RESEARCH ARTICLE

Identification of a haemolysin-like peptide with antibacterial activity using the draft genome sequence of *Staphylococcus epidermidis* strain A487

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Abstract

Our interest in *Staphylococcus epidermidis* strain A487 was prompted by the unusual nature of its inhibitory activity in screening tests against methicillin-resistant *Staphylococcus aureus* isolates. The inhibitory activity was detected in deferred antagonism tests only if the agar plate was preheated for at least 35 min at $\geq 55\,^{\circ}C$ before inoculation of the indicator bacteria, this phenomenon indicating possible involvement of a heat-labile immunity agent or protease. The inhibitor was purified to homogeneity by ammonium sulphate precipitation, followed by cation-exchange and reversed-phase chromatography. Tandem MS revealed a novel peptide of molecular weight 2588.4 Da. The draft genome sequence of strain A487 was determined using 454 GS FLX technology, allowing the identification of the structural gene (*hlp*) encoding the mature peptide MQFITDLIK KAVDFFKGLFGNK. The deduced amino acid sequence of peptide 487 exhibited 70.8% similarity to that of a putative haemolysin from *Staphylococcus cohnii*. Analysis of the genome of strain A487 showed several additional inhibitor-encoding genes, including *hld*, the determinant for staphylococcal δ-lysin . This work indicates that potentially useful inhibitors could be overlooked in agar-based inhibitor screening programmes lacking a heat pretreatment step and also highlights the utility of draft genome sequence examination in antibacterial agent discovery.

Introduction

Staphylococci remain a cause of considerable morbidity and mortality. Community- and healthcare-associated infections result in increased periods of hospitalization and escalating healthcare expenses (Navarro *et al.*, 2008; Santamour, 2008). In addition, for infections with methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin is the antibiotic of choice for therapy (Stevens, 2006), but there is a growing concern due to the emergence of vancomycin-resistant *S. aureus* in the late 1990s (Ruef, 2004) and more recently the phenomenon of vancomycin 'MIC creep' (Steinkraus *et al.*, 2007). Consequently, the development of new and effective therapeutics for MRSA remains a public health priority.

Recently, there has been renewed interest in cationic peptide antibiotics as a therapeutic alternative (Hancock, 1997; Hancock, 1998; Hancock & Lehrer, 1998; Breukink *et al.*, 1999; Breukink & de Kruijff, 2006; Brown & Hancock, 2006). Staphylococci have been identified as producers of inhibitory peptides with particular ability to inhibit other staphylococcal strains (Jack *et al.*, 1998; Daly *et al.*, 2010) and exhibiting diverse chemical compositions and modes of action (Jack *et al.*, 1998; Breukink & de Kruijff, 2006; Al-Mahrous *et al.*, 2010). These include the lantibiotics (Jack *et al.*, 1998) and the lysostaphins, members, respectively, of the bacteriocin and endopeptidase families (DeHart *et al.*, 1995; Kokai-Kun *et al.*, 2003). Staphylococcal cytolysins are a cluster of bioactive, sometimes antibacterial, proteins generally considered too toxic for use in humans (Dhople

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& Nagaraj, 2005; Verdon *et al.*, 2008), due to their haemolytic activity (Fitton *et al.*, 1980; Yianni *et al.*, 1986; Janzon & Arvidson, 1990).

The present study reports the purification and characterization of a novel cationic peptide, named peptide 487, produced by *Staphylococcus epidermidis* strain A487. This study also highlights the utility of draft genomic sequence determination for the identification of the genetic apparatus required for inhibitor production, given the availability of preliminary amino acid sequence data. This approach significantly reduces the time required for gene identification compared with more conventional reverse genetics approaches.

Materials and methods

Bacterial strains and culture conditions

The antibacterial peptide-producing *S. epidermidis* strain A487 was maintained on Columbia blood agar (CBA) (Oxoid, UK) and a brain–heart infusion (BHI) broth (Oxoid). In addition, 2 × YThs, used for growing strain A487 for the purpose of inhibitor production and purification, was made of 1.6% Bacto tryptone (Oxoid), 1% Bacto yeast extract (Difco, UK), 0.5% Bacto sodium chloride (ReAgent, UK), 1.2% biological agar (Oxoid) and 5% expired human serum (Dunedin Public Hospital, Dunedin, NZ).

