ef1097 and *ypkK* encode enterococcin V583 and corynicin JK, members of a new family of antimicrobial proteins (bacteriocins) with modular structure from Gram-positive bacteria

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Unlike the colicins, microcins and related peptide antibiotics, little is known about antibiotic proteins ($M_r > 10\,000$) from Gram-positive bacteria, since only few examples have been described to date. In this study we used heterologous expression of recombinant proteins to access the 17 kDa antibiotic protein SA-M57 from *Streptococcus pyogenes*, along with two proteins of unknown function identified in publicly available databases: EF1097 from *Enterococcus faecalis* and YpkK from *Corynebacterium jeikeium*. Here we show that all three are antibiotic proteins with different spectra of antimicrobial activity that kill sensitive bacteria at nanomolar concentrations. *In silico* structure predictions indicate that although the three proteins share little sequence similarity, they may be composed of conserved secondary structural elements: a relatively unstructured, acidic N-terminal portion and a basic C-terminal portion characterized by two helical elements separated by a loop structure and stabilized by an essential disulphide. Expression of individual segments as well as protein chimaeras revealed that, at least in the case of YpkK, the C-terminal portion is responsible for the killing action of the protein, whereas the role of the N-terminal portion remains unclear. Both *scnM57* and *ef1097* appear to be widely distributed in *Strep. pyogenes* and *Ent. faecalis* (respectively), whereas *ypkK* is found only rarely amongst clinical isolates of *C. jeikeium*. Finally, we determined that the proteins kill sensitive bacteria without lysis, a feature that distinguishes them from known murolytic proteins.

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INTRODUCTION

Bacteriocins are ribosomally synthesized, proteinaceous antibiotics produced by a variety of species of both Gram-positive and Gram-negative bacteria that inhibit the growth of other bacteria. Although from a biochemical perspective they exhibit an extraordinary diversity of structure, the bacteriocins can be loosely grouped into four categories, based on their molecular mass and whether the producing organism is Gram-positive or Gram-negative. For example, a number of species of the *Enterobacteriaceae* produce large, modular, antimicrobial proteins ($M_r > 10\,000$) known as colicins (Cascales *et al.*, 2007), whilst others produce relatively lower molecular

mass antimicrobial peptides ($M_r < 10\,000$) termed microcins (Heng & Jack, 2006). Similarly, a veritable plethora of modified and unmodified peptide bacteriocins ($M_r < 10\,000$) from Gram-positive bacteria have also been described, particularly those elaborated by the lactic acid bacteria (Bonelli *et al.*, 2006; Nes *et al.*, 2006; Heng *et al.*, 2007). In recent years considerable research interest has focussed on the low molecular mass polypeptides from both Gram-positive and Gram-negative bacteria, in part because of their potential in pharmaceutical and nutraceutical applications (Cleveland *et al.*, 2001; Ross *et al.*, 2002; Gillor *et al.*, 2004; Kirkup, 2006).

Despite the amount of research directed at the peptide bacteriocins of Gram-positive bacteria, microcins and colicins, surprisingly little is known about the higher molecular mass ($M_r > 10\,000$) bacteriocins from Gram-positive bacteria.

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Several bacteriocins that have proven to be cell-wall-hydrolysing (murolytic) enzymes, such as lysostaphin, zoocin A and stellalysin, have been reported from Gram-positive bacteria (Schindler & Schuhradt, 1964; Simmonds *et al.*, 1996; Heng *et al.*, 2006b). Historically, staphylococcin 1580 from *Staphylococcus epidermidis* (Jetten & Vogels, 1972a, b; Jetten *et al.*, 1972) was reported to be a bacteriocin with a subunit molecular mass of ~20 kDa that formed multimeric structures of 300–400 kDa in conjunction with carbohydrate and lipid. However, more recent reisolation of the inhibitory agent revealed the antimicrobial activity to be epidermin, a peptide bacteriocin (2164.6 Da) belonging to the lantibiotic subgroup, which co-purified with the higher molecular mass species (Sahl, 1994). Two additional high molecular mass bacteriocins from Gram-positive bacteria have been reported: helveticin J from *Lactobacillus helveticus* 481 (Joerger & Klaenhammer, 1986, 1990) and a potentially related, unnamed compound from *Lb. helveticus* CNRZ450 (Thompson *et al.*, 1996). The former was suggested to have a monomeric molecular mass of 37 kDa and to form aggregates of >300 kDa, whereas the latter was reported to be a bacteriocin with multimeric molecular mass of 30–50 kDa and potentially composed of ~17 kDa monomers. Helveticin J is active against other lactic acid bacteria such as lactobacilli and lactococci, is chromosomally encoded and was shown to be inactivated by proteolytic digestion and heat (Joerger & Klaenhammer, 1986, 1990), features consistent with its size and proteinaceous nature.

