on a 6% sequencing gel along with sequencing reactions performed with the same labeled primer and appropriate plasmid DNA (SequiTherm EXCEL™ II DNA Sequencing Kit, Epicenter) according to the protocol.

| Table 1. Oligonucleotides used in this study. |
|----------------------------------------------|
| | Sequence (5’–3’) |
| 1 | GACGATTCATTCATCTTCCCAGGTCGAC |
| 2 | GGTAGGCAAGTCTTAGAGCAG |
| 3 | GCAGTTGCTGAAACAGG |
| 4 | GATCGATGATTCAGAGTCATTCC |
| 5 | TTCAGATCCAGATTAGTGTATGGCG |
| 6 | CGCAAGCCTTTTAAAGTCGACCGGTTTCC |
| 7 | GAGTACTGAGAAGAAGCAGGATTTGAGG |
| 8 | CGAAGGCGAGAAGTCTTGCAG |
| 9 | CGTGGGCTGAGAATAGGAGGATCC |
| 10 | ATTCGAGATTTAAATGATTTTGCAG |
| 11 | CGGGACAGGATAGGGTATTTAAAGG |
### Table 2. Plasmids used in this study.

| Name                | Description                                                                 | Reference |
|---------------------|-----------------------------------------------------------------------------|-----------|
| pBBRxux             | Vector for generating transcriptional fusion to lux, Cm<sup>R</sup>         | [32]      |
| pBBRlux-amp         | Vector for generating transcriptional fusion to lux, bla gene was amplified  | This study|
| pET22b(+)           | IPTG-inducible expression vector allowing fusion of C-terminal His<sub>6</sub> | Novagen   |
| pET22axe            | axe gene amplified with primers 3/4, digested with NdeI-XhoI and cloned    | This study|
| pET22at_axe-txe     | at_axe-txe fragment amplified with primers 5/6, digested with BamHII-HindIII| This study|
| pluxat              | p<sub>lux</sub> promoter-operator region amplified with primers 7/8 (209 bp)| This study|
| pluxat_axe          | fragment containing p<sub>lux</sub> promoter-operator region and axe gene   | This study|
| pluxat_axe-txe      | fragment containing p<sub>lux</sub> promoter-operator region and axe-txe     | This study|
| pluxaxe             | p<sub>axe</sub> promoter-operator region amplified with primers 9/11 (353 bp)| This study|
| pluxaxemut          | p<sub>axe</sub> promoter-operator region with mutated -10 box (site-directed| This study|
| pluxaxe-txeW5C      | axe-txe genes with amino acid change in Txe protein (W5C) amplified with    | This study|
| pREGS31             | pFH450 derivative plasmid containing axe-txe cassette, used for amplifications| [24]      |
| pREGaxemut          | pREGS31 derivative with p<sub>axa</sub> promoter-operator region mutated in | This study|
| pTE103              | Vector for generating transcriptional fusion to lux, Cm<sup>R</sup>         | [33]      |
| pTEat_axe-txe       | fragment containing p<sub>axa</sub> promoter-operator region and axe-txe     | This study|
| pTEat_axe-txeW5C    | axe-txe genes with amino acid change in Txe protein (W5C) amplified with    | This study|
| pTEaxe              | axe and first 60 bp of txe genes amplified with primers 14/18, digested     | This study|
| pTEat_axe-txe_ter   | fragment containing p<sub>axa</sub> promoter-operator region and axe-txe     | This study|

### Bioinformatics

Promoter searches were performed using PromScan bioinformatic program (http://molbiol-tools.ca/promscan/). Terminator hairpin was predicted and drawn using MFOLD program (http://mfold.mla.albany.edu/).

### Results

**p<sub>axa</sub> promoter activity is inhibited by the Axe-Txe protein complex**

Type II TA genes generally are organized in operons and their expression is negatively regulated at the transcriptional level by action of antitoxin alone or in complex with its toxin partner. To assess whether the axe-txe genes show a similar scheme of regulation, primer extension analysis was first performed to determine the transcription start point(s) of the p<sub>axa</sub> promoter. Because it has been shown that the axe-txe system is fully functional as a stability cassette in *E. coli* [24], we performed experiments in this bacterium. A single major primer extension product was detected (Figure 1B). Sequences with close matches to consensus -10 (5/6 matches) and -35 (3/6 matches) boxes separated by an optimal 17 bp are located 5' of this transcription start site (Figure 1A). In addition, a sequence resembling the ribosome binding site (5'-AAAGGGG-3') located 8 nt upstream of the axe start codon was observed (Figure 1A).

