

Txe, an endoribonuclease of the enterococcal Axe–Txe toxin–antitoxin system, cleaves mRNA and inhibits protein synthesis

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The *axe-txe* operon encodes a toxin–antitoxin (TA) pair, Axe–Txe, that was initially identified on the multidrug-resistance plasmid pRUM in *Enterococcus faecium*. In *Escherichia coli*, expression of the Txe toxin is known to inhibit cell growth, and co-expression of the antitoxin, Axe, counteracts the toxic effect of Txe. Here, we report the nucleotide sequence of pS177, a 39 kb multidrug-resistant plasmid isolated from vancomycin-resistant *Ent. faecium*, which harbours the *axe-txe* operon and the *vanA* gene cluster. RT-PCR analysis revealed that the *axe-txe* transcript is produced by strain S177 as well as by other vancomycin-resistant enterococci. Moreover, we determine the mechanism by which the Txe protein exerts its toxic activity. Txe inhibits protein synthesis in *E. coli* without affecting DNA or RNA synthesis, and inhibits protein synthesis in a cell-free system. Using *in vivo* primer extension analysis, we demonstrate that Txe preferentially cleaves single-stranded mRNA at the first base after an AUG start codon. We conclude that Txe is an endoribonuclease which cleaves mRNA and inhibits protein synthesis.

INTRODUCTION

Enterococcal species such as *Enterococcus faecium* and *Enterococcus faecalis* have emerged as significant nosocomial pathogens throughout Europe and the United States. These bacteria are increasingly responsible for a variety of illnesses, particularly in immunocompromised patients, and are one of the main causes of surgical-site infections (Hidron *et al.*, 2008; Owens & Stoessel, 2008; Richards *et al.*, 2000). Clinical isolates of enterococci are often resistant to multiple antibiotics either intrinsically or through the lateral gene transfer of antibiotic resistance determinants, the most problematic of which are the genetic elements that encode vancomycin resistance (Baldassarri *et al.*, 2005; Deshpande *et al.*, 2007; Dowzicky & Park, 2008;

Guardabassi & Dalsgaard, 2004; Low *et al.*, 2001; Sader & Jones, 2009; Werner *et al.*, 2008). A recent survey of Gram-positive pathogens isolated from hospitals in the United States found that, for between 2002 and 2008, up to 33 % of enterococcal species tested as part of the surveillance network were vancomycin-resistant enterococci (VRE) (JMI, 2009). As vancomycin is a preferred antimicrobial treatment for enterococcal infections, the increasing occurrence of multidrug-resistant VRE severely limits therapeutic options and highlights the need for new antibiotics.

In the search for novel antimicrobial agents, one intriguing approach involves the explicit targeting of toxin–antitoxin (TA) systems to exploit their innate toxicity (DeNap & Hergenrother, 2005; Engelberg-Kulka *et al.*, 2004; Williams & Hergenrother, 2008). TA genes were originally discovered on plasmids, where they function as plasmid maintenance systems (Ogura & Hiraga, 1983). In plasmids containing the genes for proteic (type II) TA systems, a stable toxin and a labile antitoxin are encoded in a single operon. In a cell carrying the plasmid, both proteins are expressed and the antitoxin binds to and inhibits the toxin. However, if during cell division a plasmid-free segregant arises, the labile antitoxin is rapidly degraded, freeing the

Abbreviations: PNK, polynucleotide kinase; TA, toxin–antitoxin; TCA, trichloroacetic acid; VRE, vancomycin-resistant enterococci.

The GenBank/EMBL/DDBJ accession number for the sequence of plasmid pS177 reported in this paper is HQ115078.

Three supplementary figures, showing protein synthesis following IPTG induction of Txe–His₆, Axe–His₆ and empty pET21a vector, and the effect of Txe–His₆ expression in *Escherichia coli* Rosetta BL21(DE3), are available with the online version of this paper.

toxin to kill the cell (Aizenman *et al.*, 1996; Hayes, 2003). This post-segregational killing has led TA systems to be dubbed 'plasmid addiction' systems, as the cell will die if the plasmid is lost (Gerdes *et al.*, 1986). The first TA system identified was *ccdAB*, on the F plasmid of *Escherichia coli* (Ogura & Hiraga, 1983), and since its discovery, a number of TA systems have been identified on a variety of plasmids and bacterial chromosomes (Moritz & Hergenrother, 2007a; Pandey & Gerdes, 2005; Sletvold *et al.*, 2007). Among the type II TA systems, the toxins can be grouped into several superfamilies based on sequence and structural homology, including the RelE/ParE, MazF/CcdB, Doc, HipA, ζ and PIN domain superfamilies, and most have been shown to act as either endoribonucleases (also termed mRNA interferases) or inhibitors of DNA gyrase (Anantharaman & Aravind, 2003; Bernard & Couturier, 1992; Christensen-Dalsgaard & Gerdes, 2008; Christensen-Dalsgaard *et al.*, 2008; Fico & Mahillon, 2006; Francuski & Saenger, 2009; Jiang *et al.*, 2002; Makarova *et al.*, 2009; Miallau *et al.*, 2009; Van Melderen, 2002).

Reports of TA systems in Gram-positive bacteria have been much rarer compared with the numerous studies of TA systems in Gram-negative bacteria. One of the first proteic TA systems described in a Gram-positive organism was the Axe-Txe system, which was identified on the 24.8 kb non-conjugative plasmid pRUM, obtained from a multidrug-resistant clinical isolate of *Ent. faecium* (Grady & Hayes, 2003). pRUM confers resistance to chloramphenicol, erythromycin, streptomycin and streptothricin, and coexists in its host with a 60 kb conjugative vancomycin resistance plasmid. Sequence analysis of pRUM suggested it arose from a variety of mobile genetic elements, recombination events and smaller plasmids (Grady & Hayes, 2003). Although it was originally described in 2003, there have been no further reports on the biochemical characterization of Axe-Txe.

We have previously shown that in a collection of 75 VRE clinical isolates, 56 contained the genes for *axe-txe*, as determined by PCR analysis (Moritz & Hergenrother, 2007a). Of those 56 isolates, gel extractions and conjugative matings suggested a physical linkage between the vancomycin-resistance genes and the *axe-txe* genes in 44 isolates (Moritz & Hergenrother, 2007a). Recently, a separate analysis of a collection of *Ent. faecium* isolates found that of 42 strains positive by PCR for the pRUM replicon type, 90% were also PCR-positive for *axe-txe* (Rosvoll *et al.*, 2010). Additionally, co-hybridization studies suggested that a genetic linkage existed between the pRUM replicon and the *axe-txe* genes (Rosvoll *et al.*, 2010). Thus, the *axe-txe* genes appear to be common features of plasmids in enterococci where they may play a role in the persistence and stability of plasmid-encoded antibiotic resistance, including vancomycin resistance.

Although several TA systems have been well characterized biochemically, many of these were discovered on the chromosomes of Gram-negative bacteria (Christensen

et al., 2001; Engelberg-Kulka & Glaser, 1999). In contrast, there is a relative paucity of studies on TA systems of Gram-positive origin, and TA systems that were discovered on multidrug resistance plasmids. As the dissemination of such plasmids enables widespread resistance to antibiotics, the mechanisms by which these plasmids maintain themselves in their hosts are of critical importance. The development of toxin activation strategies for Gram-positive bacteria requires the identification of a suitable TA system that is functional in these hosts, and information about the mechanism of action of the toxin.