More recently, we have described the 17 kDa bacteriocin streptococcin A-M57 from *Streptococcus pyogenes*, identified during the sequencing of a novel plasmid (pDN571) isolated from a prototype M-57 strain (Heng *et al.*, 2004) and the detailed characterization of dysgalactin, a 21.5 kDa anionic, plasmid-encoded bacteriocin from *Streptococcus dysgalactiae* subsp. *equisimilis* (Heng *et al.*, 2006). In the former study, we also identified two further putative proteins that were recognized during interrogation of public databases and were predicted to have both sequence and structural similarity to streptococcin A-M57: EF1097 from *Enterococcus faecalis* (accession no. AAO80897; Paulsen *et al.*, 2003) and YpkK from *Corynebacterium jeikeium* (accession no. AAL85945; Tauch *et al.*, 2004). Interestingly, these proteins share no similarity with those described above, indicating that they may represent a novel family of antibiotic proteins from Gram-positive bacteria. In this study, we report the expression of these proteins in *Escherichia coli* and present evidence that EF1097 and YpkK are antimicrobial proteins with structural similarity to, but different antimicrobial spectra from, streptococcin A-M57. Moreover, we show that the C-terminal portion of YpkK is the essential killing moiety.

METHODS

Bacterial strains, culture media and culture conditions. *Escherichia coli* DH5 α (Hanahan, 1983) was the host for all expression

studies and was propagated aerobically at 37 °C, either in LB broth or on LB agar (Sambrook & Russell, 2001); when required ampicillin was added at 100 $\mu\text{g ml}^{-1}$. The bacterial strains used in spectrum of activity studies (Table 3) were routinely propagated aerobically at 37 °C on Columbia Agar Base (Becton Dickinson) containing 5 % (v/v) whole human blood or in Todd–Hewitt broth (Difco) at the same temperature. Bacterial strains were maintained as frozen stock cultures stored at –80 °C in Todd–Hewitt broth containing 20 % (v/v) glycerol (Sigma-Aldrich).

Assessment of biological activity. The biological activity of various protein preparations was determined against the bacterial indicator strains, either in a qualitative ‘spot assay’ or in a quantitative minimum inhibitory concentration (MIC) determination. In the former a 5 mg ml^{-1} protein solution for spectra studies, or a 1 mg ml^{-1} solution for the YpkK domain constructs, was sterilized by filtration using a 0.22 μm low-protein-binding PTFE syringe-driven filtration membrane (Millex-LG, Millipore), and 10 μl aliquots were applied to blood agar plates and allowed to dry in; the plates were then seeded with the indicator bacterium under test and incubated aerobically at 37 °C for ~18 h. A zone of inhibition of the indicator lawn with a diameter of at least 5 mm was arbitrarily defined as positive inhibition. The quantitative MIC determination was carried out essentially as previously described (Staubitz *et al.*, 2001) except that Tryptic Soy Broth (Difco) was used to support bacterial growth, the microtitre plates were not shaken and either *Micrococcus luteus* T-18 or *Lactococcus lactis* T-21 (Tagg & Bannister, 1979) was used to quantify the biological activity. The ability of the proteins to lyse either live or dead target bacteria using agar-immobilized or liquid culture-based assays was assessed essentially as previously described (Wirawan *et al.*, 2007).

Recombinant DNA techniques, construction of recombinant expression systems and bioinformatic analyses. PCR amplicons and plasmids from *E. coli* were purified using appropriate kits (Qiagen) according to the manufacturer’s recommendations. Protocols for molecular biological techniques (cloning, plasmid transformation, etc.) were conducted as described by Sambrook & Russell (2001). The high-fidelity enzyme Platinum *pf*x DNA polymerase (Invitrogen) was used in accordance with the manufacturer’s protocols in all PCR experiments; all primers (Table 1) were purchased from Invitrogen. DNA sequence analyses were carried out by the Allan Wilson Centre Genome Service (Palmerston North, New Zealand). Similarity searches used appropriate BLAST algorithms (Altschul *et al.*, 1997; Schäffer *et al.*, 2001), alignments were accomplished using CLUSTAL W (<http://www.ebi.ac.uk/clustalw/>; Chenna *et al.*, 2003), and assembly and basic analyses of sequence data were carried out using GeneJockey II (Biosoft). *In silico* predictions of secretion signal peptides were accomplished using SignalP 3.0 (Vullo & Frasconi, 2004), whilst structural predictions were made using the suite of programs available on the Expert Protein Analysis System (ExPASy) proteomics server (<http://expasy.org>; Gasteiger *et al.*, 2003) or the PredictProtein server (<http://predict-protein.org>; Rost *et al.*, 2004).