To assess the influence of Axe and Txe proteins on p<sub>axa</sub> promoter activity, *in vivo* and *in vitro* tests were performed. A fragment encompassing the p<sub>axa</sub> promoter and axe start codon was inserted upstream of a promoterless lux operon in the transcription fusion vector pBBRlux-amp and established in strain SC301467, which is deleted of five chromosomal toxin-antitoxin cassettes [31] to reduce any possible cross interactions from *E. coli* chromosomal TA cassettes. The yeFM-yoeB system which is homologous to axe-txe. This fusion produced ~7 x 10<sup>6</sup> RLU, whereas pBBRlux-amp alone produced ~100 units (Figure 1C, bars a and b). Thus, the region 5' of axe-txe possesses a strong promoter activity. In fact, cloning this region upstream of the lac operon in different vectors was unsuccessful, generating mutations in the promoter sequence which is a feature characteristic of very strong promoters. To compare the strength of p<sub>axa</sub>, a related promoter of the yeFM-yoeB system of *E. coli* [10,34] was also
cloned upstream of the promoterless lux operon in the same vector. This construct produced ~3.5 x 10^5 RLU. Thus, \( p_{at} \) appears to be a particularly strong promoter.

The 3’ end of \( axe \) overlaps the 5’ end of \( txe \) by 8 nt. We aimed to examine the influence of \( Axe \) and \( Txe \) on \( p_{at} \) activity in trans by cloning these overlapping genes under several different arabinose- or IPTG-inducible promoters. Despite many trials, we were not able to clone these genes (data not shown). As an alternative, it was decided to construct in cis fusions in which the \( p_{at} \) promoter, followed by \( axe \) or \( axe-txe \) genes, was fused to the lux operon. In this system, Axe alone inhibited \( p_{at} \) weakly (Figure 1C, bar c) whereas an ~5-fold decrease in \( p_{at} \) activity was observed in the presence of the Axe-Txe complex (Figure 1C, bar d).

Sequence analysis of the \( p_{at} \) promoter region previously revealed two inverted 5’-TGTACA-3’ repeats that are identical to those present in the promoter of the homologous \( yeFM-yoeB \) module and which are responsible for binding the toxin-antitoxin complex [10,34]. Moreover, in the case of \( p_{at} \), these repeats are additionally organized as a more extended inverted repeat with a single mismatch (Figure 1A). These sequences are candidate contact sites for the putative DNA binding N-terminal domain of the Axe antitoxin. To test the affinity of Axe and the Axe-Txe complex for binding to the promoter region in vitro, EMSA experiments were performed. For these experiments, BL21(DE3) crude extracts with overproduced Axe or Axe-Txe complex from the pET22(b) vector were used. BL21, like other \( E. coli \) B strains, does not possess the
An active promoter which contributes to Txe toxicity is located within the axe gene

The inability to clone the axe-txe cassette under control of an inducible promoter suggested that regulatory elements additional to $p_{\mu}$ might be present in this region. Searches using the PromScan program revealed the presence of a putative promoter within axe that might be implicated in expression of the downstream txe operon. A fragment of the axe gene encompassing this region was fused transcriptionally to the lux operon. This fusion produced $>3 \times 10^5$ RLU confirming the existence of a substantial promoter activity ($p_{\mu}$) within the axe coding sequence that might drive expression of txe (Figure 3C). This activity was comparable with that obtained for the strong yefM-yoeB promoter described above.