In this study, we report the nucleotide sequence of plasmid pS177 (39 023 bp), isolated from the vancomycin-resistant *Ent. faecium* clinical isolate S177. pS177 confers resistance to kanamycin, streptothricin, streptomycin, erythromycin and vancomycin and harbours the genes for *axe-txe* and a *relBE* homologue, *relBE_{EF}*. Additionally, we confirmed by RT-PCR analysis that the *axe-txe* transcript is synthesized in six VRE clinical isolates, including strain S177. Although *axe-txe* has been shown to function as a plasmid stabilization system in *Ent. faecium*, *Bacillus thuringiensis* and *E. coli* (Grady & Hayes, 2003), the mechanism by which Txe elicits its toxic effect on the cell is unknown. Here, we demonstrate that expression of the toxin inhibits protein synthesis in the cell but does not affect DNA or RNA synthesis. Primer extension analysis reveals that Txe is an endoribonuclease that cleaves cellular RNA three bases downstream of an AUG start codon. This is the first report, to our knowledge, of the biochemical mode of action of Txe, and one of the few studies on TA systems of Gram-positive origin.

METHODS

Plasmid DNA isolation. Plasmid DNA was isolated from *Ent. faecium* by using a modified alkaline lysis midiprep protocol (Sambrook & Russell, 2001). A 50 ml bacterial culture grown in brain heart infusion broth was harvested after 12–14 h growth and the pellet was resuspended in 2 ml solution I (25 mM Tris, pH 8.0, 50 mM glucose, 10 mM EDTA) and 200 μ l lysozyme (100 mg ml⁻¹ in 25 mM Tris, pH 8.0). The suspension was incubated at 37 °C for 1 h. Solution II (3 ml; 0.2 M NaOH, 1% SDS) was added and the tube was inverted gently six times, followed by 4.5 min incubation on ice. Finally, 3 ml solution III (5 M potassium acetate, 11.5% glacial acetic acid) was added and the tube was inverted eight times, followed by 5 min incubation on ice. Cell debris was collected by centrifuging for 20 min at 20 000 g at 4 °C. To extract the nucleic acid, 7 ml supernatant was transferred to a new tube and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, w/v/v) was added and the tube was shaken vigorously, followed by centrifugation as described previously. Nucleic acid was precipitated from 6 ml of the aqueous layer by adding an equal volume of 2-propanol and incubating at room temperature for 2 min, followed by centrifuging for 30 min at 27 000 g. The nucleic acid pellet was washed with 5 ml 70% ethanol and centrifuged for 10 min at 27 000 g at 4 °C. All ethanol was removed and the pellet was allowed to air-dry, then dissolved in 100 μ l 10 mM Tris, pH 8.0, overnight at 4 °C. The RNA was digested by incubation with 5 μ l 10 mg RNase A ml⁻¹ for 30 min at 37 °C.

Plasmid DNA isolated from *Ent. faecium* S177 was separated by electrophoresis in a 0.65% agarose gel, containing 0.5 μ g ethidium

bromide ml^{-1} . The dominant supercoiled plasmid band was excised from the gel and the DNA was recovered by electroelution, following a modification of the protocol described by Strong *et al.* (1997). Dialysis tubing with a 12–14 kDa nominal molecular mass cut-off (Spectra/Por) was prepared by boiling in 25 mM EDTA, pH 8.0, for 15 min, rinsed thoroughly in dH_2O , and rinsed in $1 \times$ Tris-acetate-EDTA (TAE) at 4°C . The gel slice and approximately 2 ml $1 \times$ TAE were secured in the dialysis tubing and electrophoresed in $1 \times$ TAE at 70 V for 2 h, at which point the polarity was reversed for 2 min. Without removing the gel slice, the solution was subjected to dialysis against 10 mM Tris, pH 8.0, overnight at 4°C . The electroeluted plasmid DNA was concentrated by centrifuging at 500 g in a 0.5 ml 30 kDa molecular mass cut-off tube (Microcon) until the volume was reduced to 100 μl .

Plasmid DNA sequencing. Shotgun cloning, sequencing and assembly of plasmid pS177 was performed by the University of Illinois W.M. Keck Center for Comparative and Functional Genomics. Briefly, DNA was sheared with a nebulizer, end-repaired and dephosphorylated. The DNA was separated by electrophoresis in a 0.8% low-melting-point agarose gel, from which DNA ranging from 1.5 to 5.0 kb was purified and cloned into the pSMART-HCKan vector (Lucigen) according to the manufacturer's instructions. Sequencing was performed on 480 subclones from both the 5' and the 3' ends of the insert by using ABI Big-Dye terminator chemistry (Applied Biosystems). Custom primers were used in PCRs and sequencing reactions to close gaps and to ensure at least $3 \times$ coverage at each nucleotide (average coverage was $12.4 \times$). Sequence data were assembled by using the Phrap (<http://www.phrap.org>) and Sequencher (GeneCodes) software programs. Analysis of the assembled plasmid via the BLAST program (Altschul *et al.*, 1990) was used to determine sequence similarity, and annotations were made accordingly.

Plasmid construction. To construct plasmid pET21a-*txe*-His₆, the *txe* gene was amplified by PCR with primers *txe*-NheI-F, 5'-CATCCCCTAGCATGATTAAGG-3', and *txe*-XhoI-R, 5'-CGCCCTCGAGTTCATAGTG-3'. The PCR product was digested with *NheI* and *XhoI* and inserted into pET21a (Novagen) digested with *NheI* and *XhoI*. His₆-Txe is expressed from pET21a-*txe*-His₆ upon addition of IPTG to *E. coli* Rosetta BL21(DE3).

To construct plasmid pACYCDuet1-His₆-*axe*, the *axe* gene was amplified by PCR with primers *axe*-start-*EcoRI*, 5'-GGGTGAATTCATGGAAGCA-3', and *axe*-stop-*Sall*, 5'-TCAGACTGCGACTT-AATCATC-3'. The PCR product was digested with *EcoRI* and *Sall* and inserted into pACYCDuet1 (Novagen) digested with *EcoRI* and *Sall*. His₆-Axe is expressed from pACYCDuet1-His₆-*axe* upon addition of IPTG to *E. coli* BL21(DE3) or Rosetta BL21(DE3).

To construct plasmid pET21a-*axe*-His₆, the *axe* gene was amplified by PCR with primers *axe*-cterm-F1, 5'-CCAGGATCCGCATATGATGGAAGC-3', and *axe*-XhoI-R, 5'-CAAGCTCGAGGACTTCATCATC-3'. The PCR product was digested with *NdeI* and *XhoI* and inserted into pET21a (Novagen) digested with *NdeI* and *XhoI*. Axe-His₆ is expressed from pET21a-*axe*-His₆ upon addition of IPTG to *E. coli* BL21(DE3) or Rosetta BL21(DE3).

To construct plasmid pET28a-*relB*-His₆, *relB* was amplified by PCR with primers *relB*-NcoI-F2, 5'-GGAGATATACCATGGGAACAATGG-3', and *relB*-HindIII-R, 5'-CCAGAAAAGCTTCCATCCGAGTTC-3'. The PCR product was digested with *NcoI* and *HindIII* and inserted into pET28a treated with *NcoI* and *HindIII*. pET28a-*relB*-His₆ produces RelB-His₆ upon addition of IPTG to *E. coli* BL21(DE3).

