Dysgalacticin: a novel, plasmid-encoded antimicrobial protein (bacteriocin) produced by *Streptococcus dysgalactiae* subsp. *equisimilis*

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Dysgalacticin is a novel bacteriocin produced by *Streptococcus dysgalactiae* subsp. *equisimilis* strain W2580 that has a narrow spectrum of antimicrobial activity directed primarily against the principal human streptococcal pathogen *Streptococcus pyogenes*. Unlike many previously described bacteriocins of Gram-positive bacteria, dysgalacticin is a heat-labile 21·5 kDa anionic protein that kills its target without inducing lysis. The N-terminal amino acid sequence of dysgalacticin [Asn-Glu-Thr-Asn-Asn-Phe-Ala-Glu-Thr-Gln-Lys-Glu-Ile-Thr-Thr-Asn-(Asn)-Glu-Ala] has no known homologue in publicly available sequence databases. The dysgalacticin structural gene, *dysA*, is located on the indigenous plasmid pW2580 of strain W2580 and encodes a 220 aa preprotein which is probably exported via a Sec-dependent transport system. Natural *dysA* variants containing conservative amino acid substitutions were also detected by sequence analyses of *dysA* elements from *S. dysgalactiae* strains displaying W2580-like inhibitory profiles. Production of recombinant dysgalacticin by *Escherichia coli* confirmed that this protein is solely responsible for the inhibitory activity exhibited by strain W2580. A combination of *in silico* secondary structure prediction and reductive alkylation was employed to demonstrate that dysgalacticin has a novel structure containing a disulphide bond essential for its biological activity. Moreover, dysgalacticin displays similarity in predicted secondary structure (but not primary amino acid sequence or inhibitory spectrum) with another plasmid-encoded streptococcal bacteriocin, streptococcin A-M57 from *S. pyogenes*, indicating that dysgalacticin represents a prototype of a new class of antimicrobial proteins.

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INTRODUCTION

Numerous genera of Eubacteria and Archaea produce proteinaceous antimicrobial substances known as bacteriocins that target related species, presumably to provide the producing organism with an ecological advantage in its micro-environment (Riley & Gordon, 1999; Riley & Wertz, 2002). Broadly speaking, bacteriocins fall into one of four main categories, depending on whether they are produced by (and therefore act primarily on) Gram-positive or Gram-negative bacteria, and whether they are of high (> 10 kDa) or relatively low molecular mass (< 10 kDa). There is a wealth of scientific literature concerning bacteriocins from three of these categories: (i) the > 20 kDa bacteriocins of Gram-negative bacteria, epitomized by the plasmid-encoded colicins from *Escherichia coli* (Gillor *et al.*, 2004; Kirkup & Riley, 2004); the < 10 kDa antimicrobial peptides or microcins produced by Gram-negative species (Gillor *et al.*, 2004); and (iii) the low-molecular-mass bacteriocins of Gram-positive

bacteria (reviewed by Jack *et al.*, 1998; Eijsink *et al.*, 2002; Fimland *et al.*, 2005; Cotter *et al.*, 2005). However, comparatively little is known about the colicin equivalents of Gram-positive bacteria, since reports of high-molecular-mass bacteriocins from such bacteria are scarce, the most extensively studied being the peptidoglycan-hydrolysing enzyme lysostaphin from *Staphylococcus simulans* (reviewed by Navarre & Schneewind, 1999).

Amongst the Gram-positive bacteria, the lactic acid bacteria are especially prodigious bacteriocin producers. Indeed, in recent years there has been a seemingly exponential increase in reports of novel inhibitory substances from these organisms (Papagianni, 2003; Fimland *et al.*, 2005). Typically, these bacteriocins are ribosomally synthesized peptides that are characterized by their small size (generally < 6 kDa) and heat stability (Jack *et al.*, 1995). On further investigation, some of these have proven to be extensively post-translationally modified, e.g. the lanthionine-containing bacteriocins, or 'lantibiotics' (Schnell *et al.*, 1988; Sahl & Bierbaum, 1998), while others remain unmodified (Chatterjee *et al.*, 2005; Fimland *et al.*, 2005).

The GenBank/EMBL/DDBJ accession number for the pW2580 sequence determined in this paper is AY907345.

Various species of the genus *Streptococcus* commonly produce bacteriocins, and those best characterized to date are from *Streptococcus pyogenes* (group A streptococcus), *Streptococcus salivarius* and *Streptococcus mutans* (Jack *et al.*, 1995, 1998; Chatterjee *et al.*, 2005), e.g. the lantibiotics streptococcin A-FF22 and salivaricin A (Ross *et al.*, 1993; Jack *et al.*, 1994), and the unmodified peptides mutacin N and mutacin IV (Balakrishnan *et al.*, 2000; Qi *et al.*, 2001). However, several relatively large, heat-labile bacteriocins have also been described, including zoocin A from *Streptococcus equi* subsp. *zooepidemicus*, millericin B from *Streptococcus milleri*, and streptococcin A-M57 from *S. pyogenes* (Simmonds *et al.*, 1996; Beukes *et al.*, 2000; Heng *et al.*, 2004).