Primer extension experiments determined the transcription start point of $p_{\mu}$ (Figure 3B). Sequences with close matches to consensus -10 (5/6 matches) and -35 (3/6 matches) motifs, separated by an optimal 17 bp, are located 5' of the transcription start site which lies ~110 bp upstream of the translation start codon for the Txe toxin (Figure 3A). To determine if the assigned promoter was responsible for the significant expression observed in the lux transcriptional reporter fusion, mutations were introduced into the -10 sequence (TATGAT$\rightarrow$TAGCAC) and the mutated sequence ($p_{\mu}$mut) was inserted upstream of lux. The mutations almost entirely abolished lux expression confirming the assignment of $p_{\mu}$ (Figure 3C). EMSA experiments showed that neither the Axe-Txe proteins nor other proteins in the E. coli extract bound detectably to a fragment bearing the wild-type $p_{\mu}$ promoter (Figure S1).

The presence of the $p_{\mu}$ Promoter internal to the axe gene may explain the inability to clone the axe-txe cassette under a heterologous promoter: the balance between axe and txe expression may be altered when $p_{\mu}$ is replaced by a different promoter. However, cloning of the axe-txe cassette was possible when the $p_{\mu}$ promoter was retained at its normal location. Nevertheless, this construct (pTEpat_axe-txe) inhibited bacterial growth, indicating that axe-txe expression was also perturbed (Figure 4). Evidence that $p_{\mu}$ drives the synthesis of Txe was provided by experiments with a strain bearing a plasmid in which the entire axe-txe cassette, including the $p_{\mu}$ promoter, was again cloned, but in which $p_{\mu}$ carried the -10 box mutations described above (pTEpat_alexemut-txe). These mutations do not change the amino acid sequence of Axe. The growth profile of the strain bearing this plasmid was very similar to strains with either the vector alone or with a plasmid producing a nontoxic version of Txe which also alleviated toxicity (pTEpat_axemut-txe) (Figure 4).

As described above, in cis fusions in which the $p_{\mu}$ promoter followed by axe or axe-txe was fused to the lux operon were used to assess repression of this promoter by Axe and Axe-Txe. The data showed that $p_{\mu}$ is down-regulated weakly by Axe and more fully by the Axe-Txe complex, although not to basal levels (Figure 1C). To examine any contribution from $p_{\mu}$ in this system, in cis fusions were designed in which this promoter

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chromosomal yefM-yoeB cassette, thus any potential cross-talk between these two homologous systems can be excluded [35]. Note that cloning of the axe-txe genes under the $p_{\mu}$ promoter was possible only if the $p_{\mu}$ promoter was included. A 295 bp biotin-labeled fragment containing the promoter region was incubated with different concentrations of E. coli BL21(DE3) crude extracts (left to right in each panel): 0, 1.25, 2.5, 5, 10, 12.5 and 25 µg/ml. Reactions were incubated for 20 min at 22°C, analyzed by native 5% PAGE, and processed further as outlined in Materials and Methods. (A) no Axe or Txe produced; (B) Axe overproduction; (C) Axe-Txe overproduction. Filled and open arrows denote positions of unbound DNA and protein-DNA complexes, respectively.

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Figure 2. Axe and Axe-Txe binding to the $p_{\mu}$ promoter-operator region. A 295-bp 5' biotinylated fragment that included the axe translation start codon and upstream promoter-operator region was subjected to EMSA. The fragment was incubated with different concentrations of E. coli BL21(DE3) crude extracts (left to right in each panel): 0, 1.25, 2.5, 5, 10, 12.5 and 25 µg/ml. Reactions were incubated for 20 min at 22°C, analyzed by native 5% PAGE, and processed further as outlined in Materials and Methods. (A) no Axe or Txe produced; (B) Axe overproduction; (C) Axe-Txe overproduction. Filled and open arrows denote positions of unbound DNA and protein-DNA complexes, respectively.
was inactivated by the TATGAT→TACGAC mutations in its -10 box. Reporter data showed that expression levels of p\textsubscript{axe} in the presence of either Axe alone or Axe-Txe were lower in comparison to those when p\textsubscript{axe} is intact (Figure 1C, bars e and f compared to bars c and d). Thus, p\textsubscript{axe} contributes significantly to expression levels when wild-type axe or axe-txe is fused to the lux operon, but this expression may not be subject to Axe-Txe regulation. These results also demonstrate that enough txe is expressed from p\textsubscript{axe} alone to produce sufficient levels of Axe-Txe complex for repression of the \textit{in cis} fusion in which p\textsubscript{axe} is mutated.