The indicator strains used for testing the biological activity of the inhibitor produced by strain A487 were *Micrococcus luteus* strain T-18 (I_1) (Tagg & Bannister, 1979), *S. aureus* strain A222 (A222), MRSA strains A269 (MRSA-A269) and A285 (MRSA-A285) and epidemic MRSA type-15 strain A208 (EMRSA15-A208). These indicators, together with the producer organism (*S. epidermidis* strain A487), were all kindly provided by staff at the Clinical Microbiology Laboratory, Central Manchester Foundation Trust, except I_1, which is an inhibitor-susceptible indicator strain commonly used for testing antibacterial peptides (Tagg & Bannister, 1979). A number of reference strains were sourced from the Microbiology Strain Collection, University of Otago. These included nine-indicator isolates (I_1–I_9) typically used for bacteriocin production-typing (P-typing) (Tagg *et al.*, 1976), *Streptococcus salivarius* strain 20P3 (producer of salivaricin A), *Streptococcus pyogenes* strain T11 (producer of salivaricin A1), *Staphylococcus simulans* strain SI10, *S. aureus* strain SI11, *S. aureus* strain C55 (producer of staphylococcin C55), *S. epidermidis* strain 5 (producer of Pep5), *Staphylococcus warneri* strain ATTC27836 (producer of warnericin), *S. epidermidis* strain 163 (producer of epidermin) and *S. epidermidis* strain K7 (producer of epilancin).

All strains were subcultured aerobically at 37 °C for 18 h on CBA before subsequent analysis, except the nine indicator strains (I_1–I_9), strain 20P3 and strain T11, which were grown in Todd–Hewitt broth (THB) (Difco) at 37 °C for 18 h in an atmosphere supplemented with 5% CO_2.

The traditional CAMP-like enhancement of haemolysis was performed as described previously (Christie *et al.*, 1944) using CBA supplemented with 5% sheep erythrocytes (TCS biosciences).

Detection of the inhibitor produced by strain A487 using antagonism tests

A modification of the simultaneous antagonism assay (Tagg & Bannister, 1979) was used to identify strain A487 as an inhibitor producer. The deferred antagonism method (Tagg & Bannister, 1979) was used to assess the inhibitory activity of strain A487 against 58 bacterial isolates belonging to 14 genera and 21 species, including known bacteriocin-producing staphylococci. Strain A487 was also cross-tested against itself to establish whether it displayed self-immunity. The deferred antagonism method was also used to test the heat stability of the inhibitor produced by strain A487 by exposing inhibitor-containing plates to a range of temperatures (dry heat) for up to 1 h before inoculating the indicator bacteria.

Purification of the inhibitory activity produced by strain A487

Lawn cultures of strain A487 were inoculated onto 50 2 × YThs agar plates. Following incubation for 18 h at 37 °C, the cells were removed and the agar plates were heated at 70 °C for 35 min. Then, the plates were frozen at − 80 °C for 3–4 h and thawed at room temperature. The frozen agar exudes liquor as it thaws. This was collected and clarified by centrifugation at 15 000 ***g*** at 4 °C for 5 min.

Solid ammonium sulphate was added to the liquor (80% saturation) at 4 °C and the precipitate was dissolved in ~5 mL of 2-(*N*-morpholino)ethanesulphonic acid (MES). This was bound to a CM-Sepharose Fast Flow weak cation-exchange column (HiTrap; GE Healthcare Bio-Sciences Corp) fitted to an ÄKTAmicro HPLC system (GE Healthcare), elution being carried out with an increasing gradient of 1 M NaCl at a flow rate of 1 mL min^{-1}. Buffer (A) consisted of 150 mM MES, pH 5.8, and buffer (B) included buffer (A) and 1 M NaCl, pH 5.8. Fractions were collected and analysed for activity using the spot-on-lawn assay. The active fractions were applied to an HPLC system (DP800 Data interface, LC1500 HPLC pumps, LC 1200 UV/Vis Detector (ICI Instruments, GBI Scientific, Australia) fitted with a Phenomenex Jupiter C_{18} RP-column (Phenomenex, Australia). Elution was performed using a gradient of increasing concentrations of acetonitrile (0–100%), over a 60-min period, at a flow rate of 1 mL min^{-1}. Fractions with activity were concentrated using a SpeedVac centrifuge and suspended in 0.1% trifluoroacetic acid before the final purification was achieved by a second cycle of HPLC with a

shallower acetonitrile gradient (45–80%) using the above parameters. The active fraction was concentrated, resuspended in dH_2O and the total activity was estimated using a spot-on-lawn assay in which doubling dilutions of the peptide solution, in dH_2O, were spotted (10 μL aliquots) onto the surface of a lawn of *M. luteus* (a susceptible indicator organism). The plates were incubated overnight and zones of clearing were observed in the *M. luteus* lawn. The amount of activity was quantified using the reciprocal of the highest dilution to yield a zone of clearing as a measure of the number of arbitrary units (AU) in the sample preparation.

