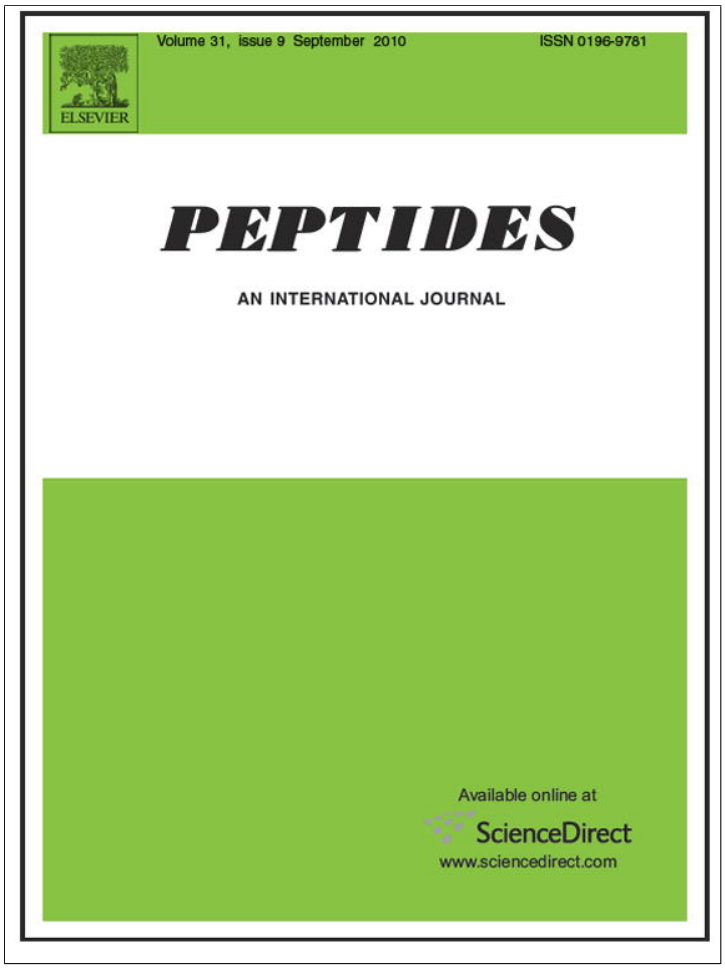

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Purification and characterization of a novel delta-lysin variant that inhibits *Staphylococcus aureus* and has limited hemolytic activity

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ABSTRACT

Delta-lysins (DL) that are produced by various species of staphylococci are not widely known for their antimicrobial activity. We have purified and characterized a novel DL variant, E229DL and examined its spectrum of inhibitory activity. The biological activity of E229DL, produced by *Staphylococcus epidermidis* strain E229, shows relatively broad-spectrum activity against Gram-positive pathogens, including representatives of MRSA and epidemic MRSA type 15. E229DL was purified to homogeneity from 95% acidified-methanol extracts of cell cultures by using a series of reversed-phase chromatographic separations. The fully processed form of E229DL is a 25-amino-acid peptide with a predicted mass of 2841.4 Da, but the purified biologically active molecule appears to be N-formylated (mass 2867.33 Da). The DL gene (hld) resembles that of other types of DL, but differs in five codons with hld in *Staphylococcus aureus* (26 residues) and one codon with the closest homolog, the *hld-II* in *S. warneri* (25 residues). The characterization of E229DL showed that its activity is stable in agar exposed to high temperatures (80 °C/45 min). In addition, biological testing of the native and synthetic peptides against a range of human and animal erythrocytes and Vero cells indicated that E229DL is an antibacterial agent with no detectable cytopathic effects at concentrations equivalent to the minimum inhibitory concentration for EMRSA15-A208. Initial investigation of the mode of action of E229DL indicated that it is rapidly lytic for target cells. This is the first description of a native form of DL having only limited cytotoxic activity for eukaryotic cells at concentrations that are inhibitory to staphylococci.

1. Introduction

The resistance of staphylococci to conventional antibiotics has risen to an alarming level [7,25,30,36]. Recently, there has been much focus on naturally produced antibacterial substances as potential novel therapeutics [2,4,23]. Amongst these is the staphylococcal toxin, delta-lysin (DL), though this has not yet been considered for use in humans [8,38]. One reason for this exclusion is the activity observed against erythrocytes [11,19,41].