Active p\textsubscript{axe} promoter is necessary for proper functioning of the axe-txe cassette as a plasmid stabilization module

The major role of toxin-antitoxin cassettes located on plasmid DNA is stable maintenance of these mobile genetic elements in bacterial populations through a post-segregational killing mechanism. Previously, the axe-txe cassette was shown to be a functional plasmid stabilization system in evolutionary diverse bacterial hosts, including \textit{E. coli} [24]. To determine whether the active p\textsubscript{axe} promoter is necessary for correct functioning of axe-txe as a plasmid stabilization module, derivatives of the segregational stability probe vector pFH450 were used [36]. This plasmid contains both moderate-copy-number ColE1 \textit{ori} and low-copy-number P1 plasmid \textit{ori}. However, replication of pFH450 proceeds only from the latter in a \textit{polA} host. As the vector contains no accessory stabilization sequences, it is unstable in this host. Plasmid pREG531 that contains axe-txe genes and flanking sequences cloned into pFH450 was used as a positive control [24]. Changes that inactivated the p\textsubscript{axe} promoter without altering the Axe amino acid sequence (TATGAT→TACGAC) were introduced by site-directed mutagenesis producing pREGpaxemut. For the
negative control, the axe-txe cassette was deleted from pREG531 to produce pREG Δaxetxe. In the absence of antibiotic selective pressure, faster plasmid loss was observed in E. coli C600 polA1 bearing pREGpaxemut relative to the strain bearing pREG531 with the wild-type axe-txe module (Figure 5). Finally, after 60 hours of discontinuous growth in the absence of selection, plasmid retention for the vector possessing the intact axe-txe module was ~55%, whereas the level of plasmid retention was only ~17% for the variant in which the paxe promoter was inactivated (Figure 5). These results clearly show that the active paxe is essential for appropriate functioning of the axe-txe cassette in stable plasmid maintenance.

Additional elements within the cassette may influence regulation of axe-txe expression

In vitro transcription analysis of the cassette was performed in the search for regulatory elements that potentially influence expression of the axe-txe operon. For this purpose pTE103 plasmid derivatives which contain a strong T7 early transcriptional terminator region were used. Thus, transcripts terminate ~280 bp downstream of the cloned fragments. Transcripts of ~850 and ~680 nt were detected that correspond to those expected to be produced from the paxe and paxe promoters, respectively (Figure 6, lane 2). Mutation of the -10 box in paxe abolished production of the smaller transcript which correlates with data presented above that paxe is a bona fide promoter that is required for txe expression (Figure 6, lane 1). In addition, these in vitro transcription experiments unexpectedly revealed the presence of a third transcript (~300 nt) which appeared only when the whole txe gene fragment was present (Figure 6, lanes 1 and 2), but not when a construct with a truncated txe gene was employed (Figure 6, lane 3). These observations suggest that this transcript must originate within the txe gene.

Comparison of cultures harbouring plasmid pTE103 containing either the complete axe-txe module (pTEpat_axe-txe) or this module with a longer downstream sequence (pTEpat_axe-txe-ter) revealed significant growth differences (Figure 7A). In the first construct, the region downstream of txe comprises ~30-bp after the stop codon. In the second construct ~90-bp longer fragment was included. As observed previously (Figure 4), the construct with short downstream sequences partially inhibited growth due to the expression of txe from paxe and paxe promoters. However, addition of the extended fragment downstream of txe alleviated this toxic effect (Figure 7A). Analysis of the sequence revealed the presence of a lengthy transcription terminator-like region starting ~20 bp downstream of the txe gene (Figure 7B). In vitro transcription assays with constructs bearing the axe-txe cassette with this stem-loop fragment showed that it functions as a transcriptional terminator/attenuator in vitro. Some of the transcripts deriving from paxe as well as from paxe promoters stop at this point, while the rest terminate further at the T7 strong terminator located...
within the vector (Figure 8, lane 3). This putative hairpin structure may have a role in transcript stability if it is recognized by RNases that decrease the stability of the mRNAs and thereby modulate Txe production. This hypothesis is being tested currently. Moreover, the axe-txe cassette without this potential terminator region cloned into a stability probe vector clearly showed impaired activity as a stability determinant indicating the importance of this element, possibly to ensure an optimal stoichiometry between toxin and antitoxin (unpublished data).