The peptide content was determined using matrix-assisted laser desorption/ionization-time-of-flight-MS. For mass determination, the analysis used was collision-induced dissociation and tandem MS (CID MS/MS) to fragment a precursor ion and determine the masses (Wells & McLuckey, 2005).

Characterization of peptide 487

Previously published methods were used to examine the sensitivity of peptide A487 to different degradative enzymes, heat and pH changes. The degradative enzymes (Sigma, York, UK) ficin (0.22 U mg^{-1}), trypsin (10.700 U mg^{-1}), β-amylase (26.8 U mg^{-1}) and lipase (type I, 7.9 U mg^{-1}) were prepared in 25 mM phosphate buffer, pH 7.0. Solutions of the antimicrobial peptide were prepared in the same buffer (He *et al.*, 2007). A change in potency was measured using the spot-on-lawn assay with twofold dilutions of the treated inhibitor. Sensitivity to heat and pH changes was determined using published methods (He *et al.*, 2007).

Partial characterization of the putative heat-labile neutralizing factor produced by A487

In an attempt to partially characterize the putative heat-labile neutralizing factor, 1 mL of unheated culture filtrate was incubated with the same amount of the semi-purified inhibitor preparation at 37 °C for 1 h, before testing against indictor strains (EMRSA15-A208 and I_1) using the spot-on-lawn assay. In addition, a separate volume of the filtrate was heated at 70 °C for 35 min and used to treat the semi-purified inhibitor preparation before activity testing, as above. The unheated filtrate was also tested independently to confirm the presence of the neutralizing agent (i.e. absence of inhibitory activity).

DNA sequence analysis and detection of the gene encoding peptide 487

A total of 5μg of genomic DNA was recovered from 18-h cultures of strain A487 grown in THB using the GenElute™ Bacterial Genomic DNA kit (Sigma). Then, sequencing of a genomic fragment library was carried out using the 454 GS FLX Titanium system on 1/2 of a picotitre plate (Roche, UK) at the University of Liverpool Centre for Genomic Research. Individual reads (4 60 269) with an average length of 389 bp were assembled into contiguous sequences (contigs) using the GS De Novo Assembler 2.0.00.20 (Roche). The assembled contigs were ordered by comparison with the genome of *S. epidermidis* strain RP62A (accession number CP000029) and data were annotated by reference to staphylococcal genomes and data held in Swissprot, RefSeq and Uniprot databases using SUGAR (SIMPLE UNFINISHED GENOME ANNOTATION RESOURCE) (J. Szubert and S.A. Beatson, unpublished data; Perl scripts available on request). A BLAST database of the draft A487 genome sequence (produced by SUGAR) was queried using the TBLASTN algorithm and the partial amino acid sequence that had been generated for the 487 inhibitor, allowing the identification of the gene encoding the peptide.

The annotation generated by SUGAR was also examined using ARTEMIS (http://www.sanger.ac.uk/resources/software/artemis/) to allow the identification of other putative inhibitors.

Results

Staphylococcus epidermidis strain A487 produces an antibacterial agent, whose activity can be masked by a heat-labile product of strain A487

Intriguingly, the inhibitory activity produced by strain A487 was detectable in deferred antagonism tests only if the agar plate on which strain A487 had been grown was heated at ≥55 °C for at least 35 min before the application of the indicator strains. Optimal results in deferred antagonism assays were obtained by heating at 70 °C for 50 min (Table 1).

Staphylococcus epidermidis strain A487 displayed varying degrees of inhibitory activity against a range of Gram-positive bacteria, although this activity was preferentially directed against other staphylococci and never included activity against itself (Table 2). In the deferred antagonism assays, activity was graded as no activity (−), or a zone of activity less wide than the area of growth of the producing isolate (+), as wide as the area of growth of the producer (++), slightly wider than the area of producer growth (+++) or markedly wider than this area (++++/+). Strain A487 inhibited the growth of staphylococci producing the bacteriocins staphylococcin C55 and warnericin, but was inactive against strains producing Pep5, epidermin and epilancin. In all plate-based assays of inhibitory activity, strain A487 did not cause haemolysis and the strain was

Table 1. Testing the inhibitory activity produced by strain A487 against various indicators at different temperatures for 45 min