In an effort to categorize streptococcal bacteriocin producer strains, a bacteriocin typing (or bacteriocin fingerprinting) scheme has been devised to discriminate inhibitory strains on the basis of differences in their inhibitory activity against a set of standard indicator bacteria (Tagg & Bannister, 1979). Using this scheme, a set of nine so-called standard producer strains with distinctive inhibitory spectra were defined (Tagg & Bannister, 1979). One of these standard producer strains, Lancefield Group G streptococcus W2580 (standard producer P4), exhibits a narrow spectrum of inhibitory activity directed almost exclusively against the large-colony β -haemolytic streptococci (Lancefield Groups A, C and G), the principal streptococcal pathogens of humans (Tagg & Bannister, 1979; Wong, 1981; Wong *et al.*, 1981; Tagg & Wong, 1983), but not against a range of oral streptococci, neisseriae, lactobacilli, *Corynebacterium diphtheriae*, *Bacillus* spp., *Listeria* spp., staphylococci, and various Gram-negative bacteria (Wong, 1981; H. J. Baird, unpublished results). Preliminary studies of the inhibitory agent from strain W2580 (originally designated streptococcin G-2580) revealed that (i) it was inactivated both by treatment with trypsin and by heating, indicating that it may be essentially proteinaceous in nature; and (ii) its elution profile during gel-permeation chromatography was consistent with that of a protein of molecular mass

~18 kDa (Wong, 1981; Wong *et al.*, 1981). Moreover, *S. pyogenes* treated with partially purified preparations of streptococcin G-2580 was shown to be killed without lysis (Wong, 1981; Wong *et al.*, 1981). This mode of action is in direct contrast to that of other large streptococcal bacteriocins such as zoocin A, which lyse target cells via proteolytic cleavage of peptidoglycan (Simmonds *et al.*, 1996). Strain W2580 has since been identified by 16S rDNA sequencing as *Streptococcus dysgalactiae* subsp. *equisimilis* (N. L. Ragland, unpublished results) and, consequently, its bacteriocin product (streptococcin G-2580) has been renamed dysgalacticin.

In this study, we have purified dysgalacticin to homogeneity, biochemically characterized the bacteriocin and determined the genetic basis for its production in strain W2580. We show that the bacteriocin is plasmid-encoded and, following heterologous expression in *E. coli*, confirm that purified recombinant dysgalacticin is solely responsible for the antimicrobial phenotype exhibited by strain W2580, and that it has a non-lytic mode of action. Furthermore, we demonstrate that dysgalacticin possesses a novel predicted secondary structure containing a single disulphide bond that is essential for its biological activity.

METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. Streptococcal strains were routinely cultivated at 37 °C in a microaerophilic (5 % CO₂ in air) atmosphere on BACa medium [Columbia blood agar base (Becton Dickinson) containing 4 % (v/v) human blood and 0.1 % (w/v) CaCO₃] or in Todd-Hewitt broth (THB; Becton Dickinson). *Escherichia coli* DH5 α (Hanahan, 1983), the host for all cloning and expression experiments, was propagated aerobically at 37 °C in LB broth or on LB agar (Sambrook & Russell, 2001). When required, antibiotics were added to the media at the following final concentrations: ampicillin, 100 μ g ml⁻¹; chloramphenicol, 15 μ g ml⁻¹.

Purification of dysgalacticin. The inhibitory activity produced by *S. dysgalactiae* strain W2580 was purified to homogeneity from a total of 4 l supernatant fluid from cultures grown in THB at 35 °C,

Table 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics	Source or reference
<i>Streptococcus dysgalactiae</i> subsp. <i>equisimilis</i>		
W2580	Dysgalacticin producer strain	Wong <i>et al.</i> (1981)
W2580C	Plasmid-cured derivative of W2580	This study
<i>Streptococcus pyogenes</i> FF22	Dysgalactacin-sensitive indicator strain	Tagg & Bannister (1979)
<i>Escherichia coli</i>		
DH5 α	Cloning and expression host	Hanahan (1983)
QE-dysA ^M	DH5 α carrying pQE-dysA	This study
Plasmids		
pW2580	Dysgalacticin-encoding indigenous plasmid of strain W2580	This study
pFX3	<i>E. coli</i> - <i>Lactococcus lactis</i> shuttle vector	Xu <i>et al.</i> (1991)
pQE-80L	<i>E. coli</i> expression vector	Qiagen
pQE-dysA ^M	pQE-80L containing the dysgalacticin expression cassette	This study

in a 2 l fermenter maintained at pH 6.5 by addition of 2 M NaOH. Briefly, the cells were removed by centrifugation, and protein in the supernatant was precipitated with ammonium sulphate (80% saturation) with gentle stirring for 18 h at 4 °C. The precipitated protein was harvested by centrifugation (12 000 g, 30 min, 4 °C) and the pellet was redissolved in 900 ml of 20 mM Tris/HCl (pH 8.0). This solution was then applied to an XK26 column (GE Healthcare Bio-Sciences) packed with 20 ml Q-MacroPrep (Bio-Rad), at a flow rate of 4 ml min⁻¹, using an ÄKTAexplorer (GE Healthcare Bio-Sciences). The column was washed with 15 bed volumes of 20 mM Tris/HCl (pH 8.0) and then developed in a linear gradient of 0–0.5 M NaCl in 20 mM Tris/HCl (pH 8.0). Fractions (12 ml) were collected and assessed for homogeneity by SDS-PAGE (see below), and biological activity was assayed as described by Wescombe & Tagg (2003) with *S. pyogenes* FF22 as the indicator strain. Suitable fractions were pooled and concentrated by membrane filtration (Microcon YM-10; Millipore), and then further fractionated by gel-permeation chromatography in 25 mM HEPES containing 150 mM NaCl (pH 7.2) through a Superdex 75 100/300 GL column (GE Healthcare Bio-Sciences) at a constant flow rate of 0.5 ml min⁻¹. Appropriate fractions (0.5 ml) containing inhibitory activity were pooled, buffer-exchanged with 5 mM HEPES (pH 7.2) and concentrated by membrane filtration (Microcon YM-10).

Bioanalytical characterization of dysgalactacin and recombinant proteins. The approximate mass and degree of homogeneity of various proteins was assessed by SDS-PAGE on pre-cast Novex 16% Tris/Tricine gels (Invitrogen) in an XCell SureLock Mini-Cell apparatus (Invitrogen), using the manufacturer's protocols and reagents. Following electrophoresis, protein bands were visualized with Coomassie Brilliant Blue G250 (Sigma). MALDI-TOF MS and N-terminal amino acid sequencing (by automated Edman degradation) of purified dysgalactacin were carried out at the Protein Microchemistry Facility (Department of Biochemistry, University of Otago). MS was conducted with a Finnigan LaserMAT 2000 (Thermo BioAnalysis) mass analyser, according to the method described by Hubbard & McHugh (1996). The N-terminal amino acid sequence of dysgalactacin was determined by automated, repetitive Edman degradation on a Procise model 492 pulsed liquid/gas-phase micro-sequencer (Applied Biosystems), using the manufacturer's reagents, protocols and analysis software.