Discussion

The toxin components of TA systems are intracellular molecular time bombs whose release from complexes with their cognate antitoxins can trigger bacterial programmed cell death or cell cycle arrest [5]. Understanding the mechanisms by which expression and activation of these modules are controlled is crucial to dissect their functioning and possible practical exploitation.

The Axe-Txe system was first discovered on the multidrug-resistant pRUM plasmid in a clinical isolate of E. faecium [24]. Preliminary analysis of Axe–Txe demonstrated that it functions as a characteristic TA system: expression of Txe is toxic to cells, Axe alleviates Txe-induced toxicity, and Axe–Txe increases plasmid maintenance [24]. It was also demonstrated that Txe is an endoribonuclease which cleaves mRNA and thereby inhibits protein synthesis [27]. Due to the prevalence of the axe–txe genes on plasmids in enterococcal isolates [29,30], artificial activation of Txe presents an attractive antimicrobial strategy. However, a complete lack of knowledge about regulation of axe-txe expression blocks potential exploration of the complex as an antimicrobial target.

The chromosomal yefM-yoeB toxin-antitoxin module of E. coli is homologous to axe-txe [24]. As is the case with most known TA systems, expression of yefM-yoeB is negatively autoregulated, with YefM being the primary transcriptional repressor and YoeB acting as a repression enhancer [10]. DNA binding is achieved by the sequential association of YefM with a pair of inverted repeats that comprise the yefM-yoeB operator site [10]. This interaction involves a pair of arginine residues in
a unique DNA binding fold within the N-terminal region of the protein [34,35]. The YoeB toxin acts as a corepressor by stabilizing the flexible C-terminal region of YefM which also conceals the toxin’s endoribonuclease fold [35].

Analysis of the nucleotide sequence of the \( p_{at} \) promoter-operator region upstream of \( axe-txe \) revealed two inverted repeats with the same 5'-TGTACA-3' core that overlap the \( yefM-yoeB \) promoter [10]. In the case of \( p_{at} \), the repression by antitoxin alone was very weak (<2-fold), whereas the Axe-Txe complex repressed more efficiently (~5-fold). However, the activity of the \( p_{at}-lux \) fusion remained very high in the repressed state. These results suggested that there might be another mechanism(s) which shut downs \( axe-txe \) expression. In agreement, an additional promoter \( (p_{axe}) \) within the \( axe \) gene directs extra synthesis of Txe protein. However, this promoter lacks overlapping 5'-TGTACA-3' boxes, is not repressed by Axe-Txe, and no detectable binding to this region was observed by Axe-Txe in vitro. The \( p_{axe} \) promoter instead may be regulated by an unknown factor(s), or may be expressed constitutively. The ~300-nt transcript produced by the \( axe-txe \) cassette may also be implicated in controlling expression of the \( p_{axe} \) promoter by an unknown mechanism. Nevertheless, the data clearly show that the active \( p_{axe} \) promoter is indispensable for proper functioning of the \( axe-txe \) cassette as a plasmid stabilization module.

The control of the synthesis of most, if not all, toxin proteins of TA complexes is likely to be multilayered. Further indications that \( axe-txe \) may be subject to additional levels of regulation came from experiments with fragments containing the \( axe-txe \) cassette but with different lengths of downstream sequence. Constructs possessing an extended fragment downstream of \( txe \) that contains a putative terminator region do not inhibit bacterial growth, whereas constructs which lack this fragment exert a pronounced growth defect. One can speculate that the potential termination hairpin may serve as an element that decreases mRNA stability and in this way lowers production of the Txe toxin. mRNA stability is one of the parameters that determine the efficiency of gene expression. mRNA turnover is mediated by a combination of endo- and exoribonucleases whose activities are modulated by structural features of the mRNA [37]. One such example is the \( kis-kid \) toxin-antitoxin system in which the intracellular levels of Kis and Kid proteins are controlled by limited degradation of a polycistronic messenger. However, in this case the presence of a stem-loop sequence located within the 5' region of \( kid \) gene shows a stabilizing effect mediated on mRNA [38]. The majority of RNA molecules are subjected to regulation and, as is the case of mRNA, their decay can be influenced by growth conditions. Moreover, the RNA degradosome can undergo changes in composition depending on growth or stress conditions [39–41]. In the case of \( axe-txe \) different regulatory mechanisms might exist to ensure a balanced production of the antitoxin relative to the toxin which is necessary for appropriate functioning of this system. The \( kis-kid \) and \( ccdAB \) operons are tightly regulated by the ratio of the toxin and the antitoxin [13,14]. It is possible that in the reporter system used here, in which the \( axe-txe \) operon lacking the terminator-like sequence downstream of \( txe \) was fused with the \( lux \) gene, the ratio of Axe and Txe was not