To construct plasmid pRSF-1b-His₆-*lpp*, the *lpp* gene was amplified by PCR with primers *lpp*-1-*BamHI*, 5'-CCCCGGATCCGGAGATTA-

ACTCAATCTAGAGG-3', and *lpp*-2-*Sall*, 5'-CCCCGTCGACGCGCCATTTTTCACCTCACAG-3'. The PCR product was digested with *BamHI* and *Sall* and inserted into pRSF-1b (Novagen) digested with *BamHI* and *Sall*. His₆-Lpp is expressed from pRSF-1b-His₆-*lpp* upon addition of IPTG to *E. coli* Rosetta BL21(DE3) or *E. coli* BL21(DE3) cells.

Macromolecular synthesis assays. For each strain tested, an overnight culture grown in Luria-Bertani (LB) with 100 μg ampicillin ml^{-1} was used to seed 10 ml filter-sterilized M9 broth (42 mM Na_2HPO_4 , 24 mM KH_2PO_4 , 9 mM NaCl, 19 mM NH_4Cl , 0.5% glucose, 1% Casamino acids) and shaken at 37°C until an OD_{600} of 0.3 was reached. To measure protein synthesis, [³H]leucine (1-[3, 4, 5-³H(N)]leucine; PerkinElmer) was added to the culture at a final concentration of 2 $\mu\text{Ci ml}^{-1}$ (74 kBq ml^{-1}) and shaken at 37°C for 5 min. To measure DNA synthesis, [³H]thymidine ([6-³H]thymidine; PerkinElmer) was added to the culture at a final concentration of 0.1 $\mu\text{Ci ml}^{-1}$ (3.7 kBq ml^{-1}) and shaken at 37°C for 1 min. To measure RNA synthesis, [³H]uridine ([5,6-³H]uridine; PerkinElmer) was added to the culture at a final concentration of 0.1 $\mu\text{Ci ml}^{-1}$ (3.7 kBq ml^{-1}) and shaken at 37°C for 1 min.

Before inducing expression, 0.5 ml was taken from the culture and added to 1 ml cold 15% trichloroacetic acid (TCA), inverted once and placed on ice for at least 30 min to precipitate (via TCA) all macromolecules in the cell. The remaining culture was split in two; one half received 0.05 mM IPTG and the other received no IPTG. The cultures were shaken again at 37°C and aliquots were TCA-precipitated, as above, 60, 120 and 180 min after IPTG was added. Precipitates were filtered on a glass microfibre disc (Whatman or Fisher Scientific), washed twice with cold 100% ethanol and allowed to dry overnight. Discs were placed in scintillation fluid and c.p.m. was measured.

Cell-free protein synthesis assays. The S30 T7 high-yield protein expression system (Promega) was used for cell-free protein synthesis according to the manufacturer's protocol with some modifications. The construct encoding the protein of interest, namely pET21-*txe*-His₆, pET21a-*axe*-His₆ or pET21a-His₆-*axe*, was incubated with 10 μl S30 Premix Plus and 9 μl T7 S30 extract (at 37°C for 20 min) to allow synthesis of Txe-His₆, Axe-His₆ or His₆-Axe. After the initial 20 min of incubation, 300 ng DNA (10 mM Tris/HCl, pH 8.0) encoding the reporter protein (pET28a-*relB*-His₆ or pRSF1b-His₆-*lpp*) was added and shaken at 37°C for 1 h. Reactions were stopped by placing tubes on ice for 5 min and by adding 1 μg RNase A to each tube and incubating at room temperature for 5 min.

Protein was isolated from each reaction by precipitation with cold 10% TCA, followed by centrifugation at 21 130 g for 15 min at room temperature. Pellets were washed with cold 100% ethanol and allowed to air-dry. Pellets were resuspended in 30 μl 50 mM Tris/HCl, pH 8.0, 300 mM NaCl and 20 μl 6 \times SDS loading dye and heated at 95°C for 5 min. For each sample, the entire volume was run on a 15% polyacrylamide Tris/HCl precast gel (Bio-Rad). After SDS-PAGE, proteins were transferred to an Immobilon-P PVDF membrane (Millipore) in cold Towbin transfer buffer. Membranes were blocked with PBS containing 5% BSA and 0.05% Tween-20 overnight with gentle shaking at 4°C . Membranes were probed with HisProbe-HRP (Pierce) at 1 $\mu\text{g ml}^{-1}$ in PBS with 0.05% Tween-20 for 1 h at room temperature. Antibody binding was detected with Supersignal West Pico chemiluminescent substrate (Pierce), followed by exposure to autoradiography film.

RNA extraction and RT-PCR analysis. Total RNA from enterococci was isolated in a manner identical to the extraction method described previously (Moritz & Hergenrother, 2007a). Intragenic primers used for *axe*-*txe* RT-PCR analysis were designed from the sequence of the plasmid pRUM. The primers used were *axe*-*txe*-RT-F,

5'-CAGTAGCTTATTCAAATTTCCGCC-3', and *axe-txe*-RT-R, 5'-GTTTCATCTGTAATTCCTTCTGGACC-3'. Control primers were based on the *tuf* gene specific to enterococcal species (Garcia-Migura *et al.*, 2007). The primers used were Ent-*tuf* F, 5'-TACTGACAAACCATTCATGATG-3', and Ent-*tuf* R, 5'-AACTTCGTCACCAACGCGAAC-3'. RT-PCR and PCR for DNA contamination were performed by using the SuperScript One-Step RT-PCR with Platinum *Taq* kit (Invitrogen) as described previously (Moritz & Hergenrother, 2007a). RT-PCR amplification products were analysed by agarose gel electrophoresis in 1% agarose and were stained with ethidium bromide.

Primer extension analysis. Primer *lpp* 21 (5'-CTGAACGTCAGGACAGCTGATCG-3') (Christensen *et al.*, 2003) was 5'-end-labelled with [γ - 32 P]ATP according to a modification of the protocol reported by Kitami & Hiwada (1999). In a 10 μ l reaction volume, 10 pmol of the *lpp* 21 primer was mixed with 1 μ l 10 \times T4 polynucleotide kinase (PNK) reaction buffer (NEB), 1 μ l 10 U T4 PNK (NEB) ml $^{-1}$ and 3 μ l [γ - 32 P]ATP [6 mCi mmol $^{-1}$ (222 MBq mmol $^{-1}$), 10 mCi ml $^{-1}$ (370 MBq ml $^{-1}$) or adjusted appropriately for decay]. The reaction mixture was incubated at 37 °C for 30 min followed by 2 min at 90 °C to inactivate the enzyme. Unincorporated nucleotides were removed with a NucAway Spin column according to the manufacturer's protocol (Ambion). The final concentration of the end-labelled primer was adjusted to 100 fmol μ l $^{-1}$ with RNase-free dH $_2$ O.

The DNA sequencing ladder was prepared with the primer *lpp* 21 and pRSF-1b-His $_6$ -*lpp* as template to map the 5'-end of the *lpp* mRNA. Sequencing reactions were prepared by using the Sequenase version 2.0 DNA sequencing kit and [α - 32 P]dATP according to the manufacturer's protocol (USB).