DL, initially discovered in *S. aureus* (the most commonly cited producer), is a member of the cytolytic and cytotoxic polypeptide family [11,19,41]. The production of DL homologs in coagulase-negative staphylococci (CNS) has also been demonstrated [26]. The DL of CNS vary in the number and sequence of amino acid residues [35]. For example, compared with the DL in *S. aureus* (26 residues), the DL in some *S. epidermidis* strains differ in at least two residues [9,24], and a truncated derivative of 25 residues has been reported [24,27]. It is suggested that DL can form pores in cell membranes and lyze erythrocytes [12,20].

In 2005, Dhople and Nagaraj reported that the DL of *S. aureus* does not exhibit antibacterial activity. They synthesized DL and several analogs wherein the N-terminal residues of the peptide were sequentially deleted, a proline residue was introduced in the native sequences as well as in the analogs and all aspartic acids (anionic residues) were replaced with lysine (cationic residues). These changes resulted in the generation of shorter peptides with antibacterial, but no hemolytic activity [8].

The present study describes the purification and antibacterial spectrum of E229DL, a cationic and highly hydrophobic member of the DL peptide family that exhibits relatively little hemolytic activity.

2. Experimental

2.1. Bacterial strains and culture conditions

The antibacterial peptide-producing *S. epidermidis* strain E229 was maintained on Columbia blood agar (CBA) (Oxoid, Basingstoke,

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Table 1
Simultaneous antagonism indicator strains.

Indicator designation	Strain specification
Staphylococcal core indicator strains	
I_1	*M. luteus* strain T-18[a]
A222	*S. aureus* strain A222
MRSA-A269	MRSA strain A269
MRSA-A285	MRSA strain A285
EMRSA15-A208	Epidemic MRSA type-15 strain A208

[a] Previously referred to as S. epidermidis strain T-18 [33].

Hampshire, UK), but was also propagated in Trypticase soy broth (TSB) (Oxoid). In addition, a modified TSB, designated TSYcat80, was made of 3% TSB, 2% Bacto yeast extract (Difco Laboratories, Moseley, UK), 0.5% Bacto calcium carbonate (Difco Laboratories), 1.2% biological agar (Oxoid) and 5% tween-80 (Difco Laboratories). TSYcat80 was used for growing strain E229 for the purpose of inhibitor purification.

Indicator strains used for testing the biological activity of the inhibitor produced by strain E229 are listed in Table 1. These indicators, together with the producer organism (*S. epidermidis* strain E229), were all provided by staff at the Clinical Microbiology Laboratory, Central Manchester Foundation Trust, except I_1 which is a biologically sensitive indicator generally used for testing antibacterial peptides [33] and *Legionella pneumophila*, which were from the University of Manchester Medical Microbiology Bacterial Collection. In addition, a number of reference strains were obtained from the University of Otago culture collection, and these included nine indicator isolates (I_1–I_9) usually used for bacteriocin production typing (P-typing) [34], *Streptococcus salivarius* strain 20P3, *Streptococcus pyogenes* strain T11, *Staphylococcus simulans* strain SI10 and *S. aureus* strain SI11.

All strains were sub-cultured aerobically at 37 °C for 18 h on CBA (Oxoid) prior to 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.

Legionella isolates that were included in this study were maintained on Legionella CYE agar base medium (Oxoid) and incubated at 37 °C for 48 h in an atmosphere supplemented with 5% CO_2.

2.2. Detection and identification of inhibitor produced by strain E229 using antagonism tests

The deferred antagonism test, as described by Tagg and Bannister in 1979, was used to screen a collection of isolates for potential inhibitory activity against I_1 and *S. aureus* indicator isolates. Through these tests, strain E229 was identified as a producer of a potent inhibitor and this isolate was investigated in more detail.

The deferred antagonism method was also used to test for heat stability (by exposing inhibitor-containing plates to 80 °C dry heat for up to 1 h prior to inoculating the indicator bacteria) and to assess the activity of strain E229 against 60 clinical bacterial isolates of 22 species belonging to 15 genera (Table 2). Deferred antagonism was also used to test the amount of inhibitor present in cultures of strain E229 that had been incubated for various times between 4 and 49 h.

Strain E229 was also cross-tested against itself to establish whether it displayed self-immunity to its own inhibitor.