Plasmid curing experiments. Plasmid-cured mutants of *S. dysgalactiae* strain W2580 were derived according to the method of Tagg & Wannamaker (1976) by growing the strain in THB containing doubling dilutions of novobiocin (1250 µg ml⁻¹ to 5 µg ml⁻¹) and 0.5 µg trypsin ml⁻¹ (to destroy any residual bacteriocin activity). A total of approximately 200 colonies were picked from cultures grown in the presence of 5 and 10 µg novobiocin ml⁻¹ and subjected to a deferred antagonism assay (Tagg & Wannamaker, 1976) using *S. pyogenes* FF22 as the indicator strain. Colonies displaying loss of inhibitory activity were then retested using a standard bacteriocin typing scheme (Tagg & Bannister, 1979). One plasmid-cured mutant, designated W2580C, which was indistinguishable from the wild-type strain by biochemical (API 20 Strep, bioMérieux) and serological (Oxoid Streptococcal Grouping Kit) testing, and by 16S rDNA sequencing (Wilson *et al.*, 1990), was selected for further study.

Recombinant DNA techniques, sequencing of pW2580 and bioinformatic tools. Genomic DNA from streptococcal strains, plasmid DNA (from *E. coli*) and PCR amplicons were purified using the appropriate Qiagen spin-column-based kits, according to the manufacturer's instructions. Plasmid DNA from *S. dysgalactiae* strain W2580 was obtained using the TempliPhi DNA amplification kit (Amersham Biosciences). Established protocols for molecular cloning in *E. coli* and genetic analysis (e.g. Southern hybridization) were conducted as described by Sambrook & Russell (2001). For

PCR experiments, the high-fidelity Platinum *Pfx* DNA polymerase (Invitrogen) was used according to the supplier's recommendations. All nucleotide sequencing requirements were met either by the Waikato DNA Sequencing Facility (Hamilton, New Zealand) or by the Allan Wilson Centre Genome Service (Palmerston North, New Zealand).

pW2580 was completely sequenced using the strategy described by Heng *et al.* (2004). After initial enrichment of pW2580 DNA using TempliPhi DNA polymerase, the 2.2 kb *Pst*I–*Xba*I and 1.8 kb *Eco*RI–*Xba*I plasmid-derived fragments were individually cloned into pFX3 (Xu *et al.*, 1991) and partially sequenced. The remainder of the plasmid sequence was obtained by PCR-based primer walking, using a total of six oligonucleotide primers (Table 2). Assembly and basic analyses of nucleotide sequence data were accomplished using the GeneJockey II software package (Biosoft). Homology searches were conducted with the appropriate BLAST algorithms (Altschul *et al.*, 1997). *In silico* predictions of (i) the secretion signal peptide and (ii) potential secondary structures of dysgalactacin were achieved using SignalP version 3.0 (Bendtsen *et al.*, 2004) and various algorithms (Rost, 1996; McGuffin *et al.*, 2000; Pollastri *et al.*, 2002; Vullo & Frasconi, 2004) available on the PredictProtein server (<http://predictprotein.org>; Rost *et al.*, 2004), respectively.

Expression and purification of recombinant dysgalactacin in *E. coli*. An expression cassette incorporating nucleotides encoding a short linker sequence (Gly-Ser-Gly-Ser) and a recognition site for factor Xa protease (Ile-Glu-Gly-Arg), followed by the segment of the *dysA* gene encoding the amino acid sequence of mature dysgalactacin (i.e. without the 28 aa leader peptide), was amplified by PCR using primers *DysA*-ExpF and *DysA*-ExpR (Table 2). Purified PCR products were then digested with *Bam*HI and *Hind*III, and inserted into the corresponding sites of the expression vector pQE-80L (Qiagen), yielding pQE-dysA^M (Table 1). The presence of the desired coding sequences was verified by sequencing of the cloned insert with primers QIAF and QIAR (Table 2). *E. coli* QE-dysA (DH5α carrying pQE-dysA^M) was grown in 2 × yeast tryptone (YT) broth (Sambrook & Russell, 2001) containing 0.5% (w/v) glucose to OD₅₉₀ 0.6, after which IPTG was added to a final concentration of 1 mM to induce expression. Following incubation for a further 4 h, cells were harvested by centrifugation (4000 g, 5 min), resuspended in one-thirtieth of their original volume of lysis buffer [50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 1 mg lysozyme ml⁻¹ (pH 8.0)] and disrupted by repetitive sonication (15 bursts of 30 s; Ultrasonics model W-220F). Cell debris was then removed by centrifugation (31 000 g, 30 min, 4 °C) and the supernatant (crude protein preparation) was subjected to further purification by chromatographic separation.

Recombinant dysgalactacin was purified by: (i) initial enrichment of hexahistidine-tagged recombinant dysgalactacin using nickel-nitriloacetate (Ni-NTA) agarose (Qiagen) under native (non-denaturing) conditions, according to the manufacturer's protocol; (ii) anion-exchange chromatography; (iii) removal of the hexahistidine tag by treatment with factor Xa protease (New England Biolabs) at a 50:1 substrate:enzyme ratio for 4 h at 22 °C; and (iv) gel-permeation chromatography. The anion-exchange and gel-permeation chromatographic steps were the same as those used for the purification of native dysgalactacin, except that the anion-exchange buffers contained 2 mM CaCl₂ to facilitate factor Xa digestion without the need for buffer exchange. The purity of recombinant protein was assessed by SDS-PAGE.