The principal features of the pQE80L-based expression system (Qiagen) used for all recombinant proteins produced in this study has been previously described (Heng *et al.*, 2004, 2006). In order to express full-length constructs, appropriate forward and reverse primers were used to amplify the correct product from the respective genomic DNA template, and the products were purified, digested, cloned into pQE80L and transformed into *E. coli* DH5 α . Plasmids were recovered from appropriate transformants, nucleotide sequenced to confirm their identity, and those with the correct inserts were used further as template in PCR for the generation of N- and C-terminal segment constructs and the expression of protein chimaeras. In order to construct the former, N-terminal segments

Table 1. Sequences and characteristics of the primers used in the construction of various recombinant protein expression systems

Primer	Sequence (5'–3')
Full-length constructs*	
SAM57-F1	ACCAGGATCCGGCAGCATTGAAGGCCGTAAGTAGTAGCTGACGAAAAAACTTC
SAM57-R1	CCCAAGCTTTTAGAAGTTGATTCCGCATTG
EF1097-F1	ACCAAGGATCCGGCAGCATTGAAGGCCGTTCTGACCAGCTAGAGGATTGAGAGTTGAGGC
EF1097-R1	CTATCGAAGCTTACACAAAAGCGATGATAAACT
YpkK-F1	ACCAGGATCCGGCAGCATTGAAGGCCGTCAGAGACTCCTGCCGCTGTGCAATCTGCA
YpkK-R1	ATCCTCCAAGCTTACGTCTCTCTGTTCTG
N-terminal segments†	
QIAF	CGGATAACAATTTACACAG
ScnM57-R2	CCCAAGCTTTTAACTTTATACATGATTGC
EF1097-R2	CCCAAGCTTTTATACTGTTGAAGCATCAGA
YpkK-R2	CCCAAGCTTTTACACGGACTGACCGGTGGC
C-terminal segments‡	
QIAR	ATTGGGATATATCAACGGTGG
ScnM57-F2	ACCAGGATCCGGCAGCATTGAAGGCCGTTGTTGATTGGGGCGCACTAGGAAAC
EF1097-F2	ACCAAGGATCCGGCAGCATTGAAGGCCGTTGTTGATTGGGGCGCACTAGGAAAC
YpkK-F2	ACCAAGGATCCGGCAGCATTGAAGGCCGTTGTTGATTGGGGCGCACTAGGAAAC
Chimaeras§	
ScnM57-SalF	ATGTATAAAGTCGACTGGGGCGCACTAGGAAAC
ScnM57-SalR	GCCCCAATCGTCGACTTTATACATGATTGCATTTTC
EF1097-SalF	GCTTCAACAGTCGACCAAGCAGCTTTTAATTGG
EF1097-SalR	TGCTTGAACGTCGACTGTTGAAGCATCAGAAATATT
YpkK-SalF	CAGTCCGTGTCGACGCGGGGGCTGGAACA
YpkK-SalR	GCGCGTGAGTCGACGACTGACCGGTGGCAGAATT
Screening primers	
EF1097-F3	GGCGATGGCATTACTAATGACATTAGG
EF1097-R3	CTTAGCCCACATTGAACTGCCCATAAAGC
YpkK-F3	CGCTGTGCAATCTGCAGGAA
YpkK-R3	GACGCCTTGCTTAGCTAGGT

*The engineered *Bam*HI restriction site is underlined, the engineered *Hind*III restriction site is double underlined and the engineered flexi-linker/factor Xa cleavage site (GSGSIEGR) is in bold.

†The engineered *Hind*III restriction site is underlined.

‡The engineered *Bam*HI restriction site is underlined and the engineered flexi-linker/factor Xa cleavage site (GSGSIEGR) is in bold.

§The engineered *Sall* restriction site is underlined.

were amplified using QIAF and appropriate reverse primers, whereas C-terminal segments were amplified using appropriate forward primers and QIAR; in each case the products were purified, digested with *Bam*HI and *Hind*III, cloned into pQE80L and transformed into *E. coli* DH5 α and appropriate transformants were selected and characterized prior to expression of the protein. In order to construct expression systems for chimaeras, the appropriate N-terminal segment was amplified by PCR using forward primers for the full-length constructs and an appropriate reverse primer incorporating a *Sall* restriction site, whereas the C-terminal segments were amplified using the appropriate forward primer incorporating a *Sall* restriction site and QIAR. Purified PCR products were restricted with *Sall* and ligated. The ligation mix was then used as template in a PCR amplification carried out using a forward primer appropriate for full-length amplification of the N-terminal segment (incorporating a *Bam*HI restriction site) and a reverse primer appropriate for amplification of the C-terminal segment (incorporating a *Hind*III restriction site). The product was purified, restricted with *Bam*HI and *Hind*III, ligated into pQE80L and then transformed into *E. coli* DH5 α . The identity of the inserts obtained was verified by sequencing of selected transformants.