2.3. Characterization of the inhibitor produced by strain E229

The methods of He et al. [16] were used to examine sensitivity to different degradative enzymes, heat and pH changes. In brief, semi-pure HPLC preparations of the inhibitor were used for these tests. Four degradative enzymes, ficin (0.22 U/mg), trypsin (10.700 U/mg), β-amylase (26.8 U/mg) and lipase (type I, 7.9 U/mg; all from Sigma, York, UK) were prepared in 25 mM phosphate buffer, pH 7.0. Solutions of the antimicrobial peptide were prepared in the same buffer. All stock solutions were separately sterilized by filtration through low-protein binding filters (MILLEX-GV 0.22 μm filter unit; Millipore, Carrigtwohill, County Cork, Ireland). Then the qualitative spot-on-lawn assay was used to examine alterations in antimicrobial potency post-treatment. For sensitivity to heat and pH changes, the method of He et al. was adopted [15].

2.4. Purification of the inhibitor produced by strain E229

Lawn cultures of strain E229 were grown on 50 TSYcat80 agar plates following inoculation with cells from 18 h TSB cultures of *S. epidermidis* strain E229. Following incubation for 18 h at 37 °C, the cells were harvested using a sterile spatula and re-suspended in 25 ml 95% acidified-methanol (pH 2). After 18 h at 4 °C on an orbital shaker at 180 rpm, the cells were removed by centrifugation (15,300 × *g* for 5 min). Then the supernatant was assayed for inhibitory activity against the core indicator strains, using the spot-on-lawn assay [34]. The methanol was removed using a SpeedVac centrifuge (Eppendorf) and then 400 μl of the sample was applied to an Ettan™ LC system (GE Healthcare) fitted with a Synergi C_{18} RP-column at 50 °C. Elution was performed using a gradient of increasing concentrations of 0–100% acetonitrile [with 0.05% (v/v) trifluoroacetic acid (TFA)] over 60 min at a flow rate of 1 ml/min and the content of the eluate was monitored at 215, 206 and 280 nm.

Fractions of the eluate were assayed by spotting 10 μl volumes onto a lawn of I_1 cells seeded onto a CBA plate, which was incubated for 18 h at 37 °C. Fractions demonstrating inhibitory activity were concentrated and final purification was achieved by a second cycle of HPLC with a shallower acetonitrile/TFA gradient of 60–80% over 60 min. The peak fraction of inhibitory activity was concentrated and then re-suspended in 400 μl of purified dH_2O then the activity was determined using the spot-on-lawn assay. The concentration of pure peptide was measured using a NanoDrop® ND-1000 UV-V Spectrophotometer.

2.5. Mass-spectrometric analysis of inhibitor produced by strain E229

The initial analysis was achieved using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-ToF MS). In brief, the purified HPLC fraction showing inhibitory activity was mixed with an internal standard of glucagon or mellitin and matrix (0.5 μL of 10 mg ml^{-1} α-cyano-4-hydroxy cinnamic acid in 0.1% TFA and 60% acetonitrile) directly on the stainless steel slide and left to dry at room temperature. Then, a Lasermat 2000 device (Finningan Mat, Hemel Hempstead, England) was used for the collection of mass data. In addition, the samples were digested with trypsin and then analyzed inline by positive ion electrospray ionization mass spectrometry/mass spectrometry (ES-IMS/MS) using a Quadrupole-Time of Flight Micromass spectrometer (Q-ToF MS) (Waters, Manchester, UK). This allowed identification of peptide fragments and de novo determination of the amino acid sequence of the inhibitor.

Data acquired were analyzed using ProteinLynx global server 1.1 analytical software (Waters) at SWISS-PROT/TrEMBL (http://www.expasy.ch/tools/protparam.html).

2.6. DNA extraction and PCR amplification of the gene encoding E229DL

The amino acid sequence obtained for E229DL was highly homologous (81% identity) to the product of the *hld-II* gene in *S. warneri* [35]. For this reason, the DNA sequence of the latter (accession number AJ223776) was used to design PCR

primers for amplification of the target gene from strain E229, using OligoPerfect software (Invitrogen, Paisley, UK). The sequence of the synthesized primers (Eurofins MWG GmbH, Germany) was 5′-ATTTAGGATTTACAATAATTCAAATGG-3′ (forward) and 5′-TGGGATGGCTTAATAACTCACC-3′ (reverse). These primers were used to amplify a DNA fragment of 183 bp containing the gene of interest.