Preliminary mode-of-action studies. In order to ascertain whether purified recombinant dysgalactacin kills target cells by lysis, we tested the effect of dysgalactacin on actively growing *S. pyogenes* strain FF22 by measuring changes in OD₅₉₀ and cell viability, and compared this with cells treated with the known bacteriolytic agent

Table 2. Oligonucleotides used in this study

Oligonucleotide	Nucleotide sequence (5'→3')	Purpose
OP4-A	5'-AA(C/T)GAAAC(A/T)AA(C/T)AA(C/T)TTTGC-3'	Probe for dysgalactacin structural gene
P4-P1	5'-ATAACTACGATGATGAAAGTATAAAAGGGG-3'	Sequencing of pW2580
P4-P2	5'-GCGTAAACAAGTCTGGAAGTGTACTTGAA-3'	Sequencing of pW2580
P4-PR1	5'-TGCTCTATCCAGAATCAATCCCGAACGATTG-3'	Sequencing of pW2580
P4-PR2	5'-CCATAGTAGCACAAATACGCATTGAATGCA-3'	Sequencing of pW2580
P4-PR3	5'-GTTATTTGTCGCCCTTTGTTTGGCCACATGCG-3'	Sequencing of pW2580
P4-PR4	5'-GGTGATACTTGCTTTGTCTCGGTCGGTCGAACAA-3'	Sequencing of pW2580
DysA-ExpF*	5'-CAGGATCCGGCAGCATTGAAGGCCGTAATGAAACAA ATAACTTTGCAGAACTCAAAAAG-3'	Dysgalactacin expression cassette forward primer
DysA-ExpR	5'-GCGTAGAAAAAGAGGTGGTGAAATGGATAGATACG-3'	Dysgalactacin expression cassette reverse primer
QIAF	5'-CCCGAAAAGTGCCACCTG-3'	Forward sequencing primer for pQE-dysA*
QIAR	5'-ATTGGGATATATCAACGGTGG-3'	Reverse sequencing primer for pQE-dysA*
DysA-intF	5'-GGAAATGACAAGTAGTATAATGAAAGCGGA-3'	<i>dysA</i> internal forward primer
DysA-intR	5'-TCGTTCAAACATCTTTCATGAGATAAACCC-3'	<i>dysA</i> internal reverse primer
DysA-UpF†	5'-GCTTAGTTGAATTCCATATTGCGACTA-3'	Amplification of <i>dysA</i> locus
RepB-R†	5'-CGTATCTATCCATTTCCGCCACCTCTTTTCTACGC-3'	<i>repB</i> -specific primer

*The nucleotides in italics encode the linker-factor Xa cleavage site (NH₂-GSGSIEGR-COOH; see text). The *Bam*HI restriction site used for cloning into pQE-80L is underlined.

†The partners for the DysA-UpF and RepB-R primers during PCR were DysA-ExpR and P4-PR1, respectively.

zoocin A (Simmonds *et al.*, 1996). Briefly, 50 ml pre-warmed THB broth was inoculated with 50 µl overnight culture of *S. pyogenes* strain FF22 and incubated at 37 °C without shaking. Samples were removed every hour for 4 h, after which, the culture was divided into three aliquots: the first was treated with the addition of 80 nM recombinant dysgalactacin, the second was treated with 10 nM purified recombinant zoocin A (kindly provided by R. Simmonds, University of Otago), and the third was untreated (negative control). Samples were then removed from each of the three aliquots after 10, 20, 40, 60 and 120 min. The OD₅₉₀ was measured using a Novaspec-II spectrophotometer (Biochrom). Bacterial viability, expressed as c.f.u. ml⁻¹, was assessed by spotting (in triplicate) 5 µl aliquots of serial dilutions (100 µl samples in 900 µl fresh THB) on blood agar plates. Following overnight incubation at 37 °C, colonies were enumerated.

Reductive alkylation of dysgalactacin. In order to determine the oxidation state of the predicted cysteine residues of dysgalactacin, alkylation in the absence and presence of a reducing agent was carried out essentially as previously described (Jack *et al.*, 1996). Briefly, recombinant dysgalactacin (~3 µmol) was dissolved in 120 µl TA buffer [100 mM Tris/HCl (pH 8.0) containing 30 % (v/v) acetonitrile] and the sample was divided into three equal aliquots. The reducing agent 2-mercaptoethanol (Sigma) was then added in fivefold molar excess to one aliquot, and an equivalent volume of TA buffer was added to the remaining two portions of protein. After the samples had been mixed by gentle vortexing, they were incubated at room temperature for 1 h. Subsequently, 4-vinylpyridine (Sigma) was added in 20-fold molar excess to the reduced and one additional aliquot; an equivalent volume of TA buffer was added to the third portion and served as an untreated control. Following mixing, all samples were incubated for 2 h at room temperature. All three samples were desalted by gel-permeation chromatography in 5 mM HEPES (pH 7.2) on a 5 ml HiTrap desalting column (GE Healthcare Bio-Sciences), according to the manufacturer's instructions, and then assayed for antimicrobial activity (see above). Appropriate fractions were also analysed by MALDI-TOF MS to confirm incorporation of the 4-vinylpyridine moiety.

RESULTS AND DISCUSSION

Purification and N-terminal amino acid sequence of dysgalactacin

The antimicrobial activity elaborated by *S. dysgalactiae* strain W2580 was purified to apparent homogeneity by ammonium sulphate precipitation followed by anion-exchange and size-exclusion chromatography to yield a protein of ~22 kDa as judged by SDS-PAGE (data not shown). MALDI-TOF MS analysis revealed a single product with a mass of 21 492.5 ± 19.3 Da, and N-terminal amino acid sequence analysis by automated Edman degradation generated the single sequence NETNNFAETQKEITT(N)-EA. A BLASTP search of publicly available sequence databases revealed no significant similarity between the N-terminal sequence of dysgalactacin and those of proteins of either known or unknown function, indicating that dysgalactacin is a novel antimicrobial agent.