E. coli Rosetta BL21(DE3) carrying pET21-*txe*-His $_6$ was grown in LB containing 100 μ g ampicillin ml $^{-1}$ to an OD $_{600}$ of 0.3. Before adding 0.5 mM IPTG, a 1 ml aliquot was centrifuged for 5 min at room temperature and the pellet was frozen at -80 °C. Following addition of IPTG, aliquots were also taken at 30, 60 and 120 min post-induction. RNA was extracted by using the RNeasy mini kit for isolation of total RNA from bacteria and treated with RNase-free DNase (Qiagen). The 32 P-labelled *lpp* 21 primer was hybridized with 10 μ g total RNA from each time point according to a modification of the protocol reported by Kitami & Hiwada (1999). In a 40 μ l reaction volume, 10 μ g total RNA, 200 fmol 32 P-labelled *lpp* 21 primer, 4 μ l 10 \times avian Myeloblastosis virus reverse transcriptase (AMV RT) reaction buffer (NEB) and 4 μ l 10 \times dNTP solution (10 mM each dNTP) were added together. The reaction mixture was heated at 90 °C for 2 min and then incubated at 54 °C overnight (18–20 h).

The next day, 20 μ l of a primer extension mastermix (6 μ l 10 \times AMV RT reaction buffer, 6 μ l 10 \times dNTP solution, 4.2 μ l 40 mM sodium pyrophosphate, 1.8 μ l dH $_2$ O and 2 μ l 10 U AMV RT μ l $^{-1}$) was added to each hybridization reaction, mixed gently and incubated at 42 °C for 1 h. Reactions were then treated with 2 μ l 10 mg RNase A ml $^{-1}$ at 37 °C for 30 min and extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1, w/v/v). DNA was precipitated by adding 0.1 vols 3 M sodium acetate, pH 5.5, and 2.5 vols 70% ethanol and storing at -80 °C for 30 min. Precipitates were centrifuged at room temperature for 15 min, washed with 70% ethanol and allowed to air-dry before resuspending in 10 μ l stop solution (USB).

Gel analysis was performed by using a 6% polyacrylamide TBE-urea gel prepared with 6% PAGE/urea and complete buffer solutions (Ambion). The gel was pre-electrophoresed at 45 W in 1 \times Tris-borate-EDTA (TBE) buffer for 30 min. Primer extension samples (10 μ l) were heated at 90 °C for 5 min before loading onto the gel, and the 32 P-labelled sequencing reactions (2 μ l) were heated at 75 °C for 2 min before loading onto the gel. After running the gel at 45 W in 1 \times TBE buffer, it was dried under vacuum at 80 °C for 1 h. The

gel was cooled before exposing to a storage phosphor screen cassette (Molecular Dynamics) at room temperature overnight. Primer extension products and the DNA sequencing ladder were detected by using the Storm PhosphorImager 840 (Molecular Dynamics/GE Lifesciences).

RESULTS

Although it has been suggested that activation of toxins of TA pairs could be a viable antibacterial strategy, the success of such an approach is dependent on the TA system being present and functional in the bacteria of interest (Williams & Hergenrother, 2008). The *axe-txe* operon has been shown to reside on plasmids within VRE (Moritz & Hergenrother, 2007a; Rosvoll *et al.*, 2010), although PCR alone was used to characterize *axe-txe* in these studies. In an effort to link *axe-txe* definitively to plasmid-encoded vancomycin resistance genes and to establish the functionality of *axe-txe* within VRE, we first sought to characterize fully an enterococcal plasmid harbouring *axe-txe*, and to determine if VRE clinical isolates synthesize the *axe-txe* mRNA transcript. For this, a collection of VRE clinical isolates previously shown to harbour a plasmid with *axe-txe* was used (Moritz & Hergenrother, 2007a).

Sequence analysis of pS177

The nucleotide sequence of plasmid pS177 was determined by shotgun cloning and sequencing, with average coverage of 12.4 \times . pS177 is a 39 032 bp non-conjugative plasmid isolated from *Ent. faecium* clinical strain S177. Its DNA G+C content is 35.5 mol%, consistent with that of other enterococcal plasmids and genomes (Sletvold *et al.*, 2007). Nucleotide BLAST analysis of pS177 revealed that 37 358 bp (95.7%) shared significant (99–100%) similarity with sequences deposited in the GenBank database (Altschul *et al.*, 1990). Forty ORFs were identified and annotated based on these known sequences. For ORFs annotated in the database as hypothetical proteins, BLASTP searches were performed to assign a putative function for that potential protein based on amino acid similarity (Altschul *et al.*, 1990). pS177 has a mosaic structure comprising a pRUM backbone, two resistance gene cassettes and five insertion elements (Fig. 1, Table 1). Interestingly, pS177 has 12 748 bp (32.6%) and 18 930 bp (48.4%) in common with plasmids p5753cA (GenBank accession no. GQ900435) and p5753cB (GenBank accession no. GQ900487), respectively, both isolated from *Ent. faecium*.

pS177 shares 12 341 bp (31.6%) of its sequence with pRUM and contains 75% of the total pRUM plasmid (GenBank accession no. AF50797). It harbours a pRUM-like replicon consisting of the putative RepA replication protein observed in various enterococcal plasmids (Grady & Hayes, 2003; Rosvoll *et al.*, 2010). In addition to genes encoding stability mechanisms such as plasmid replication and partitioning, pS177 carries the TA system *axe-txe* (Grady & Hayes, 2003). The *axe-txe* genes and upstream promoter region share 100% homology with the pRUM

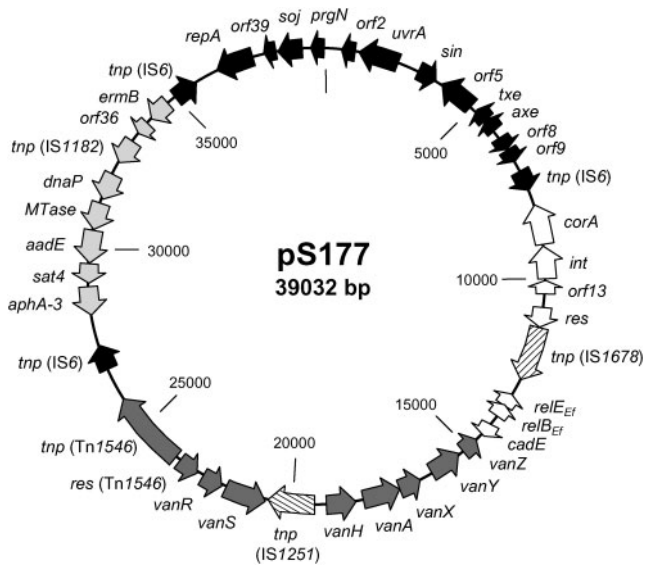


Fig. 1. Genetic organization and mosaic structure of multidrug-resistant plasmid pS177. Coding sequences are indicated by arrows showing the predicted direction of transcription. Position 1 of pS177 was assigned as the same nucleotide position 1 of pRUM. The origins of the components of pS177 are shown by black, white or grey arrows: genes from pRUM, black; genes from p5753cA, white; solitary insertion elements, hatched; VanA-type glycopeptide resistance gene cassette, dark grey; *S. intermedius* resistance gene cassette, light grey.

sequence, suggesting that this TA system is functional (Grady & Hayes, 2003). Plasmid pS177 harbours a gene cassette from *Staphylococcus intermedius* (GenBank accession no. AF299292), which confers resistance to streptothricin, streptomycin, kanamycin and erythromycin (Boerlin *et al.*, 2001). pRUM carries a nearly identical gene cassette in which the *aphA-3* gene encoding kanamycin resistance is truncated (Grady & Hayes, 2003). The full-length *S. intermedius* resistance gene cassette is also carried on the enterococcal plasmids pRE25 (Schwarz *et al.*, 2001) and p5753cB.