DNA extraction was performed using GenElute™ Bacterial Genomic DNA kit (Sigma) from *S. epidermidis* strain E229 cells grown in TSB for 18 h at 37 °C. PCR reactions contained 1 μl DNA, 2 μl (10 pmol) of each primer, 25 μl ReadyMix™ PCR Master Mix (ABgene, Surry, UK) and molecular grade water (to a final volume of 50 μl). For PCR, an Eppendorf-Mastercycler thermal cycler was used with the following amplification conditions: 94 °C for 5 min; followed by 35 cycles of 94 °C for 30 s; 55 °C for 30 s; and 72 °C for 1 min; with a final extension of 7 min at 72 °C.

2.7. DNA sequencing and analysis of the E229DL gene

PCR clean up was performed using GenElute™ PCR Clean-Up kit (Sigma) according to the provided instructions. The sequencing reaction was prepared using 2 μl BigDye Master Mix

Table 2
The inhibitory activity produced by strain E229 against a wide range of bacteria using the deferred antagonism test.

Strain designation	Strain specification	Activity
EMRSA15-A208	Epidemic MRSA-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	*S. aureus* strain A222	+
A224	*S. aureus* strain A224	+++
A251	*S. aureus* strain A251	+
A371	*S. aureus* strain A371	++
A457	*S. aureus* strain A457	+
A459	*S. aureus* strain A459	+
A475	*S. aureus* strain A475	+
A491	*S. epidermidis* strain A491	+
A487	*S. epidermidis* strain A487	–
E22	*S. epidermidis* strain E22	+
E54	*S. epidermidis* strain E54	++
E133	*S. epidermidis* strain E133	++
E204	*S. epidermidis* strain E204	+++
E224	*S. epidermidis* strain E224	+++
E226	*S. epidermidis* strain E226	+
E229	*S. epidermidis* strain E229	–
E230	*S. epidermidis* strain E230	++++++
C673	*Streptococcus salivarius* strain C603	+++
C603	coagulase-negative staphylococci	+++
C672	coagulase-negative staphylococci	++
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* subsp. *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* spp.	++
C650	Vancomycin-resistant *Entero. faecalis*	–
C724	*Enterobacter cloacae*^{ESBL}	–
C692	*Enterococcus* spp.	–
C644	*Neisseria gonorrhoeae*	–
C712	*Neisseria gonorrhoeae*	–
C559	*Eikenella corrodens*	–
C744	*Escherichia 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*	–
C750	*Candida albicans*	–
Cl1	*Legionella pneumophila*	+
ATCC 33152	*Legionella pneumophila*	++

ESBL, extended spectrum beta-lactamase.

Terminator (Applied Biosystems, Foster City, CA, USA), 3 μl 1,2:3,4-diepoxybutane (BDE) Buffer, 2 μl of template DNA, 1 μl of primer (10 pmol) (Eurofins MWG GmbH) and 12 μl of molecular grade water (total volume of 20 μl) (Oxoide). Cycle sequencing was carried out at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a final extension of 7 min at 72 °C. The sequencing products were precipitated using 2 μl of 3 M sodium acetate and 50 μl of 100% ethanol and then were analyzed using an ABI-Prism 377 DNA sequencer (Applied Biosystems) at the DNA Sequencing Facility, Faculty of Life Sciences, the University of Manchester.

2.8. Synthesis of E229DL and native delta-lysin from S. aureus

DNA sequence analysis of the amplified E229DL gene allowed confirmation of the amino acid sequence of the peptide, which was then chemically synthesized (Activotec, Cambridge, UK). The peptide was obtained at 95% purity, as determined by HPLC by the supplier. The DL of *S. aureus* strain W46M (26 amino acids; MAQDIISTIGDLVKWIIDTVNKFTKK) [8,11,20] was also synthesized for use as a positive control.

2.9. Determination of the MIC of delta-lysin peptides for strain EMRSA15A-208

The minimum inhibitory concentrations (MIC) of native and synthetic DL from strain E229 and synthetic DL from *S. aureus* strain W46M towards EMRSA15-A208 were determined using a broth microdilution assay according to NCCLS recommendations [37]. Briefly, each microtitre plate well contained 100 μl of TSB. Double dilutions were performed with the stock solutions E229DL and W46M DL. Then, 1 μl of EMRSA15-A208 (from a stock with a concentration of approximately 10^5 cfu/ml) was administered to each well. The plates were then incubated at 37 °C for 24 h.