The dysgalactacin structural gene is plasmid-encoded

In order to localize the dysgalactacin structural gene, oligonucleotide OP4-A (Table 2), synthesized based on the first seven amino acid residues of dysgalactacin, was used as a probe in Southern hybridization analyses. When *Eco*RI-digested genomic DNA of strain W2580 was probed, OP4-A hybridized not only to a fragment of ~3.0 kb but also to a band migrating below that of chromosomal DNA in an undigested sample (data not shown), indicating an extra-chromosomal location for the dysgalactacin structural gene. Furthermore, when W2580 genomic DNA treated

with TempliPhi DNA polymerase (which preferentially amplifies plasmid DNA as concatemers) was subjected to restriction endonuclease analysis (yielding unit-length plasmid fragments), a single ~ 3.1 kb band was observed by agarose gel electrophoresis (data not shown). Recognition sites for the restriction enzymes *EcoRI*, *PstI* and *HindIII*, but not *BamHI*, were present in the plasmid (Fig. 1).

In order to further verify that the antimicrobial phenotype of *S. dysgalactiae* W2580 is plasmid-encoded, the strain was grown in the presence of the plasmid-curing agent novobiocin. Using a colony-based deferred-antagonism assay against the indicator *S. pyogenes* FF22, a bacteriocin-negative derivative (*S. dysgalactiae* W2580C) that did not exhibit any antimicrobial activity against a standard set of indicator strains (Tagg & Bannister, 1979) was isolated following novobiocin treatment. Together, these results demonstrate that the genetic determinant responsible for dysgalactacin production is located on the indigenous plasmid of *S. dysgalactiae* W2580, designated pW2580.

Nucleotide sequence analysis of pW2580 and identification of *dysA*, the dysgalactacin structural gene

The complete nucleotide sequence of pW2580 was determined and totalled 3043 bp with a mol% G + C content of 35 mol%, slightly lower than the 39–41 mol% determined

for *S. dysgalactiae* (Hardie, 1986). To the best of our knowledge, pW2580 is the first plasmid from this organism to be completely sequenced. We arbitrarily assigned the first nucleotide of the unique *EcoRI* site (Fig. 1) as the starting point for plasmid annotation. BLAST searches identified four genetic elements (Fig. 1), all associated with plasmid replication, and which classify pW2580 as a new member of the pMV158 rolling-circle plasmid family (Moscoso *et al.*, 1995): (i) *copG* (nt 86–220) encodes a putative 5 kDa CopG repressor protein, probably responsible for maintaining plasmid copy number; (ii) *repB* (nt 283–906) which specifies the 24 kDa RepB replication initiation protein; (iii) *dso* (nt 2893–2991) or double-strand origin of replication, which is nicked by RepB during initiation of plasmid replication; and (iv) *sso* (nt 2394–2567) or single-strand (lagging-strand) origin, essential for conversion of the ssDNA intermediate to its double-stranded form (del Solar *et al.*, 1998; Khan, 2005). The putative *sso* of pW2580 contains a highly conserved 14 bp sequence, termed RS_B (5'-TTTA-TGCCGTGAAA-3'; nt 2399–2412) that is believed to be the site of interaction with RNA polymerase during initiation of lagging-strand synthesis (Kramer *et al.*, 1998; Khan, 2005). The RS_B is also a distinctive feature of *ssoA*, the most commonly encountered *sso* in rolling-circle plasmids isolated from Gram-positive species, irrespective of plasmid family (Khan, 2005). Each component of the replication machinery of pW2580 displays significant homology (between 86 and 97% identity) with its respective counterpart from the prototype of the plasmid family, pMV158 (Moscoso *et al.*, 1995), as well as other streptococcal members of the pMV158 family, including pSSU1 from *Streptococcus suis* (Takamatsu *et al.*, 2000), and pER13 and pSMQ172 from *Streptococcus thermophilus* (Solow & Somkuti, 2000; Turgeon & Moineau, 2000).

The dysgalactacin structural gene, designated *dysA*, was located between nt 1102 and 1764 but in the opposite transcriptional orientation with respect to the other ORFs identified in pW2580 (Fig. 1). The *dysA* gene, which encodes the 220 aa (24.5 kDa) dysgalactacin preprotein DysA, was flanked (i) upstream by a potential promoter consisting of hexanucleotide motifs resembling -35 and -10 regions (separated by a 17 bp spacer), and (ii) downstream by an inverted repeat sequence that could function as a rho-independent transcriptional terminator (Fig. 2). As anticipated, DysA contained the sequence obtained by N-terminal sequencing of dysgalactacin (Fig. 2). However, the first amino acid of mature dysgalactacin corresponds to the twenty-ninth amino acid of DysA, indicating that the first 28 aa may constitute a secretion signal peptide. This finding was not unexpected, as all streptococcal bacteriocins characterized to date are exported either by dedicated ATP-binding cassette transporters, e.g. streptin (Wescombe & Tagg, 2003) and the non-lantibiotic mutacins (Hale *et al.*, 2005), or by the general secretory (Sec-dependent) pathway, e.g. zoocin A (Simmonds *et al.*, 1997). Indeed, the putative signal peptide of DysA displayed the typical hallmarks of proteins secreted by the Sec-dependent translocation

Fig. 1. Genetic map of pW2580. Numbering of the nucleotide sequence starts from the unique *EcoRI* site. The various genetic features and their respective transcriptional orientations are depicted with the replication-associated elements coloured black. Restriction endonuclease sites relevant to the preliminary sequencing experiments (see text) are also shown. *copG*, copy control protein; *repB*, replication initiation protein; *dysA*, dysgalactacin preprotein; *sso*, single-strand origin of replication; *dso*, double-strand origin of replication.