	Temperature tested (°C)											
Indicator	20 *	40	45	50	55	60	65	70	75	80	85	90
Epidemic MRSA type 15 strain A208	–	–	–	–	–	++	+++	+++++	++	++	+	+
MRSA strain A269	–	–	–	–	++	++	+++	+++++	+++	+++	+++	++
MRSA strain A285	–	–	–	–	++	++	+++	+++++	+++	+++	+++	+++
MRSA strain A345	–	–	–	–	++	++++	++++	++++++	++++	++++	++++	++++
Staphylococcus aureus strain A222	–	–	–	–	++	+++	+++	+++++	+++	+++	+++	++
Staphylococcus aureus strain A251	–	–	–	–	–	++	+++	+++++	+++	+++	+++	++
Staphylococcus aureus strain A371	–	–	–	–	++	++++	++++	++++++	++++	++++	++++	+++
Staphylococcus epidermidis strain A487†	–	–	–	–	–	–	–	–	–	–	–	–
Staphylococcus epidermidis strain A491‡	–	–	–	–	–	–	–	–	–	–	–	–

*Room temperature.

†Against itself.

‡Negative control isolate, not susceptible to the peptide produced by strain A487.

negative in the CAMP assay for the detection of enhancement of *S. aureus* β-lysin haemolysis (Fig. 1).

The inhibitory activity produced by *S. epidermidis* strain A487 is a stable peptide

The semi-purified preparations of inhibitor 487 were estimated to contain 64 or 1024 AU mL^{-1} when assayed against *M. luteus* and EMRSA15-A208, respectively (Table 3). The semi-purified inhibitory substance retained most of its antimicrobial activity when held at 30–80 °C for 45 min and was also stable to heating at 90–100 °C for 30 min (data not shown). In addition, the semi-purified preparations showed no loss of activity when stored for 3 months at either 4 or −20 °C. Semi-purified preparations were stable under acidic conditions (pH 2) for long periods, but only retained full activity for ≤ 24 h at pH 9 (data not shown). The inhibitor from strain A487 lost activity totally and partially (50% reduction) when digested with ficin and trypsin, respectively, but it was not affected upon treatment with β-amylase or lipase, indicating that the inhibitor is a peptide, with no essential polysaccharide or lipid moieties (Sweeney & Walker, 1993)

Peptide 487 can be purified to homogeneity using HPLC

Much of the inhibitory activity produced by strain A487 existed in an extracellular form, which was purified from the freeze–thaw liquor of 2 × YThs agar cultures. The mass of the peptide associated with the single peak seen following HPLC was 2588.49 Da.

The purified peptide preparation contained 4096 AU mL^{-1} of inhibitory activity against strain EMRSA15-A208, representing a 256-fold increase in titre. The purified peptide inhibited the growth of various Gram-positive bacteria, including all tested MRSA (Table 3). Notably, many isolates appearing to be resistant in deferred-antagonism assays were inhibited by the pure, high-titre inhibitor preparation. This activity spectrum included the producers of the known staphylococcal bacteriocins staphylococcin C55, epidermin, warnericin, epilancin and Pep5 (Table 3).

Strain A487 produces a neutralizing agent that reversibly binds to peptide 487

The inhibitory activity against EMRSA15-A208 and I_1 decreased by approximately 50% when incubated with a filtrate from a culture of the producer (Table 4). When the same mixture was heated, the activity was restored, indicating the release of peptide 487 from the neutralizing agent, probably through heat inactivation of the latter molecule. In addition, no changes in the activity were detected when the inhibitor preparation was incubated with a preheated filtrate (control). The filtrate from the culture of the producer that was used as a source for the neutralizing agent did not exhibit inhibitory activity.

Draft genome sequence determination facilitates the rapid identification of the structural gene encoding peptide 487, which shares homology to haemolysins

Using CID MS/MS, the partial amino acid sequence (MR)FLTDLLKKAVDFFQGLF(GNK) was obtained for peptide 487. This showed a high confidence internal sequence tag. The draft genome sequence of *S. epidermidis* strain A487 (2 523 252 bp) was obtained in 71 contigs (average contig length of 35 538 bp), with 50% of the genome sequence contained in fragments at least 1 15 205 bp (i.e. N_{50} = 1 15 205 bp) and an average depth of coverage > 70-fold.