Additionally, vancomycin resistance is encoded by the VanA-type glycopeptide resistance determinant Tn1546, observed on many plasmids identified in VRE, including plasmids pVEF3 (Sletvold *et al.*, 2008), p5753cA and pIP816 (GenBank accession no. AM932524). However, the Tn1546 cassette on pS177 contains the insertion element IS1251 between *vanS* and *vanH*. This unusual *vanA* cluster type has been observed previously (Camargo *et al.*, 2004; Handwerger & Skoble, 1995; Handwerger *et al.*, 1995) and is also present on the enterococcal plasmid p5753cA, which carries a truncated *vanA*-type gene cassette with IS1251 in the same position as observed in pS177.

Plasmid pS177 shares 12 748 bp (32.6%) with p5753cA, including the putative RelBE TA system homologue from *Ent. faecium*, which we designate *relBE_{Ef}*. The ORFs encoding *relB_{Ef}* and *relE_{Ef}* were identified based on their

homology with p5753cA, in which their putative gene products were annotated as hypothetical proteins. A BLASTP search of the amino acids encoded by *relE_{Ef}* showed up to 100% identity with a variety of *Ent. faecium* hypothetical proteins, as well as 53% amino acid identity with the RelE protein from *Lactobacillus antri* (Altschul *et al.*, 1990). The BLASTP search of the amino acids encoded by *relB_{Ef}* resulted in hits sharing up to 100% homology with other *Ent. faecium* hypothetical proteins, as well as 79% amino acid identity with the *Ent. faecalis* AbrB family antitoxin component.

Transcription of *axe-txe* in VRE clinical isolates

To determine whether the mRNA transcript coding for the Axe-Txe proteins is produced in VRE strains previously shown by PCR to carry *axe-txe* (Moritz & Hergenrother, 2007a), RT-PCR was performed from total mRNA isolated from six VRE clinical isolates. As shown in Fig. 2(a), all six isolates (C29113, S34, S177, SL242, SL266 and SL745) produce the *axe-txe* transcript. RT-PCR analysis was also performed on total RNA isolated from the plasmid-free *Ent. faecium* strain BM4105-RF, which does not contain the *axe-txe* genes, and no *axe-txe* transcript was detected (Fig. 2a). To ensure that the observed RT-PCR products were not due to DNA contamination, controls were performed in which the reverse transcriptase enzyme was not added, but all other components (including thermostable DNA polymerase and primers for *axe-txe*) were included. As shown in Fig. 2(b), no amplification product is observed under these conditions, confirming that the products seen by RT-PCR were due to the presence of transcript and not DNA contamination. Finally, RT-PCR with primers for the enterococcal elongation factor *tuf* gave the expected positive result for all seven strains examined (Fig. 2c) (Garcia-Migura *et al.*, 2007). Thus, RT-PCR analysis demonstrated that the *axe-txe* transcript is synthesized in VRE clinical isolates carrying the *axe-txe* genes.

Txe expression inhibits global protein synthesis

As sequencing of pS177 and RT-PCR analysis of six VRE isolates revealed that *axe-txe* is present and transcribed in VRE, we sought to determine the mechanism by which Txe exerts its toxic activity. Such information could be exploited in the development of TA-specific antimicrobial therapies. Txe mechanistic experiments began by assessing the toxin's effect on global biosynthetic pathways in the cell. Radiolabel incorporation assays were carried out with [³H]thymidine to measure DNA synthesis, [³H]uridine to measure RNA synthesis and [³H]leucine to measure protein synthesis upon induction of Txe-His₆ expression with IPTG in *E. coli* Rosetta BL21(DE3). As shown in Fig. 3(a, b), levels of DNA and RNA synthesis were not inhibited by the expression of Txe-His₆ in *E. coli*. In contrast, the level of protein synthesis was significantly inhibited by the expression of Txe-His₆ in *E. coli* (Fig. 3c). By 2 h post-induction,

Table 1. ORFs of plasmid pS177

ORF*	Gene	Nucleotide position		Protein length (aa)	Database match (GenBank accession no.)	Amino acid identity (%)
		5'	3'			
1c	<i>prgN</i>	1	291	97	pRUM PrgN (AAO52827)	100
2c	<i>orf2</i>	603	980	126	pRUM conserved hypothetical protein, <i>Ent. faecium</i> (AAO52828)	100
3c	<i>uvrA</i>	946	2271	442	pRUM UvrA, structural gene for UV resistance (AAO52829)	100
4	<i>sin</i>	2784	3335	184	pRUM Sin recombinase (AAO52830)	100
5c	<i>orf5</i>	3500	4504	335	pRUM hypothetical protein (AAO52831)	100
6c	<i>txe</i>	4963	5220	86	pRUM Txe, toxin component of TA system (AAO52832)	100
7c	<i>axe</i>	5213	5482	90	pRUM Axe, antitoxin component of TA system (AAO52833)	100
8	<i>orf8</i>	5824	6126	101	pRUM conserved hypothetical protein (AAO52834)	100
9	<i>orf9</i>	6169	6387	73	pRUM conserved hypothetical protein (AAO52835)	100
10	<i>tnp</i> (IS6)	6559	7245	229	pRUM IS6 transposase (AAO52848)	100
11c	<i>corA</i>	7536	8924	463	<i>Ent. faecium</i> magnesium/nickel/cobalt transporter CorA (ZP_05660433)	100
12c	<i>int</i>	8989	9942	318	<i>Ent. faecium</i> integrase, catalytic region (ZP_00603158)	100
13c	<i>orf13</i>	10 026	10 156	43	p5753cA hypothetical protein (ADA62235)	100
14	<i>res</i>	10 585	11 148	188	p5753cA resolvase, N-terminal domain (ADA62236)	100
15	<i>tnp</i> (IS1678)	11 336	12 655	440	<i>Ent. faecium</i> transposase, IS1678 (AAW32124)	99
16c	<i>relEEf</i>	13 111	13 398	96	<i>Lactobacillus antri</i> RelE-like toxin component of TA system (ZP_05745775)	53
17c	<i>relBEf</i>	13 388	13 717	110	<i>Ent. faecalis</i> Abr-family antitoxin component of TA system (ZP_06623423)	79
18	<i>cadE</i>	14 022	14 351	110	p5753cA cadmium efflux system accessory protein (ADA62239)	100
19c	<i>vanZ</i>	14 638	15 123	162	Tn1546 VanZ, teicoplanin resistance protein (AAA65959)	100
20c	<i>vanY</i>	15 276	16 187	304	Tn1546 VanY, truncated carboxypeptidase (AAA65958)	99
21c	<i>vanX</i>	16 615	17 223	203	Tn1546 VanX, D-Ala-D-Ala dipeptidase (AAA65957)	99
22c	<i>vanA</i>	17 229	18 260	344	Tn1546 VanA, D-Ala-D-Lac ligase (AAA65956)	99
23c	<i>vanH</i>	18 253	19 221	323	Tn1546 VanH, pyruvate dehydrogenase (AAA65955)	100
24	<i>tnp</i> (IS1251)	19 553	20 845	431	<i>Ent. faecium</i> IS1251-like transposase (AAF73111)	100
25c	<i>vanS</i>	20 943	22 097	385	Tn1546 VanS, truncated histidine kinase (AAA65954)	100
26c	<i>vanR</i>	22 075	22 770	232	Tn1546 VanR, regulator of two-component regulatory system (AAA65953)	100
27c	<i>res</i>	22 984	23 559	192	Tn1546 resolvase (AAA65952)	100
28	<i>tnp</i> (Tn1546)	23 705	25 856	717	Tn1546 transposase (AAA65951)	100
29	<i>tnp</i> (IS6)	26 661	27 347	229	pRUM IS6 transposase (AAO52854)	100
30c	<i>aph-3</i>	28 195	28 980	262	<i>S. intermedius</i> Aph-3, aminoglycoside phosphotransferase (AAG42234)	100
31c	<i>sat4</i>	29 082	29 612	177	<i>S. intermedius</i> Sat4, streptothricin acetyltransferase (AAG42233)	100
32c	<i>aadE</i>	29 621	30 529	303	<i>S. intermedius</i> AadE, streptomycin adenylyltransferase (AAG42232)	100
33c	<i>MTase</i>	30 562	31 296	245	<i>Ent. faecium</i> methyltransferase (ZP_00603123)	100
34c	<i>dnaP</i>	31 277	32 146	290	<i>Ent. faecium</i> DNA polymerase, beta-like region (ZP_00603124)	100
35c	<i>tnp</i> (IS1182)	32 521	33 195	225	<i>S. intermedius</i> transposase, IS1182 (AAG42229)	99
36c	<i>orf36</i>	33 652	33 783	44	<i>S. intermedius</i> orf3 (AAG42228)	100
37c	<i>ermB</i>	33 788	34 525	246	<i>S. intermedius</i> ErmB, erythromycin resistance methylase (AAG42227)	100
38	<i>tnp</i> (IS6)	34 873	35 559	229	pRUM IS6 transposase (AAO52854)	100
39c	<i>repA</i>	36 175	37 215	347	pRUM putative RepA replication protein (AAO52855)	100
40c	<i>orf40</i>	37 564	37 890	109	pRUM hypothetical protein (AAO52856)	100
41c	<i>soj</i>	37 877	38 680	268	pRUM Soj partitioning protein (AAO52857)	100