nt		aa
	GCGACTATTTTTGTGCCTTCCGCTAAGTCATTATTTGTTGGATAAAAC	
	-35 -10	
	CTTAAATAATAGACATCGGAAATGACAAGTAGTATAATGAAAGCGG	
	ATTCATATATAAAATATTTAGGTTAGAAAGGAAATAATCATAAA	
	TCCTAAATATAACAATTACCAAAAAACACAGGAAGGAAAGCAGTTT	
	M K K L K R L V I S L V	-17
1764	ATG AAA AAA TTA AAA CGT CTT GTT ATC TCT CTT GTT	
	T S L L V I S S T V P A	-5
1728	ACT TCA TTA CTA GTA ATT TCA AGT ACA GTT CCA GCA	
	-1 +1	
	L V Y A N E T N N F A E	+8
1692	CTT GTT TAC GCT AAT GAA ACA AAT AAC TTT GCA GAA	
	<u>T Q K E I T T N S E A T</u>	+20
1656	ACT CAA AAA GAA ATT ACA ACA AAT TCA GAA GCA ACA	
	L T N E D Y T K L T S E	+32
1620	TTA ACC AAT GAA GAC TAC ACT AAA TTA ACT TCC GAA	
	<u>V K T I Y T N L I Q Y D</u>	+44
1584	GTA AAA ACA ATT TAT ACA AAT CTG ATT CAA TAC GAC	
	Q T K N K F Y V D E D K	+56
1548	CAA ACA AAA AAC AAA TTT TAC GTC GAT GAA GAC AAA	
	T E Q Y Y N Y D D E S I	+68
1512	ACT GAA CAA TAT TAT AAC TAC GAT GAT GAA AGT ATA	
	K G V Y L M L D S L N D	+80
1476	AAA GGG GTT TAT CTC ATG AAA GAT AGT TTG AAC GAT	
	E L N N N N S S N Y S E	+92
1440	GAG TTA AAC AAT AAT AAC TCT TCA AAC TAT TCT GAA	
	<u>I I N O K I S E I D Y V</u>	+104
1404	ATA ATT AAT CAA AAA ATC TCT GAA ATT GAC TAT GTC	
	L Q G N D I N N L I P S	+116
1368	CTT CAA GGA AAC GAT ATA AAT AAT TTA ATT CCT AGC	
	N T R V K R S A D F S W	+128
1332	AAT ACC AGA GTA AAA AGA TCA GCA GAT TTT TCT TGG	
	<u>I Q R C L E E A W G Y A</u>	+140
1296	ATT CAA AGA TGT CTA GAA GAA GCA TGG GGA TAT GCT	
	<u>I S L V T L K G I L N L</u>	+152
1260	ATT AGT CTA GTT ACT CTA AAA GGA ATA ATC AAT CTA	
	<u>F K A G K F E A A A K</u>	+164
1224	TTT AAA GCA GGA AAA TTT GAA GCT GCT GCT GCT AAA	
	<u>I A S A T A G R I A G M</u>	+176
1188	TTA GCT TCT GCT ACA GCA GGT AGA ATC GCT GGA ATG	
	<u>A A L F A F V A T C G A</u>	+188
1152	GCT GCC TTA TTT GCT TTC GTA GCA ACT TGC GGT GCG	
	T T V S *	+192
1116	ACA ACT GTA TCA TAA AAATAAGGAAGTCTATCTGAGTTCCCTT	
1074	TATTTTTTTTTGAAATTATAGTAAGTCTCAAAAATATCATAAATAAC	
1027	TAACAAAGTACCTAAACTATAAAAAATAAATTTATAGTTTAAATTTT	

Fig. 2. Nucleotide sequence of the *dysA* locus. The numbering of the sequence is in reverse order due to the transcriptional orientation of the locus. The hexanucleotide motifs TAGACA and TATAAT which could function as -35 and -10 promoter sequences, respectively, are depicted by the raised text. The putative ribosome-binding site is double-underlined. The deduced amino acid sequence of the dysgalactacin preprotein, DysA, is numbered with negative and positive values assigned to the secretion signal peptide and mature bacteriocin, respectively. The N-terminal amino acid sequence obtained from automated Edman degradation is underlined and the putative signal peptidase cleavage site is indicated by the inverted arrow. The amino acid tracts predicted to form helical motifs (see text) are highlighted by the dotted underlining. The two cysteine residues which form the disulphide bond essential for biological activity are circled. The putative rho-independent transcription terminator is delineated by the facing arrows.

pathway (Tjalsma *et al.*, 2004; van Roosmalen *et al.*, 2004) including: (i) several positively charged amino acids at the N-terminal (N-domain) end; (ii) a central core (H-domain) of primarily hydrophobic amino acids; and (iii) a C-terminal (C-domain) portion that contains a conserved proline residue at position -6 (Fig. 2). These observations were in perfect agreement with the results independently obtained using the SignalP signal peptide prediction algorithm (Bendtsen *et al.*, 2004).

The 192 residue deduced primary amino acid sequence of dysgalactacin had a calculated molecular mass of 21 504.4 Da, which corresponds very well to that obtained for the dysgalactacin molecule by MALDI-TOF MS

(21 492.5 $\pm$ 19.3 Da), indicating that dysgalactacin is probably not further modified post-translationally. Furthermore, dysgalactacin had a predicted pI_{calc} of 4.67, a characteristic consistent with the experimental parameters used to effect purification of the protein. BLAST homology searches did not reveal any significant similarity between the amino acid sequence of dysgalactacin and those of proteins of known function, or to any other previously detected hypothetical proteins.

In addition to the bacteriocin structural gene, most (if not all) bacteriocin-associated genetic loci usually contain genes that encode immunity factors conferring producer self-protection. For example, *zif*, which encodes the zoocin A

immunity factor, is located adjacent to *zooA*, the zoocin A structural gene (Simmonds *et al.*, 1997). Interestingly, the pW2580-free derivative, *S. dysgalactiae* strain W2580C, was not only defective in its ability to produce dysgalactacin, but also sensitive to the effects of the bacteriocin (either in a deferred-antagonism assay or with exogenously added inhibitor), implying that the gene(s) encoding the dysgalactacin immunity factor is (are) also plasmid-borne. However, as pW2580 appeared to contain only *dysA* and those genetic elements necessary for its replication, the mechanism of immunity to dysgalactacin may be atypical and thus remains enigmatic.