The peptide 487 sequence was used to query a BLAST database of the draft A487 genome, enabling the identification of the gene encoding peptide 487 on a contig of

Table 2. The inhibitory activity produced by strain A487 against a wide range of bacteria as determined using the deferred antagonism test

Strain designation	Strain specification	Activity*
EMRSA15-A208	Epidemic MRSA type 15 strain A208	++++++
MRSA-A195	MRSA strain A195	+++++
MRSA-A269	MRSA strain A269	++++++
MRSA-A285	MRSA strain A285	++++++
MRSA-A345	MRSA strain A345	++++++
MRSA-A415	MRSA strain A415	–
MRSA-C755	MRSA strain C755	–
MRSA-n14	MRSA strain n14	++
A222	*Staphylococcus aureus* strain A222	+++++
A224	*Staphylococcus aureus* strain A224	+++
A251	*Staphylococcus aureus* strain A251	+++++
A371	*Staphylococcus aureus* strain A371	++++++
A457	*Staphylococcus aureus* strain A457	+++
A459	*Staphylococcus aureus* strain A459	+++
A475	*Staphylococcus aureus* strain A475	+
A491	*Staphylococcus epidermidis* strain A491	–
A487	*Staphylococcus epidermidis* strain A487	–
E22	*Staphylococcus epidermidis* strain E22	+
E54	*Staphylococcus epidermidis* strain E54	+
E133	*Staphylococcus epidermidis* strain E133	–
E204	*Staphylococcus epidermidis* strain E204	++
E224	*Staphylococcus epidermidis* strain E224	–
E226	*Staphylococcus epidermidis* strain E226	–
E229	*Staphylococcus epidermidis* strain E229	–
E230	*Staphylococcus epidermidis* strain E230	+++
C673	*Streptococcus salivarius* strain C603	++
C603	Coagulase-negative *Staphylococcus*	+++
C672	coagulase-negative *Staphylococcus*	++
20P3	*Streptococcus salivarius* strain 20P3	–
T11	*Streptococcus pyogenes* strain T11	–
I_1	*Micrococcus luteus* strain T-18	++
I_2	*Streptococcus pyogenes* strain FF22 (M type 52, T-pattern 3/13)	–
I_3	*Streptococcus anginosus* strain T29	–
I_4	*Streptococcus uberis* strain T-6 (ATCC 27958)	–
I_5	*Streptococcus pyogenes* strain 71–679 (M-type 4, T-pattern 4)	–
I_6	*Lactococcus lactis* ssp. *lactis* strain T-21	–
I_7	*Streptococcus pyogenes* strain 71–698 (M-type 28, T-pattern 28)	–
I_8	*Streptococcus pyogenes* strain W-1 (PT2841, T-pattern 6)	–
I_9	*Streptococcus equisimilis* strain T-148	–
C727	*Streptococcus pyogenes*	–
C601	*Lactobacillus* species	–
C650	Vancomycin-resistant *Enterococcus faecalis*	–
C724	*Enterobacter chloacae*^{ESBL}	–
C692	*Enterococcus* species	–
C644	*Neisseria gonorrhoeae*	–
C712	*Neisseria gonorrhoeae*	–
C559	*Eikenella corrodens*	–
C744	*E. coli*^{ESBL}	–
C743	*Klebsiella pneumoniae*	–
C748	*Klebsiella pneumoniae*^{ESBL}	–
C742	*Klebsiella oxytoca*	–
C732	*Pseudomonas aeruginosa*	–
C747	*Pseudomonas aeruginosa*^{ESBL}	–
C701	*Pseudomonas aeruginosa*	–
C669	*Stenotrophomonas maltophilia*	–
C605	*Stenotrophomonas maltophilia*	–
C586	*Pasteurella maltocida*	–

Table 2. Continued.

Strain designation	Strain specification	Activity*
C750	*Candida albicans*	–
C55	C55-producing *S. aureus* strain C55	++++++
5	PepA-producing *S. epidermidis* strain 5	–
27836	Warnericin-producing *S. warneri* strain ATTC 27836	++
163	Epidermin-producing *S. epidermidis* strain 163	–
K7	Epilancin K7-producing *S. epidermidis* strain K7	–

ESBL, extended-spectrum β-lactamase-producing strain.
*At 70 °C for 50 min.