2.10. Determination of mutant frequency and calculation of the mutation rate

A modification of the Rosche and Foster method was adopted for this purpose [29]. The core indicator strains were grown in TSB to early stationary phase (18 h) with the viable cell number being approximately 10^9 cfu/ml. This culture was diluted 10^{-4} in TSB and divided into 15, 0.5 ml aliquots. The 15 aliquots were allowed to grow for 18 h at 37 °C to obtain parallel, independent cultures. The number of resistant mutants that emerged in each culture was determined by plating the entire culture on TSB agar containing the selective inhibitor (500 μl of a concentration of E229DL that was equivalent to the MIC for EMRSA15-A208). The total number of cells was determined by plating appropriate dilutions of three cultures (10^{-5}, 10^{-6} and 10^{-7}) on non-selective medium (no addition of E229DL).

The frequency of resistant mutants was expressed as the mean number of resistant cells divided by the total number of viable cells per culture. For calculation of the mutation rate per cell division, the most likely number of mutations per culture (m) was first calculated from the distribution of numbers of resistant mutants in the independent cultures using the equation: [$m = -\ln$(zero fraction of resistant bacteria)] [29]. Then, the mutation rate (μ) per cell division was calculated as $\mu = m/\mathrm{Nt}$, where Nt is the total cell number per culture [29].

2.11. Investigation of mechanism of action

Study of the mode of action was carried out using electron microscopy. A spot-on-lawn assay of E229DL was carried out against EMRSA15-A208 with a concentration equivalent to 0.5× MIC on TSYcat80 for 18 h at 37 °C. EMRSA15-A208 cells that were just at the edge of the inhibition zone were carefully scraped off, suspended in buffered glutaraldehyde and then submitted to the Electron Microscopy Unit, Central Manchester Foundation Trust, Manchester, UK. The Electron microscopy grids were made using resin embedding/thin sectioning. In brief, bacteria were fixed for a minimum of 4 h in 2.5% cacodylate buffered glutaraldehyde. Post fixation incubation in 2% aqueous osmium tetroxide was for 1 h, followed by dehydration in a graded series of ethanol and embedding in Araldite resin (TAAB). After polymerization, 100 nm thick sections were cut from the resin blocks on a Reichert OMU4 Ultracut ultramicrotome. Sections were mounted onto 400 mesh copper grids, stained in uranyl acetate and lead citrate and examined under a Philips CM10 electron microscope. Images were recorded digitally using an AMT LR44 digital camera.

2.12. Examination of hemolytic activity of E229DL

For hemolysis assays, a modification of the Birkbeck and Freer method [1] was adopted. Erythrocytes [expired human samples from the blood bank at the Manchester Royal Infirmary and horse blood (Oxoid)] were centrifuged (3400 × g for 10 min at 4 °C), washed three times in double volumes of sterile 20 mM phosphate-buffered saline (PBS, pH 7.2). Synthetic and native E229DL (10 μl of a concentration equivalent to the MIC for EMRSA15-A208) was mixed with 90 μl of a 1% (v/v) suspension of the washed erythrocytes in a 96-well U-shaped microtitre plate (Sigma). An erythrocyte suspension in PBS was included as a negative control and DL of *S. aureus* strain W46M was used as a positive control [8,11,20]. The mixture was incubated for 1 h at 37 °C and hemolysis was recorded by visual inspection.

In addition, the traditional CAMP-like enhancement of the hemolysis for the inhibitory activity of strain E229 was assessed, as described by Christie et al. [5].

2.13. Cytopathic effects of E229DL against the Vero cell-line

The African green monkey kidney cell-line (Vero; ATCC CRL 1587) was grown in Eagle's minimal essential medium (EMEM) (Sigma) in 96-well microtitre plates supplemented with 10% foetal bovine serum and 10 mg/ml gentamicin (Gibco Laboratories, Paisley, UK). Cell suspensions (100 μl) were seeded 24 h before the assay at concentrations allowing the formation of a nearly confluent monolayer (approximately 20,000 cells/ml) at the time the test was performed. EMEM was discarded and another volume (200 μl) of fresh of EMEM was immediately added, followed by 200 μl of serially diluted of the native and synthesized E229DL (starting at 65 μg/ml, which was 2-fold higher than the MIC for EMRSA15-A208) and then incubated for 7 days at 37 °C under 5% CO_2. The occurrence of any cytopathic effect (CPE) was noted by microscopic observation twice a day. Sterile PBS was used as negative control and DL of *S. aureus* strain W46M was used as a positive control.