Figure 7. The role of a putative terminator region downstream of the \( txe \) gene. (A) \( E. col i \) SC301467 harbouring derivatives of \( pTE103 \) bearing the \( axe-txe \) cassette with \( (pat_axe-txe\_ter) \) or without \( (pat_axe-txe) \) the putative downstream transcription terminator were grown at 37°C. Absorbance readings at 600 nm were taken at 60 minutes intervals. (B) The terminator in the region downstream of the \( txe \) gene was predicted and drawn by the MFOLD program. doi: 10.1371/journal.pone.0073569.g007
optimal for full repression of $p_{at}$ promoter due to the excess of the toxin arising from altered mRNA stability. This agrees with other data showing that an excess of toxin can abolish transcriptional repression by releasing the TA complex from the operator site [15,16].

It should be emphasized that observations about $axe$-$txe$ regulation presented in this paper are true for $E. coli$ and may differ in the natural host, $E. faecium$. On the other hand, study of TA systems that derive from different bacterial species, including Streptococcus, Staphylococcus, Synechocystis, Streptomyces and Vibrio, in an $E. coli$ model is common [42–46]. Nevertheless, studies of $axe$-$txe$ regulation in the natural host will reveal whether different regulatory mechanisms operate in $E. faecium$ compared to $E. coli$.

In conclusion, the data presented here show that the regulation of expression of the $axe$-$txe$ module appears to be very complex. The $p_{at}$ promoter activity is very high and is only partially repressed by the concerted action of the Axe-Txe complex. Moreover, another promoter, $p_{axe}$, provides additional expression of the $txe$ gene. Therefore, the expression of the toxin gene requires additional negative regulation. This may be achieved by two means: (i) decreased stability of $txe$ mRNA due to its degradation starting after formation of a specific hairpin structure at the 3' end of the transcript; and (ii) the action of a counter transcript derived from the promoter located within $txe$ gene. Our experiments clearly indicate that both the active $p_{axe}$ promoter and the region downstream of $txe$ gene with the putative terminator region are necessary for proper functioning and tight regulation of the $axe$-$txe$ cassette.

One might ask why did such a complicated regulatory system evolve in the $axe$-$txe$ module? We speculate that additional regulatory elements provide more possibilities to optimize toxin and antitoxin production under diverse environmental conditions, e.g., nutrient availability or different temperatures. This may be especially important for bacteria living under conditions with potentially rapid fluctuations, including enterococci occupying the mammalian intestine that are suddenly excreted outside their host in stools. The balance between the amounts of toxin and antitoxin is of particular importance for cell survival.

**Supporting Information**

Figure S1. Neither Axe-Txe proteins nor other proteins in the $E. coli$ extract bound detectably to a fragment bearing the wild-type $p_{axe}$ promoter. A 126 bp 5' biotinylated fragment that includes $p_{axe}$ was subjected to EMSA. DNA samples were incubated with the different crude extracts concentrations of $E. coli$ BL21(DE3) harbouring pET22at_axe-txe plasmid (left to right): 0, 1.25, 2.5, 5, 10, 12.5 and 25 µg/ml for 20 min at 22°C.
and analyzed by a native 5% PAGE. Reactions were processed as outlined in Materials and Methods.

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Author Contributions

Conceived and designed the experiments: LB GW FH BK. Performed the experiments: LB LL BK. Analyzed the data: LB LL GW FH BK. Wrote the manuscript: BK GW FH.

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