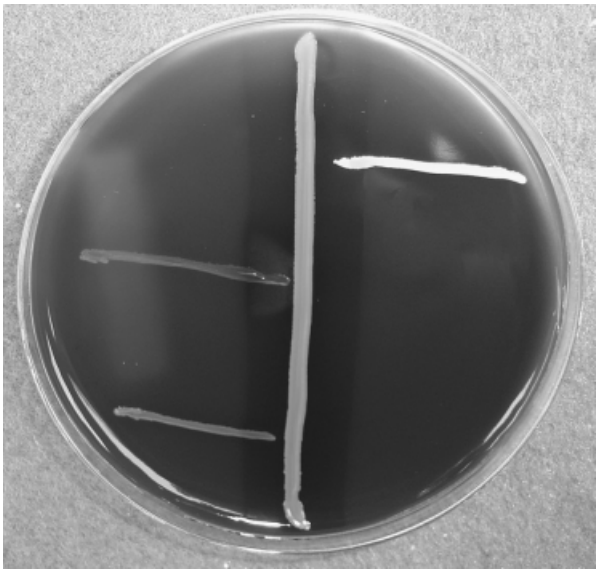

Fig. 1. *Staphylococcus epidermidis* strain A487 showing a negative result on the CAMP test. Top right, *S. epidermidis* strain A487; middle left, Group B streptococcus (positive control); bottom left, Group D *Streptococcus* (negative control).

approximately 36 kbp. This contig shared overall synteny with the chromosome of the *S. epidermidis* RP62A reference genome across its entire length, with the peptide 487 encoded within a 1.8-kb region of difference located between upstream copies of genes encoding an *O*-succinylbenzoic acid synthetase and 2-succinylbenzoate-CoA ligase, both on the complementary strand, and a downstream hypothetical protein of 160 amino acids length (data not shown). These proteins do not appear to play a role in the production or activity of peptide 487.

The deduced amino acid sequence for peptide 487 (22 residues) was determined to be MQFITDLIK KAVDFFKGLFGNK. The predicted mass of the deduced peptide was 2561.051 Da, a difference of 27.35 from the mass observed using MS/MS, which indicated that the native peptide may be N-formylated.

The first 19 residues of the 487 peptide sequence shared 57% identity and 63% similarity to the first 15 residues of the known haemolysin H2C (accession number P85220) of *Staphylococcus cohnii* ssp. *cohnii* (Mak *et al*., 2008) (Fig. 2). The H2C haemolysin is one of three synergistic haemolysins (H1C, H2C and H3C) concurrently produced by over 90% of a selection of *S. cohnii* isolates from Poland (Mak *et al*., 2008). This group of haemolysins is related to the SLUSH peptides of *Staphylococcus lugdenensis* and a putative antibacterial peptide (of unknown haemolytic nature) identified in the genome of *Staphylococcus saprophyticus* (Mak *et al*., 2008). Accordingly, the peptide identified in this study has been named haemolysin-like peptide (Hlp) and the corresponding gene, *hlp*.

The *hlp* gene shares 97% identity to those encoding putative haemolysins in the genome sequences of *S. epidermidis* strains RP62A and 12228 (accession numbers AAW53110 and AAO05868, respectively), which indicates the possibility of a wide distribution of peptide 487 homologues in *S. epidermidis*.

The sequences of *hlp* and the mature peptide have been submitted to GenBank and assigned accession number HM357252. The draft genome sequence and 454 read data have been submitted to EMBL Whole-Genome Shotgun division and Sequence Read Archive, respectively (accessions pending).

Examination of the genomic sequence of strain A487 indicates the presence of additional inhibitors

Visual inspection of the preliminary annotation of the draft genome sequence and additional BLAST searches with sequences of known inhibitors and lysins indicated that strain A487 potentially has the capacity to produce at least four additional molecules that may have inhibitory or lytic properties. These included a 44-residue peptide with 73% similarity to *S. cohnii* haemolysin H1C (P85219) (Fig. 2). Although the H3C haemolysin is typically produced in combination with the H1C and H2C peptides (Mak *et al*.,

Table 3. Quantitative measurements (AU mL^{-1}) of the inhibitory activity produced by strain A487 during the purification process

	Indicator strains										
Fraction	I_1*	EMRSA15-A208†	A269‡	A487§	A224‡	E230§	C55‡	163§	27836§	K7§	5§
Freeze–thaw liquor	8	16	8	–	8	4	32	4	4	4	4
Ammonium sulphate	64	1024	128	–	256	64	1024	128	128	512	64
Cation-exchange	256	2048	512	–	512	128	2048	256	256	512	256
HPLC	1024	4096	1024	–	1024	1024	4096	512	512	1024	512

**Micrococcus luteus*.
†Epidemic MRSA type 15 strain A208.
‡*Staphylococcus aureus*.
§*Staphylococcus epidermidis*.

Table 4. Partial characterization of the substance that neutralizes the activity of peptide 487

	Activity (AU mL^{-1}) against	
Sample	I_1	A208
Culture filtrate*	0	0
Inhibitor preparation†	8	16
Inhibitor preparation†+unheated culture filtrate*	4	8
Inhibitor preparation†+heated culture filtrate*	8	16
Heated (inhibitor preparation†+unheated culture filtrate*)	8	16

*From freeze–thaw of strain A487.
†Partially purified inhibitor that had been subjected to cation exchange chromatography.