Detection of *dysA* variants in *S. dysgalactiae* subsp. *equisimilis*

Forty-two presumptive *S. dysgalactiae* strains available in our culture collection, which were isolated from clinical sources (Dunedin Public Hospital and the University of Minnesota, Minneapolis, MN, USA) and from Dunedin schoolchildren (Ragland & Tagg, 1990) were screened for the production of inhibitory activity using a standard deferred-antagonism protocol (Tagg & Bannister, 1979). Of these, nine exhibited inhibitory activity similar to that of strain W2580, whilst the remainder were non-inhibitory. All 42 strains were subjected to PCR analyses to detect the presence of *dysA* or pW2580-like plasmids using the following primer pairs (Table 2): (i) *DysA*-intF/*DysA*-intR, which amplifies an internal fragment of *dysA*; (ii) *DysA*UpF/*DysA*-ExpR, yielding the entire *dysA* locus; and (iii) P4-PR1/RepB-R, specific for *repB* of pW2580. Whereas PCR amplicons were obtained with all primer combinations from the nine inhibitory strains (data not shown), none was detected from non-inhibitory strains. In order to ascertain if these nine dysgalactacin-producing strains harboured *dysA* elements identical to that of strain W2580, nucleotide sequence analysis of these nine *dysA* homologues was conducted. Three nucleotide changes were observed in the dysgalactacin-coding sequence, but only resulting in two conservative amino acid changes (Leu⁻²⁵→Ile, Thr⁺²²→Ser), in each case apparently not affecting the inhibitory activity displayed by the producer strains. Taken collectively, our results demonstrate that: (i) the production of dysgalactacin is correlated with the presence of pW2580-like plasmids, and (ii) dysgalactacin appears to be highly conserved.

Production of recombinant dysgalactacin by *E. coli*

As a means of obtaining larger amounts of dysgalactacin to facilitate subsequent biochemical studies, and also to establish whether the 21.5 kDa protein isolated from *S. dysgalactiae* W2580 is the sole agent responsible for the antimicrobial activity of this strain, recombinant dysgalactacin was produced by heterologous expression in *E. coli*. In order to accomplish this, an expression cassette consisting of the *dysA* gene (excluding the nucleotide tract encoding the signal peptide), preceded by a 5' nucleotide extension encoding a short linker sequence followed by a

recognition site for factor Xa protease, was cloned into the hexahistidine-tag-encoding vector pQE-80L (Qiagen). The resulting construct, pQE-dysA^M, facilitated the production of a recombinant protein that could be purified using successive chromatographic separations. Hexahistidine-tagged recombinant dysgalactacin was enriched by nickel ion affinity and subsequent anion-exchange chromatography and, following removal of the hexahistidine tag with factor Xa protease, recombinant dysgalactacin was obtained after fractionation by gel-permeation chromatography. Purified recombinant dysgalactacin was biologically active and its activity spectrum was indistinguishable from that of *S. dysgalactiae* strain W2580 in a deferred antagonism test (data not shown). Taken together, these results conclusively demonstrate that: (i) dysgalactacin is solely responsible for the antibacterial activity exhibited by *S. dysgalactiae* strain W2580, and (ii) purified recombinant dysgalactacin is suitable for subsequent biochemical characterization experiments.

Dysgalactacin kills sensitive *S. pyogenes* without inducing lysis

The large bacteriocins of Gram-positive bacteria can generally be divided into the bacteriolytic enzymes, such as lysostaphin and zoocin A (Simmonds *et al.*, 1997; Navarre & Schneewind, 1999), and the bacteriocins that kill by non-lytic means. However, to date, only one large bacteriocin, namely helveticin J from *Lactobacillus helveticus* (Joerger & Klaenhammer, 1986), appears to fulfil the criteria of the latter category, although nothing is known of its structure and physical properties. It has previously been suggested that partially purified dysgalactacin acts to kill *S. pyogenes* strain FF22 in a non-lytic manner, based on examination of treated cells by Gram staining and by electron microscopy (Wong *et al.*, 1981; Wong, 1981). In the present study, we treated *S. pyogenes* strain FF22 with either purified recombinant dysgalactacin or the bacteriolytic protein zoocin A (Simmonds *et al.*, 1996) and monitored both the OD₅₉₀ and the viable cell count of the culture. As shown in Fig. 3, the OD₅₉₀ of dysgalactacin-treated cells remained static, whilst the cell viability decreased dramatically immediately after addition of the bacteriocin. In contrast, zoocin A treatment of *S. pyogenes* resulted in a rapid decrease in both OD₅₉₀ and viable cell count, consistent with the bacteriolytic mode of action of this bacteriocin (Simmonds *et al.*, 1996). Taken collectively, these observations confirm that dysgalactacin kills sensitive target cells by a non-lytic mechanism.

Dysgalactacin contains a disulphide bond essential for biological activity

Using various algorithms available at the PredictProtein server (www.predictprotein.org), the secondary structure of dysgalactacin is predicted to be composed of: (i) a relatively unstructured N-terminal region, and (ii) a primarily helical C-terminal segment. Furthermore, the deduced amino acid sequence of dysgalactacin contained two cysteine residues

Fig. 3. Non-lytic mode of action of dysgalactacin on *S. pyogenes* FF22 cells. (a) Effect of dysgalactacin on OD₅₉₀ of *S. pyogenes* cultures; (b) effect of addition of dysgalactacin on the viable cell count (c.f.u. ml⁻¹). The positive control in both sets of experiments was zoocin A, a known bacteriolytic enzyme. The arrows indicate the time point at which dysgalactacin or zoocin A was added. ○, untreated *S. pyogenes* culture (negative control); ●, *S. pyogenes* treated with dysgalactacin; ◆, *S. pyogenes* treated with zoocin A.

at positions 132 and 186 (Fig. 2) and these, according to the DISULFIND algorithm (Vullo & Frascioni, 2004), are predicted to form a disulphide bond. It is interesting to note that the putative disulphide (cystine) in dysgalactacin flanked the predicted strongly helical region (Fig. 2) and could, therefore, play a role in maintaining the structural stability of the C-terminal portion of the bacteriocin.