Expression, purification, modification and bioanalytical characterization of recombinant proteins. The expression and purification of all recombinant proteins, as well as the reductive alkylation of selected proteins, was carried out as previously described (Jack *et al.*, 1996; Heng *et al.*, 2004, 2006). SDS-PAGE analyses were carried out by electrophoretic separation on precast 16 % Tris-Tricine gels (Invitrogen) using the manufacturer's apparatus, chemicals and protocols, and protein bands were visualized with Coomassie brilliant blue G250 (Bio-Rad). Bioanalytical characterization of recombinant proteins involved mass spectrometry and, where necessary, N-terminal amino acid sequence analysis; this work was undertaken by the Centre for Protein Research (University of Otago).

Assessment of the distribution of *ef1097* and *ypkK*. Various clinical isolates of *Enterococcus faecalis* and *Enterococcus faecium* from our laboratory collection were used in colony PCR incorporating the primer pair EF1097-F3 and EF1097-R3 to detect *ef1097*. In order to detect *ypkK*, PCR were performed on clinical isolates of the nosocomial pathogen *C. jeikeium* obtained from the University Hospitals, Leuven (Belgium), and at the Université Louis Pasteur, Strasbourg (France), with the primer pair YpkK-F3/YpkK-R3

Table 2. Description of the recombinant proteins constructed in this study

Recombinant protein	Description
Streptococcin A-M57 (SA-M57)*	ScnM57† [Lys28–Phe179] (i.e. the 152 amino acid mature protein)
Enterococcin V583	EF1097‡ [Ser35–Ser170] (i.e. the 136 amino acid mature protein)
Corynicin JK	YpkK§ [Ala28–Trp164] (i.e. the 137 amino acid mature protein)
ScnM57[Lys28–Lys105]	N-terminal segment of SA-M57
EF1097[Ser35–Val93]	N-terminal segment of enterococcin V583
YpkK[Ala28–Ser87]	N-terminal segment of corynicin JK
ScnM57[Val106–Phe179]	C-terminal segment of SA-M57
EF1097[Val94–Ser170]	C-terminal segment of enterococcin V583
YpkK[Val88–Trp164]	C-terminal segment of corynicin JK
ScnM57[Lys28–Lys105]/EF1097[Val94–Ser170]	Chimaera (SA-M57 N-terminal segment fused to enterococcin V583 C-terminal segment)
ScnM57[Lys28–Lys105]/YpkK[Val88–Trp164]	Chimaera (SA-M57 N-terminal segment fused to corynicin JK C-terminal segment)
EF1097[Ser35–Val93]/SA-M57[Val106–Phe179]	Chimaera (enterococcin V583 N-terminal segment fused to SA-M57 C-terminal segment)
EF1097[Ser35–Val93]/YpkK[Val88–Trp164]	Chimaera (enterococcin V583 N-terminal segment fused to corynicin JK C-terminal segment)
YpkK[Ala28–Ser87]/SA-M57[Val106–Phe179]	Chimaera (corynicin JK N-terminal segment fused to SA-M57 C-terminal segment)
YpkK[Ala28–Ser87]/EF1097[Val94–Ser170]	Chimaera (corynicin JK N-terminal segment fused to enterococcin V583 C-terminal segment)

*Reported in a previous study (Heng *et al.*, 2004).

†GenBank accession no. AAT72798.

‡GenBank accession no. NP_814827.

§GenBank accession no. AAL85945.

(Table 1), obtained from Operon Biotechnologies (Cologne, Germany). In each case, PCR amplification was carried out using *Taq* DNA polymerase according to standard procedures, and products generated were separated by electrophoresis on 1% agarose gels and visualized by transillumination following staining with ethidium bromide. *E. coli* DH5 α with and without pQE80L-enterococcin V583 served as controls for *ef1097* amplifications, whilst purified plasmid DNA of pB85766 and pK64 (Tauch *et al.*, 2004) served as controls for the identification of *ypkK*.

RESULTS AND DISCUSSION

The hypothetical proteins EF1097 and YpkK are precursors of two novel protein antibiotics

We have previously shown that recombinant expression of antibacterial proteins is a practical route to obtaining proteins in sufficient quantities for further study, as well as in clearly demonstrating that the antibiotic protein is solely responsible for the activity observed (Heng *et al.*, 2006a, b). In the present study the predicted mature forms of ScnM57 (i.e. ScnM57[Lys28–Phe179] or SA-M57), EF1097 (i.e. EF1097[Ser35–Ser170]) and YpkK (i.e. YpkK[Ala28–Trp164]; Table 2) were individually expressed as recombinant proteins in *E. coli*, purified and characterized to confirm their identity. All three recombinant proteins had demonstrable antimicrobial activity against a variety of

bacterial strains (Table 3) with MICs against *Lactococcus lactis* of ~64 nM, 4 nM and 2 nM for SA-M57, EF1097 and YpkK, respectively. Interestingly, some bacterial strains such as *Micrococcus luteus*, *Lc. lactis* and several species of listeriae appeared susceptible to all three antibiotics, whilst other indicators were targeted in a highly specific manner. Still others, for example all staphylococcal species tested, were insensitive to the effects of the three antibiotics. Similarly, no antimicrobial activity could be detected when SA-M57, EF1097[Ser35–Ser170] or YpkK[Ala28–Trp164] were tested against a variety of Gram-negative bacteria (not shown). Since their current names reflect their unknown status, as well as the observation that the mature, biologically active proteins are not the primary translation product of their respective gene but are revealed only after cleavage of a leader peptide, we suggest that EF1097[Ser35–Ser170] be renamed enterococcin V583 and YpkK[Ala28–Trp164] be renamed corynicin JK to adequately reflect their bacteriocinogenic nature.