*ORFs predicted to be transcribed on the complementary strand are denoted with 'c'.

the amount of [³H]leucine incorporation measured was greatly reduced in cells receiving IPTG compared with cells that did not receive IPTG. Shorter time points (5, 10, 15 and

30 min) were investigated to determine how quickly expression of Txe-His₆ inhibited protein synthesis; however, differences in [³H]leucine incorporation were not observed

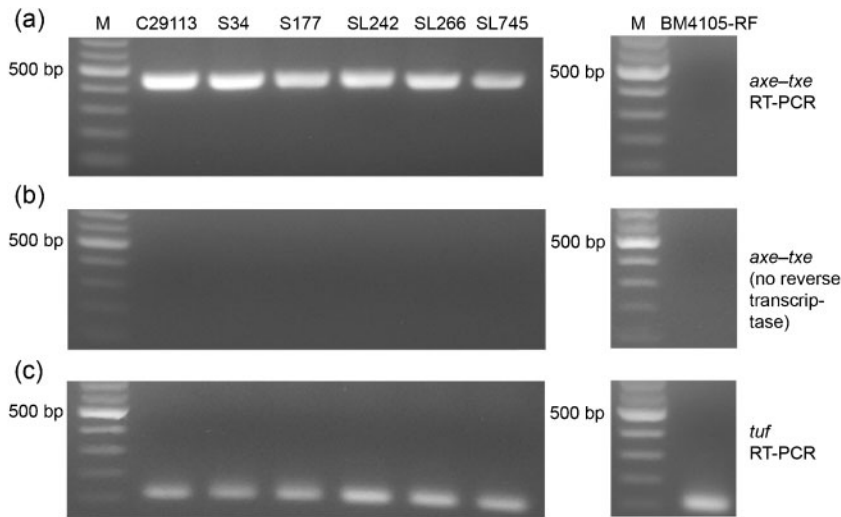


Fig. 2. RT-PCR analysis of six VRE isolates and a plasmid-free enterococcal strain (BM4105-RF). The VRE isolates tested were C29113, S34, S177, SL242, SL266 and SL745. (a) RT-PCR with primers complementary to *axe-txe* (product size=447 bp). (b) Controls for DNA contamination, in which reverse transcriptase was omitted from the reaction mix. (c) RT-PCR with primers complementary to the enterococci *tuf* gene (product size=112 bp). Lane M, DNA molecular mass markers.

until 60 min post-induction (Supplementary Fig. S1, available with the online version of this paper). Growth of *E. coli* harbouring the pET21a-*txe*-His₆ plasmid in liquid culture was also examined; induction of Txe-His₆ expression with IPTG in *E. coli* resulted in a decrease in the viable cell count (Supplementary Fig. S2).

To determine whether the inhibition of protein synthesis observed was specific for the expression of Txe-His₆, radiolabel incorporation assays were performed with [³H]leucine and *E. coli* carrying the pET21a-*axe*-His₆ plasmid or the empty pET21a vector. As shown in Supplementary Fig. S3(a, b), the level of protein synthesis was not inhibited by the expression of Axe-His₆ in *E. coli* or when IPTG was added to cells carrying the empty vector. A direct comparison of levels of [³H]leucine incorporation following addition of IPTG to *E. coli* with pET21a, pET21a-*axe*-His₆ and pET21a-*txe*-His₆ at the various time points examined indicated that there is considerable inhibition of protein synthesis by Txe-His₆ (Fig. 4).

Txe inhibits protein synthesis in a cell-free system

A cell-free system was used to examine further the inhibitory effect of Txe-His₆ on protein synthesis. An *E. coli* T7 S30 extract system was preincubated with the pET21a-*txe*-His₆ plasmid to begin synthesis of the Txe-His₆ protein, followed by the addition of DNA encoding the reporter (pET28a-*relB*-His₆ or pRSF1b-*His*₆-*lpp*) to allow synthesis of RelB-His₆ (the antitoxin of the RelBE TA system) or the major lipoprotein, Lpp, of the *E. coli* outer membrane (Christensen & Gerdes, 2003; Gotfredsen & Gerdes, 1998). Txe-His₆ inhibited the synthesis of RelB-His₆ (Fig. 5a, lane 3) and His₆-Lpp (Fig. 5b, lane 3). When His₆-Axe or Axe-His₆ was synthesized instead of Txe-His₆, protein synthesis of RelB-His₆ and His₆-Lpp was not inhibited (Fig. 5a, b, lane 4). Interestingly, both tagged versions of the antitoxin were unable to significantly prevent protein synthesis inhibition by Txe-His₆ (data not

shown). This may be the result of Txe-His₆ inhibiting protein synthesis of the antitoxin as well as RelB-His₆ and His₆-Lpp, or interference of the His₆-tag on Axe with its ability to bind the toxin.