2008), we could find no evidence of an H3C homologue within the draft genome assembly of *S. epidermidis* A487. The genes encoding the H1C and H2C homologues in strain A487 were located on separate contigs (data not shown).

Two tandemly arranged δ-lysin genes (separated by 66 bp) were detected in the putative *agr* locus of strain A487, in a manner identical to that observed in *S. warneri*, where *hld*-I and *hld*-II encode near-identical paralogous peptides (CAA11543.1 and CAA11544.1, respectively) (Fig. 2). Notably, the A487 region shares 100% nucleotide identity to the 758 bp *hld* gene region from *S. warneri* (AJ223776), but substantially less identity to the same region of other *S. epidermidis* strains, which typically contain only one δ-lysin homologue (Tegmark *et al.*, 1998).

Also detected was an ORF with 87% similarity to a putative antibacterial peptide (SSP1597/YP_301687) from *S. saprophyticus*, which appears to be widely conserved in staphylococci (Fig. 2). Finally, a small contig encoding a truncated 34-residue peptide (MTKLAEAIANTVQAAQGHDGAKLGTSIVSIVENG) with high identity ($>$ 80%) to peptides described as phenol-soluble modulins in a significant proportion of staphylococcal genomes was also identified in the draft assembly of A487.

Discussion

We have purified a novel inhibitory peptide from *S. epidermidis* strain A487 that exhibited homology to a previously described haemolytic peptide. Strain A487, however, is not haemolytic either on isolation or assay plates or in the CAMP assay.

Interestingly, the inhibitory activity in deferred-antagonism assays was only detected when the plates were heated, at $\geq$55 °C for at least 35 min, before indicator strain application. This indicated that a heat-labile neutralizing agent is coproduced in these cultures. The nature of the putative neutralizing agent was not fully established, but it is unlikely to be a protease as this would have irreversibly digested the inhibitor. The availability of the genome sequence of strain 487 will facilitate the identification of this agent, if the partial amino acid sequence of a purified compound can be obtained. It is possible that the neutralizing substance stoichiometrically binds to the inhibitor, rendering it unable to interact with target cells. However, even highly concentrated preparations of peptide 487 were not active against the producing strain, indicating the role of additional mechanisms of producer self-immunity. If this inhibitor-neutralizing phenomenon is more widely distributed, it may be that other potentially useful inhibitory agents have been overlooked in conventional screening assays that lack a heat pretreatment step.

The inhibitor produced by strain A487 showed characteristics comparable to those of bacteriocins, because it appeared to be a small, cationic, hydrophobic, proteinaceous, heat-stable molecule, highly active against strains of closely related species (Jack *et al.*, 1995; Crupper & Iandolo, 1996).