In silico alignments of the respective sequences of SA-M57, enterococcin V583 and corynicin JK (Fig. 1) indicate that the polypeptides share little similarity in the N-terminal half of their sequences and only minimal similarity in the C-terminal portion. However, it has previously been noted that they do contain conserved predicted secondary structural similarity in the latter portion (Heng *et al.*,

Table 3. Spectrum of antimicrobial activity of purified, recombinant SA-M57, EF1097 and YpkK

Bacterium	No. of strains tested:	No. of strains sensitive to recombinant:		
		SA-M57	Enterococcin V583	Corynicin JK
<i>Corynebacterium diphtheriae</i>	1	0	0	1
<i>C. ulcerans</i>	1	0	0	1
<i>Enterococcus faecalis</i>	11	0	11	0
<i>Ent. hirae</i>	2	1	2	0
<i>Lactobacillus acidophilus</i>	1	0	1	1
<i>Lb. casei</i>	1	0	1	0
<i>Lactococcus lactis</i>	2	2	2	2
<i>Listeria grayi</i>	1	1	1	1
<i>Lis. innocua</i>	1	0	1	1
<i>Lis. ivanovii</i>	1	1	1	0
<i>Lis. monocytogenes</i>	3	1	3	1
<i>Lis. murrayi</i>	1	1	1	1
<i>Lis. seeligeri</i>	1	1	1	1
<i>Lis. welshimeri</i>	1	0	1	0
<i>Micrococcus luteus</i>	2	2	2	2
<i>Staphylococcus aureus</i>	8	0	0	0
<i>Staph. cohnii</i>	1	0	0	0
<i>Staph. epidermidis</i>	1	0	0	0
<i>Staph. hominis</i>	1	0	0	0
<i>Staph. saprophyticus</i>	1	0	0	0
<i>Staph. simulans</i>	1	0	0	0
<i>Staph. warneri</i>	1	0	0	0
<i>Stomatococcus</i> spp.	3	0	0	0
<i>Streptococcus constellatus</i>	2	0	0	0
<i>Strep. dysgalactiae</i>	2	0	1	0
<i>Strep. equisimilis</i>	1	0	0	0
<i>Strep. gordonii</i>	3	0	3	0
<i>Strep. mitis</i>	3	0	3	0
<i>Strep. mutans</i>	2	0	0	0
<i>Strep. oralis</i>	1	0	1	0
<i>Strep. pyogenes</i>	8	0	8	0
<i>Strep. salivarius</i>	13	0	0	0
<i>Strep. sanguinis</i>	3	0	3	0
<i>Strep. uberis</i>	1	0	0	0

2004). Moreover, whilst the full-length SA-M57, enterococcin V583 and corynicin JK have predicted acidic pI values of 4.8, 4.9 and 5.4, respectively, the N-terminal portions of each appear to be more acidic (pI 4.1, 3.8 and 4.0, respectively) whilst the individual C-terminal segments are basic (pI 9.6, 10.0 and 9.8, respectively). These observations lead us to hypothesize that such antibiotic proteins may be composed of two portions: N-terminal segments with diverse structure and sequences responsible for targeting the diverse ranges of susceptible bacteria, and C-terminal portions with highly conserved secondary

structure (but not primary structure) responsible for the killing action of the proteins.

SA-M57, enterococcin V583 and corynicin JK contain an essential disulphide bond

One of the few highly conserved features of the C-terminal portions of all three antibiotics is a pair of Cys residues (Fig. 1) that are predicted *in silico* to form a disulphide. We have previously shown that the related antibiotic protein dysgalactacin contains a disulphide that is essential for biological activity (Heng *et al.*, 2006). In the present study, differential S-alkylation in the presence or absence of a reducing agent demonstrated that (i) recombinant SA-M57, enterococcin V583 and corynicin JK each contain a single disulphide bond and (ii) this bond is essential for biological activity (Table 4). This result further demonstrates the structural similarities conserved amongst the C-terminal portions of all members of this novel family of antimicrobial proteins and also supports the hypothesis that the C-terminal portion is essential for killing activity. *In silico* structure predictions for all three polypeptides indicate that, whilst the N-terminal segment is relatively unstructured, the C-terminal region may be composed of two helical elements, separated by a flexible loop region (Heng *et al.*, 2004, 2006). Since the conserved Cys residues are located immediately before the first and immediately after the second putative helix, it seems reasonable to assume that the covalent bridge formed by the disulphide holds the helices parallel to one another and stabilizes the overall conformation of this region.