Disulphides are thought to be important to the biological activity of a number of antimicrobial proteins from Gram-positive bacteria (Ennahar *et al.*, 2000). Alkylation with 4-vinylpyridine in the absence and presence of a reducing agent has been shown to be a useful technique in determining both the oxidation state of cysteine residues and the role they play in the biological activity of bacteriocins such as piscicolin 126 (Jack *et al.*, 1996). Under the conditions employed, alkylation only occurs when the polypeptide being studied contains cysteine (free thiol); thus, if cystine is naturally present, S-pyridethyl adducts are observed (e.g. by MS) only on alkylation following reduction. Conversely, if the encoded Cys residues are naturally present as cysteine, alkylation will occur irrespective of prior reduction.

In order to assess the oxidation state of Cys¹³² and Cys¹⁸⁶, recombinant dysgalactacin was exposed to the alkylating agent 4-vinylpyridine, in either the absence or presence of the reducing agent 2-mercaptoethanol (Jack *et al.*, 1996). Samples that were treated with 2-mercaptoethanol prior to alkylation exhibited a marked loss of biological activity against the indicator organism *S. pyogenes* strain FF22 (Fig. 4). In contrast, untreated dysgalactacin, or that treated with the 4-vinylpyridine alone, showed essentially the same level of biological activity (Fig. 4). Furthermore, MS of the reaction products generated showed an increase in mass of 210 Da (indicating alkylation at two sites) only in protein that had been reduced prior to exposure to the alkylating agent. These results provide experimental evidence that dysgalactacin contains a disulphide essential for its biological activity.

Dysgalactacin: the prototype of a new family of antimicrobial proteins?

Dysgalactacin is the second plasmid-encoded Sec-dependent bacteriocin to be characterized from a member of the genus *Streptococcus*, the first being the recently described streptococcin A-M57 (SA-M57) from M-type 57 *S. pyogenes* (Heng *et al.*, 2004). These two bacteriocins do not share any similarity with respect to their primary amino acid sequences but, surprisingly, the predicted secondary

Fig. 4. Reductive alkylation abolished the biological activity of dysgalactacin. The various reaction mixtures (applied as 15 µl spots) were: (a) dysgalactacin plus 4-vinylpyridine (without reducing agent); (b) untreated dysgalactacin (positive control); (c) reagent-only negative control consisting of 2-mercaptoethanol and 4-vinylpyridine; and (d) dysgalactacin pre-reduced with 2-mercaptoethanol and alkylated with 4-vinylpyridine. Reaction mixtures (a), (b) and (d) each contained ~20 µg purified recombinant dysgalactacin. The indicator strain was *S. pyogenes* FF22.

structure of SA-M57 is similar to that of dysgalactacin, i.e. a flexible N-terminal portion and a predominantly helical C-terminal region containing a putative disulphide (between Cys⁸⁷ and Cys¹⁴⁸). Reductive alkylation experiments with purified recombinant SA-M57 confirmed the presence of an essential disulphide (data not shown), strongly supporting the proposed function of the disulphide in facilitating a biologically active protein conformation. Unlike dysgalactacin, however, SA-M57 is not active against *S. pyogenes*, but rather has a spectrum of activity that includes *Micrococcus luteus*, *Lactococcus lactis* (and its different subspecies), certain *Listeria* spp., *Bacillus megaterium* and *Staphylococcus simulans* (Heng *et al.*, 2004). Interestingly, SA-M57 (but not dysgalactacin) shares limited sequence similarity with two hypothetical proteins of unknown function (Heng *et al.*, 2004): EF1097 from the genome sequence of *Enterococcus faecalis* V583 (Paulsen *et al.*, 2003), and YpkK from the sequence of a large virulence-associated plasmid harboured by *Corynebacterium jeikeium* (Tauch *et al.*, 2004). It remains to be determined whether EF1097 and YpkK possess antimicrobial activity.

Based on the similarity of their predicted secondary structures, dysgalactacin and SA-M57 (and possibly EF1097 and YpkK) may represent the first members of a novel family of antimicrobial agents, the target specificity of which may be dictated by their primary amino acid sequences (i.e. the variable N-terminal segment), whilst their killing function could be determined by their shared secondary structure, especially of the C-terminal portion. Such a model is compatible with that proposed for the well-studied lytic bacteriocin zoocin A, which is composed of an N-terminal peptidase (catalytic) domain and a C-terminal substrate-binding (targeting) domain, separated by a short linker sequence (Simmonds *et al.*, 1997). Recent studies with recombinant fragments of zoocin A have also shown that each individual domain can function independently (Lai *et al.*, 2002), indicating that a similar approach may be of value in determining whether dysgalactacin and its related proteins are indeed composed of distinct domains.

Conclusion

In conclusion, we have utilized a combination of protein purification, reverse genetics and heterologous expression (in *E. coli*) to characterize dysgalactacin, a novel plasmid-encoded, large non-lytic bacteriocin that is presumably exported via a Sec-dependent protein secretion system. In light of the observation that strain W2580C (the plasmid-cured mutant) is sensitive to the effects of the bacteriocin, it is tempting to speculate that the ecological role of dysgalactacin is analogous to that of the colicins, i.e. as a selective agent to maintain pW2580 in the bacterial population (Kirkup & Riley, 2004). In addition, dysgalactacin may represent the prototype of a new family of antimicrobial proteins, differing in their antimicrobial spectra, but possessing similar predicted secondary structures. Our ongoing studies are now focused on

obtaining a greater understanding of structure–function relationships within this unusual new class of antimicrobial proteins.

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