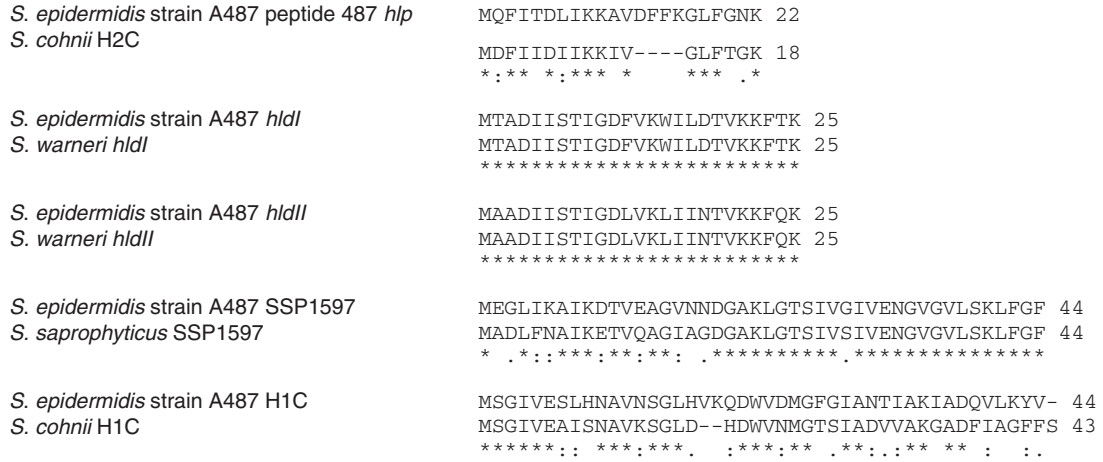

Fig. 2. CLUSTALW2 alignment of peptides produced by *Staphylococcus epidermidis* strain A487, including peptide 487, with homologues (from top of alignment) of *Staphylococcus cohnii* ssp. *cohnii* haemolysin H2C, *hld*I and *hld*II of *Staphylococcus warneri*, a putative antibacterial protein (SSP1597) of *Staphylococcus saprophyticus* ssp. *saprophyticus* strain ATCC 15305/DSM 20229 and *S. cohnii* ssp. *cohnii* haemolysin H1C. Identity is indicated by '*' and conserved or semi-conserved substitutions by ':' or '.', respectively.

The partial amino acid sequence data obtained contained a sequence tag that did not have any significant homology to previously identified peptides in the GenBank database. Previously, in similar situations, partial amino acid sequence data would be used to initiate a program of reverse genetics that, usually after some considerable investment in time and resources, would yield the genetic apparatus underlying the production of a novel inhibitor. In the current work, we took advantage of high-throughput genome sequence determination methods, allowing the identification of the gene encoding peptide 487 in < 4 weeks. The gene was found to be chromosomally located, as reported previously for staphylococcal haemolysins (Waalwijk *et al*., 1984; Boerlin *et al*., 1998; Piccinini & Zecconi, 2001). Some haemolysin genes are plasmid located in *Escherichia coli* (Avgustin & Grabnar, 2007) and *Enterococcus faecalis* (Leclercq *et al*., 1988; Gilmore *et al*., 1994). The deduced amino acid sequence of peptide 487 was most similar to a peptide from *S. cohnii* previously described as the H2C haemolysin (Mak *et al*., 2008). H2C was shown to be haemolytic and to be toxic to human fibroblasts, but the antibacterial activity of the peptide was not reported.

There have been a number of recent reports of nonhaemolytic molecules that share high levels of similarity or identity to known haemolysins (Verdon *et al*., 2008; Verdon *et al*., 2009; Al-Mahrous *et al*., 2010). A key example is the work of Dhople & Nagaraj (2005), demonstrating that slight modifications in the amino acid sequence of a haemolysin from *S. warneri* abolished haemolytic activity and conferred antimicrobial properties to the resulting synthetic peptides. In addition, we have demonstrated recently that a native antibacterial peptide from *S. epidermidis* that has relatively little haemolytic activity differs only at one residue when compared with a haemolytic peptide produced by *S. warneri* (Al-Mahrous *et al*., 2010).

It is probable that the classification of a number of novel or putative peptides as haemolysins, based solely on homology to experimentally proven haemolysins, should be revisited and that their antimicrobial activity should also be assessed. A number of potentially useful novel antimicrobial agents may unnecessarily have been classified as toxic and not investigated in more detail.

Homologues of *hlp* are present in the genomes of *S. epidermidis* strains RP62A and 12228, indicating that this molecule is probably widely distributed in *S. epidermidis*. Their annotation as putative haemolysins in the two previously published *S. epidermidis* genomes may be inappropriate, given their similarity to peptide 487. However, confirmation of this will require specific investigation of the activity of the peptide 487 homologues. The lack of an H3C homologue in strain 487 may explain, at least in part, the lack of haemolytic activity as the H1C, H2C and H3C peptides are synergistic haemolysins (Mak *et al*., 2008). In *S. cohnii*, the H2C haemolysins have been implicated as potential virulence determinants in this usually benign organism (Mak *et al*., 2008). It is possible that in *S. epidermidis*, the Hlp homologues play a role in niche competition (Daly *et al*., 2010), with activity predominantly directed against *S. aureus* strains.

The availability of the draft genome sequence for strain A487 enabled us to search for ORFs encoding additional putative inhibitory agents, and these are now undergoing follow-up examination in our laboratory. The importance of DNA sequence databases as starting points in the search for new antimicrobial agents is increasingly evident (Begley *et al*., 2009; Daly *et al*., 2010) and the current work

demonstrates the utility of this approach at the level of an individual strain.

In this study, we observed inhibitor production by *S. epidermidis* strain A487. Characterization of the inhibitor using chromatographic separation, partial amino acid sequencing and draft genome sequence determination allowed the deduction of the full amino acid sequence for peptide 487, which is novel and shares the highest homology with a previously described haemolysin from *S. cohnii*. Peptide 487 has potential for application as a topical treatment for staphylococcal infections and may play a role in niche competition.

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