Txe cleaves mRNA in a sequence-specific manner

In vivo primer extension analysis was used to determine if Txe-mediated protein synthesis inhibition is achieved via mRNA cleavage in the cell. Cleavage of the 5' end of *lpp* mRNA was chosen for analysis given its frequent use as an RNA substrate for both Northern blot analysis and primer extension analysis in mechanistic investigations of other TA system toxins (Christensen-Dalsgaard & Gerdes, 2008; Christensen & Gerdes, 2003; Christensen *et al.*, 2003; Zhang *et al.*, 2004).

To perform primer extension analysis in cell culture, RNA was isolated from *E. coli* Rosetta BL21(DE3) cultures before and after induction of *txe* expression by IPTG. As shown in Fig. 6, induction of Txe-His₆ expression led to the disappearance of the full-length *lpp* mRNA band and the appearance of a major cleavage band as early as 30 min post-induction (Fig. 6). The major cleavage band appeared three bases downstream of the A of the AUG start codon. These results suggest that Txe-His₆ cleaves mRNA and that this RNase activity may lead to the protein synthesis inhibition observed upon induction of Txe-His₆ expression in the macromolecular synthesis assays and cell-free protein synthesis assays.

DISCUSSION

The Axe-Txe TA system was first discovered on a multidrug-resistant plasmid, pRUM, in a vancomycin-resistant *Ent. faecium* clinical isolate (Grady & Hayes, 2003). Axe-Txe was one of the first proteic TA systems to be characterized from Gram-positive bacteria; it was also notable in that *axe-txe* was discovered on a bacterial

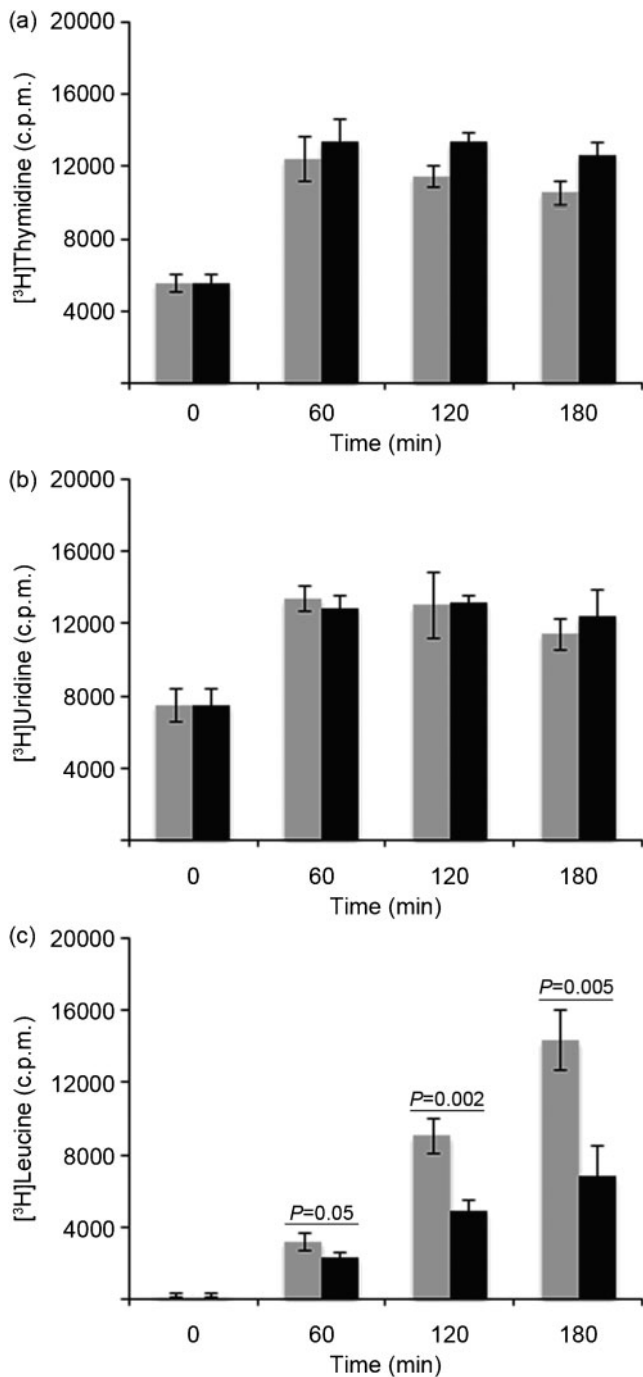


Fig. 3. DNA, RNA and protein synthesis following IPTG induction of Txe-His₆, as assessed immediately before and following addition of 0 (grey bars) or 0.05 (black bars) mM IPTG to *E. coli* Rosetta BL21(DE3) carrying the pET21a-txe-His₆ plasmid. Incorporation of [³H]thymidine (a), [³H]uridine (b) and [³H]leucine (c). Results are the mean of three independent experiments. Error bars, SD; *P*, statistical significance between designated groups.

plasmid, whereas most TA systems were discovered on bacterial chromosomes. Preliminary analysis of Axe-Txe demonstrated that it functions as a characteristic TA

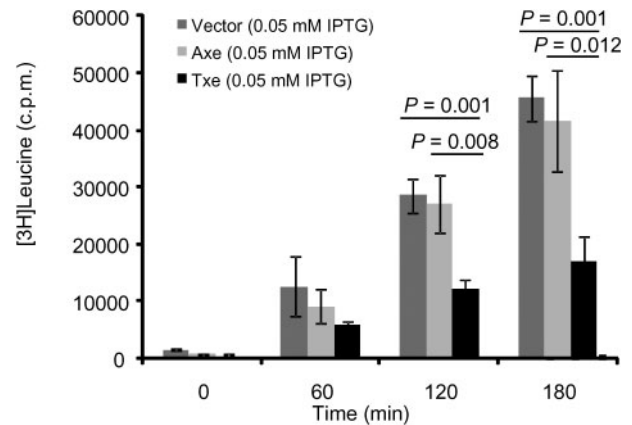


Fig. 4. Protein synthesis following IPTG induction of Txe-His₆, Axe-His₆ and empty pET21a vector. Incorporation of [³H]leucine immediately before and following addition of 0.05 mM IPTG to *E. coli* Rosetta BL21(DE3) carrying pET21a, pET21a-txe-His₆ or pET21a-axe-His₆. Results are the mean of three independent experiments. Error bars, SD; *P*, statistical significance between designated groups.

system: expression of Txe is toxic to cells, expression of Axe alleviates Txe-induced toxicity and Axe-Txe increases the segregational stability of plasmids (Grady & Hayes, 2003). Due to the prevalence of the *axe-txe* genes encoded on plasmids in enterococcal isolates (Moritz & Hergenrother, 2007a; Rosvoll *et al.*, 2010), activation of Txe presents an attractive antimicrobial strategy. However, a lack of biochemical characterization of the Axe-Txe proteins, including knowledge of the biological target or mechanism of action of Txe, hampers exploration of Axe-Txe as an antimicrobial target.