Dissection of the molecular architecture of SA-M57, enterococcin V583 and corynicin JK

In order to gain insight into the molecular architecture of these antibiotic proteins, we expressed the respective N-terminal portions of each antibiotic in the same manner as the mature full-length proteins (Table 2). None of the purified recombinant N-terminal segments showed antibacterial activity at any concentration tested. In addition, we observed neither agonistic nor antagonistic effects when susceptible cells were either pre- or concomitantly treated with the N-terminal segment and its cognate full-length polypeptide. This latter result may indicate that the N-terminal portion is not (solely) responsible for target specificity.

By contrast, attempts to express either ScnM57[Val106–Phe179] or EF1097[Val94–Ser170] were unsuccessful; the reasons for this were not further investigated, but appeared to involve toxicity to the heterologous host as it failed to thrive after induction. We did however successfully express, isolate and characterize the C-terminal portion of YpkK. Interestingly, YpkK[Val88–Trp164] was biologically active in a spot assay (Fig. 2A) and had a MIC against *M. luteus* of ~72 nM, a value only sixfold less than that for corynicin JK (MIC 12 nM). In a further experiment, approximately equal amounts of purified, recombinant

SA-M57	KVVADEKTSITSEYITIEEQRQIDEVAAVLEKMFADGVTEENLKQYAQANYSEEELIIDN	60
Enterococcin V583	-----SDQLEDS-EVEAVAKGLEEMYANGVTEDNFKNYVKNNFAQQEISSVEE	47
Corynicin JK	-----AETPAAVESAGTVDEQALAQYFAKVDNAQTEKAHDALVDLFGED	45
	* :. . * : * : . : : : : . : :	
SA-M57	ELNTNLSQIQDENAIMYKVDWGALGNCMANKIKDELLAMISVGTIIKYAQKKAWKELAKI	120
Enterococcin V583	ELNVNISDAS--TVVQARFNWNLGSCVANKIKDEFFAMISISAIVKAAQKKAWKELAVT	105
Corynicin JK	AANQAIKENSATGQS ¹ VSTRGAGTFLTICIKGASDDIKSVFDVNVVAAAIGQKDYAKAAKE	105
	* :. . . . :. : * : * : : : : : : : : * : : *	
SAM-57	VIKYVAKAGVKTNAALIAGQLAIWGLQCGINF	152
Enterococcin V583	VLRFAKANGLKTN ² AIIVAGQLALWAVQCGLS-	136
Corynicin JK	AVKYLAKQGVKRNAAALAGMFAYWGWQCRGSW	137
	: : : * : * * : * : * : * : .	

Fig. 1. CLUSTAL W-generated (Chenna *et al.*, 2003), gapped alignment of SA-M57, enterococcin V583 and corynicin JK. The conserved cysteines involved in disulphide formation are highlighted and the arbitrarily assigned first amino acid (Val) of the expressed C-terminal portions is shown in bold and underlined; * indicates identical amino acids; : indicates functionally conserved amino acids; . indicates partially conserved residues.

YpkK[Ala28–Ser87] and YpkK[Val88–Trp164] were mixed, but this had neither an agonistic nor an antagonistic effect on the biological activity of the mixture. Similarly, YpkK[Ala28–Ser87] was neither agonistic nor antagonistic to corynicin JK when the two were mixed in approximately equal proportions. Surprisingly, YpkK[Val88–Trp164] also showed the same spectrum of activity as corynicin JK (not shown), indicating that the determinants of target specificity lie, at least partially, within this portion of the polypeptide and not the N-terminal region as hypothesized. The reduced antimicrobial activity of YpkK[Val88–Trp164] compared with corynicin JK also indicates that the N-terminal portion is at least indirectly involved in biological activity. Since the N- and C-terminal segments have opposite (and therefore attractive) pI, it is possible that the N-terminal segment interacts with and stabilizes the C-terminal portion or that it interacts with a possible ‘receptor’, thereby better localizing the C-terminal region to its target.

In order to gain further understanding of the molecular architecture of these antibiotic proteins, we also constructed expression systems for six chimaeras incorporating each of the three N-terminal portions fused to each possible C-terminal segment (Table 2). These experiments were based on the premise that if target specificity is defined by the N-terminal portion, then swapping potential ‘domains’ might alter the spectrum of activity of a given C-terminal segment and further clarify the role of the individual N-terminal portions. Surprisingly, only two of these recombinant chimaeras, namely ScnM57[Lys28–Lys105]/EF1097[Val94–Ser170] and ScnM57[Lys28–Lys105]/YpkK[Val88–Trp164], were expressed in the heterologous host as judged by SDS-PAGE. Again, the reasons for the failure of the remaining four to express in *E. coli* were not further investigated, but in this case it did not appear to involve host toxicity as the host grew as well as controls expressing full-length constructs during the expression phase. Both expressed chimaeras were isolated and analytically characterized to confirm their

Table 4. Effect on mass and antimicrobial activity of exposure of recombinant SAM-57, EF1097 and YpkK to the *S*-alkylating agent 4-vinylpyridine, with or without prior reduction by 2-mercaptoethanol

Treatment	SA-M57		Enterococcin V583		Corynicin JK	
	Mass (Da)	MIC (nM)*	Mass (Da)	MIC (nM)	Mass (Da)	MIC (nM)
None (control)	16 876 ± 7	64	14 832 ± 2	4	14 436 ± 2	2
4-Vinylpyridine	16 879 ± 4	64	14 833 ± 5	4	14 439 ± 7	2
2-Mercaptoethanol/ 4-vinylpyridine†	17 085 ± 4	>1024	15 044 ± 6	>1024	14 649 ± 7	>1024

*MIC was determined against *Lc. lactis* strain T-21 (Tagg & Bannister, 1979).

†Samples were treated with 2-mercaptoethanol for 1 h at room temperature prior to S-alkylation.

Fig. 2. Comparison of the killing action of corynicin JK and YpkK[Val88–Trp164]. (A) Both recombinant corynicin JK and YpkK [Val88–Trp164] are biologically active against *Micrococcus luteus* in a spot test assay: a, 10 µg purified recombinant corynicin JK; b, 10 µg purified recombinant YpkK[Val88–Trp164]; c, 10 µg purified recombinant YpkK[Ala28–Ser87]. (B) Corynicin JK and YpkK[Val88–Trp164] inhibit the growth of *Lc. lactis* T-21 in liquid culture. *Lc. lactis* T-21 was treated at time 0 with: water (untreated control, ○); 10 nM corynicin JK (●); 60 nM YpkK[Val88–Trp164] (□); or 1 mg lysozyme ml⁻¹ (lysis control, ■). (C) Corynicin JK and YpkK[Val88–Trp164] do not lyse heat-killed *Lc. lactis* T-21 in suspension. Heat-killed *Lc. lactis* T-21 was treated at time 0 with: water (untreated control, ○); 10 nM corynicin JK (●); 60 nM YpkK[Val88–Trp164] (□); or 2 mg lysozyme ml⁻¹ (lysis control, ■).

identity, and their potential antimicrobial activity was assessed. Surprisingly, neither showed any detectable antibacterial activity against any of the indicator strains tested. This result could indicate that there is an intimate and customized association between the N- and C-terminal

segments of these antibiotics that is critical for full antimicrobial activity.

SA-M57, enterococcin V583, corynicin JK and YpkK[Val88–Trp164] kill sensitive bacteria without lysis

A small number of high molecular mass bacteriocins have been reported that lyse susceptible bacteria. For example, the glycyl-glycine endopeptidase lysostaphin from *Staphylococcus simulans* hydrolyses the transpeptide bridges of certain staphylococcal cell walls (Schindler & Schuhardt, 1964; Zygmunt & Tavormina, 1972), whereas the bacteriocins zoocin A from *Streptococcus zooepidemicus* (Simmonds *et al.*, 1996, 1997) and stellalysin from *Streptococcus constellatus* (Heng *et al.*, 2006b) are thought to act in a similar manner against various susceptible streptococci. In addition, the cyclic antimicrobial peptide uberolysin from various *Streptococcus uberis* strains kills susceptible cells by inducing their lysis (Wirawan *et al.*, 2007). By contrast, we have previously shown that the 21.5 kDa antibiotic protein dysgalactacin kills susceptible cells without lysis (Heng *et al.*, 2006). In the present study we assessed the killing action of SA-M57, enterococcin V583, corynicin JK and YpkK[Val 88–Trp164]. When the proteins were applied to agar-immobilized cell suspensions (Wirawan *et al.*, 2007) of a variety of either live or heat-killed sensitive and non-sensitive indicator strains, we observed no indications of target cell lysis, although further growth of live, sensitive indicators was inhibited as expected. Moreover, addition of either 20 nM corynicin JK or 60 nM YpkK[Val88–Trp164] to a mid-exponential-phase liquid culture of *Lc. lactis* T-21 inhibited further growth but did not result in a decrease in optical density (Fig. 2B). Similarly, the optical density of a heat-killed suspension of *Lc. lactis* T-21 did not change after treatment with either corynicin JK or YpkK[Val 88–Trp164] at the same concentrations (Fig. 2C). In both cases, controls consisting of lysozyme-treated *Lc. lactis* T-21 showed characteristic loss of optical density, consistent with the mureolytic mode of action of this enzyme. Taken together these results indicate that the tested proteins are neither mureolytic enzymes nor mediators of lysis induction in sensitive cells; however, the mechanism by which they kill susceptible bacteria, like the basis for their target specificity, remains enigmatic.