In this study, the complete nucleotide sequence of the 39 kb non-conjugative plasmid pS177 isolated from a VRE clinical strain S177 was determined. A BLAST analysis revealed extensive homology to known sequences, including the enterococcal plasmid pRUM, the *vanA*-type glycopeptide resistance determinant Tn1546 and the *S. intermedius* resistance gene cassette (Altschul *et al.*, 1990). Thus, pS177 appears to have arisen from multiple recombination events between smaller plasmids and mobile genetic elements. This is the first report, to our knowledge, of a completely sequenced VRE plasmid that harbours a full-length *vanA* cassette containing the insertion element IS1251 between *vanS* and *vanH*. Additionally, plasmid pS177 confers resistance to vancomycin, kanamycin, streptomycin, streptothricin and erythromycin and, indeed, VRE strain S177 was resistant to gentamicin, erythromycin and vancomycin (Moritz & Hergenrother, 2007a). The presence of TA systems on pS177, including *axe-txe* and *relBE_{E β}* probably enhances plasmid stability and enables the persistence of this multidrug-resistant plasmid in clinical isolates of VRE. This is also the first report of a completely sequenced plasmid carrying both the VanA-

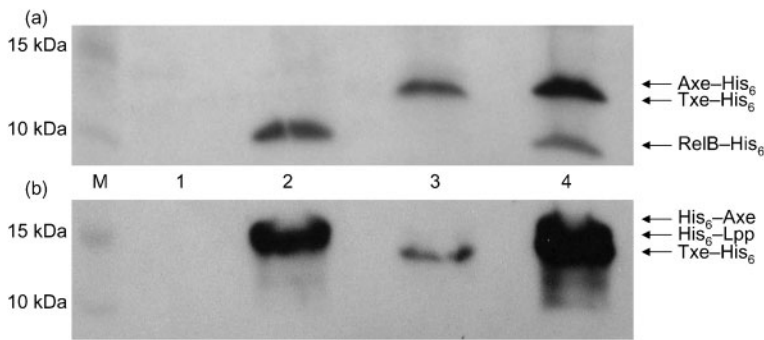


Fig. 5. Effect of Txe-His₆, Axe-His₆ and His₆-Axe on cell-free protein synthesis. (a) Western blot with HisProbe-HRP of cell-free protein synthesis reactions with no DNA added (lane 1), pET28a-reIB-His₆ DNA added (2), pET28a-reIB-His₆ and pET21a-txe-His₆ DNA added (3), and pET28a-reIB-His₆ and pET21a-axe-His₆ DNA added (4). (b) Western blot with HisProbe-HRP of cell-free protein synthesis reactions with no DNA added (lane 1), pRSF1b-His₆-lpp DNA added (2), pRSF1b-His₆-lpp and pET21a-txe-His₆ DNA added (3), and pRSF1b-His₆-lpp and pACYCDuet1-His₆-axe DNA added (4). M, Marker lane.

type resistance determinant and *axe-txe*. In addition, it is shown here for the first time to our knowledge that the *axe-txe* transcript is produced in VRE clinical isolates. The conclusive link between the pRUM-like replicon, *axe-txe*, the *vanA*-type resistance determinant (as demonstrated from sequencing) and the presence of *axe-txe* transcripts in VRE clinical isolates further supports the importance of *axe-txe* in the maintenance of plasmids coding for multidrug resistance, and bolsters the notion of targeting *axe-txe* for antimicrobial development (Moritz & Hergenrother, 2007b; Rosvoll *et al.*, 2010).

Txe shares significant sequence similarity with the YoeB toxin, which is part of the RelE toxin superfamily, and is structurally related to the archaeal RelE monomer (Francuski & Saenger,

2009; Kamada, 2005). The RelE and YoeB toxins from *E. coli* and two YoeB homologues in *Staphylococcus aureus* have been shown to inhibit protein synthesis through mRNA cleavage (Grady & Hayes, 2003; Zhang & Inouye, 2009; Yoshizumi *et al.*, 2009; Kamada, 2005). The results of the macromolecular synthesis assays and cell-free protein synthesis assays described herein indicate that Txe significantly inhibits protein synthesis. The results of primer extension analysis indicate that Txe cleaves mRNA, and this activity is probably the mechanism by which Txe inhibits protein synthesis.

The two major mechanisms of action for toxins of TA systems are the inhibition of DNA gyrase and the cleavage of cellular RNA. There is reasonable amino acid sequence similarity between Txe and the YoeB RNase (Grady & Hayes, 2003); however, it has been noted that these two toxins have very different effects on cells, with *yoeB* expression dramatically reducing viable cell numbers, whereas *txe* expression halts growth but does not induce death (Grady & Hayes, 2003). Although structural and sequence similarity to other toxins can be informative in discerning mode of action, it can be misleading as well. For example, significant structural similarity exists between the gyrase poison CcdB and the RNase MazF (Buts *et al.*, 2005). Thus, it was not obvious *a priori* under which category Txe would fall.

With the completion of the experiments described herein, it is now apparent that Txe is an RNase harbouring similarities to YoeB and sharing some, but not all, characteristics with other members of the RelE superfamily. Txe cleaved *lpp* mRNA three bases downstream of the A of the AUG start codon, similar to what has been observed with the YoeB toxin (Yoshizumi *et al.*, 2009; Zhang & Inouye, 2009). In contrast, the RelE toxin has been shown to cleave mRNA at both sense and stop codons, with a strong preference for cleavage of the stop codon UAG between the second and third bases (Christensen & Gerdes, 2003; Pedersen *et al.*, 2003).

The data presented here indicate that the Txe toxin is an RNase and inhibits protein synthesis, activities which may play a role in the ability of *axe-txe* to stabilize plasmids in

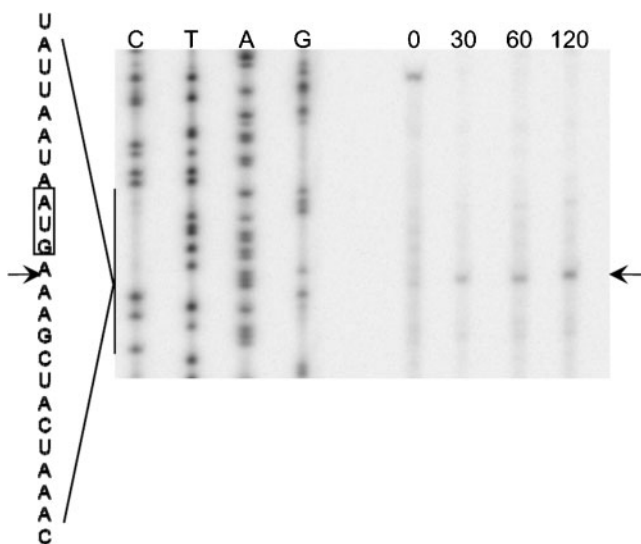


Fig. 6. Txe-induced cleavage of *lpp* mRNA. The 5' end of *lpp* mRNA was mapped by using the primer *lpp* 21. Numbers indicate times (min) at which mRNA was harvested after the addition of IPTG. The major cleavage site is indicated by an arrow. The sequence around the major cleavage site is shown to the side, with the cleavage site again indicated by an arrow.

enterococci, including plasmids harbouring the genes for antibiotic resistance (Grady & Hayes, 2003). As *axe-txe* appears to be widespread in VRE clinical isolates (Moritz & Hergenrother, 2007a; Rosvoll *et al.*, 2010), the artificial activation of *Txe* could be an effective antibacterial strategy.

ACKNOWLEDGEMENTS

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