Distribution of the genes encoding SA-M57, enterococcin V583 and corynicin JK

SA-M57 has been previously reported to be widely distributed amongst M type 57 *Streptococcus pyogenes*, as both the gene (*scnM57*) and plasmid on which it is carried (pDN571), as well as biologically active SA-M57, were identified in all 37 clinical isolates tested (Heng *et al.*, 2004). In contrast, PCR screening of 33 independent clinical isolates of *C. jeikeium* identified *ypkK* in just two strains, indicating a relatively restricted distribution of this

gene in corynebacterial isolates. This finding is surprising given that the plasmid on which *ypkK* may be carried (pK43-like family) is common among clinical isolates (Tauch *et al.*, 2004). Furthermore, antibacterial activity consistent with corynicin JK could not be detected from *C. jeikeium* K64 in a modified deferred antagonism test, raising the possibility that *ypkK* either is not expressed in this strain, or is only expressed under as yet undefined culture conditions.

Using a diagnostic PCR with appropriate primers (Table 1) we have screened 18 independent isolates and found *efl097* in 10 of 11 strains of *Ent. faecalis* (including the genome sequence reference strain *Ent. faecalis* V583: Paulsen *et al.*, 2003) and four of seven strains of *Ent. faecium*. This result would indicate that, like *scnM57*, *efl097* is widely distributed amongst natural isolates of enterococci. Moreover, antibacterial activity consistent with enterococin V583 could also be detected from *Ent. faecalis* V583 in a modified deferred antagonism test, confirming that the gene is expressed, at least in this strain. In a recent study (Bourgogne *et al.*, 2006), the FsrABC system of enterococci, which is a quorum-sensing regulator of enterococcal virulence (Qin *et al.*, 2000, 2001), was shown to upregulate *efl097* expression (at the level of mRNA) upon entry into stationary phase. The broad distribution of *efl097* in enterococci, coupled with the observation that its expression is tightly regulated together with known virulence factors, may indicate that it is of importance to the producing bacterium and could also indicate that its production is related to enterococcal virulence.

SA-M57, enterococin V583 and corynicin JK may be part of a broader family of antibiotic proteins from Gram-positive bacteria

Interestingly, recent interrogation of currently available databases would suggest that at least four further potential family members may also be currently documented as proteins of unknown function: the hypothetical proteins BCZK5154 from *Bacillus cereus* E33L (accession no. YP_086722; Han *et al.*, 2006), BcerKBAB4DRAFT_0813 from *Bacillus weihenstephanensis* KBAB4 (accession no. ZP_01187444; A. Lapidus and others, 2006, DOE Joint Genome Institute, unpublished) and BL05338 from *Bacillus licheniformis* ATCC 14580 (accession no. YP_080680; Rey *et al.*, 2004), as well as the putative 'integral membrane protein' from *Lactobacillus plantarum* WCFS1 (accession no. NP_786248; Kleerebezem *et al.*, 2004). Although they share little sequence similarity, *in silico* analysis of these four sequences (not shown) indicates that their translated products are all likely to yield mature proteins of approximately 150 amino acids following cleavage of ~30 amino acid signal peptides. Moreover, the predicted proteins are composed of two distinct portions, the C-terminal segments of which share similar predicted secondary structure and contain two conserved

cysteine residues. Thus, it would seem that the hitherto unrecognized ability of Gram-positive bacteria to produce large proteinaceous antibiotics (bacteriocins) is relatively widely distributed. Indeed, it is tempting to speculate that this family of protein antibiotics may represent the Gram-positive equivalent of the colicin family in Gram-negative bacteria (Cascales *et al.*, 2007).

Conclusion

We have demonstrated that the hypothetical proteins Efl097 and YpkK previously identified in the genome sequences of *Ent. faecalis* and *C. jeikeium* (Paulsen *et al.*, 2003; Tauch *et al.*, 2004) are precursors of antimicrobial proteins with similarities to the bacteriocins streptococin A-M57 (Heng *et al.*, 2004) and dysgalactin (Heng *et al.*, 2006). We have also shown that dysgalactin, SA-M57, enterococin V583 and corynicin JK may form (part of) a family of antibiotic proteins from Gram-positive bacteria that share little sequence similarity whilst maintaining conserved secondary structural elements. Information gained from the variety of recombinant protein segments and chimaeras used in this study indicates that the conserved secondary structure of the C-terminal portion of this family of protein antibiotics may be critical in mediating their killing activity and that target cell death does not involve lysis. Thus, our future studies will be directed at dissecting the basis for their target specificity and defining the mechanism by which the proteins kill susceptible cells.

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