3. Results

3.1. Detection and identification of inhibitor produced by strain E229 using antagonism tests

The inhibitory activity was continuously produced during growth of strain E229 in batch culture, but maximum activity was found at the very end of the exponential and early stationary phase (10–16 h) of the growth cycle (data not shown). In addition, when the producing cells were removed, the inhibitory substance that had accumulated in the agar was stable to heating at 80 °C for 45 min.

Strain E229 displayed varying degrees of inhibitory activity against a range of Gram-positive bacteria, and strong activity was especially evident against other staphylococci (Table 2). Given the reported activity of DL-I from *S. warneri* against *Legionella* strains [38], we observed the deferred activity of strain E229 against two isolates of *L. pneumophila*, both of which were weakly inhibited (Table 2).

It is worth noting that in deferred antagonism test plates, strain E229 was not hemolytic against either horse or human erythrocytes (data not shown).

3.2. Characterization of the inhibitor produced by strain E229

Propagation on TSYcat80 agar resulted in production of more inhibitory activity than on other media. The activity appeared to be mostly retained on the cell surface, but the bound inhibitor could be eluted by acidified-methanol extraction and these semi-purified preparations were estimated to contain 2 Arbitrary Units (AU)/ml or 4 AU/ml when assayed against *M. luteus* and EMRSA15-A208, respectively.

The inhibitory substance retained most of its antimicrobial activity when held at 30, 37, 50, or 80 °C for 45 min and was also stable to heating at 100 °C for 10 min. In addition, the crude preparations showed no loss of activity when stored for 3 months at either 4 or 20 °C. When subjected to different pH values from 2.0 to 9.0, the extract was more stable under acidic conditions (pH 2) and only retained full activity for less than 24 h under alkaline conditions.

Upon treatment with various enzymes, the inhibitor from strain E229 lost its activity totally and partially (~2-fold) when digested with ficin and trypsin, respectively, but it was not affected by treatment with β-amylase or lipase.

From the above observations, it was not possible to further establish the nature of the inhibitor produced by strain E229, so purification and further characterization were initiated.

3.3. Purification of the inhibitory activity produced by strain E229

The titre of the strain E229 inhibitory agent against EMRSA15-A208 (4 AU/ml), as recovered in acidified-methanol extraction of cells grown on TSYcat80 agar medium, showed an 8-fold increase when the extract was concentrated using SpeedVac. During HPLC separation, the biologically active substance demonstrated a distinct absorbance peak (at 53.6–56.4 ml) equating to elution at approximately 70% acetonitrile. Although only approximately 5% of the initial inhibitor activity obtained from strain E229 was recovered in the last step of the purification process, the specific activity against EMRSA15-A208 was 256-fold higher than that of the original extract and 32-fold higher against *M. luteus*.

3.4. Determination of the amino acid sequence of the inhibitor produced by strain E229

MALDI-ToF MS analysis indicated that the mass of the inhibitor was 2867.332 Da. Further analysis using Q-ToF MS identified the sequence of the 25 amino acids of the native peptide as N-MTAD(L/I)(L/I)ST(L/I)GDFVKW(L/I)(L/I)DTVKKFAK-C. Interrogation of the NCBI non-redundant protein sequences database using the Blastp algorithm indicated 66.4% identity with the DL-II of *S. warneri* [35,38] indicating that the inhibitor produced by strain E229 was, in fact, a DL variant.

3.5. Physicochemical properties of E229DL

Analysis of the 25 residues of E229DL using ProteinLynx global server 1.1 software and SWISS-PROT/TrEMBL showed a theoretical pI of 8.19. The instability index (II) was computed to be 1.42. This classifies E229DL as stable. The aliphatic index was 109.20 and the grand average of the hydropathicity (GRAVY) was 0.440.

3.6. DNA sequence analysis of the gene encoding E229DL

PCR primers were designed based on the sequence of *hld-II* from *S. warneri*. The amino acid sequence of E229DL was deduced following amplification of the E229DL gene from strain E229. The DNA sequence of *hld* in strain E229 (5′-ATG ACT GCA GAT ATC ATT TCA ACA ATT GGT GAT TTT GTA AAA TGG ATT TTA GAT ACA GTA AAA AAA TTC GCT AAA-3′) confirmed that the amino acid sequence was N-MTADIISTIGDFVKWILDTVKKFAK-C (Fig. 1). The predicted mass of this peptide was 2841.4 Da, indicating that the native form may be formylated, as has been previously reported for similar peptides [36].

This DNA sequence differed in six residues (76% identity) from *S. warneri* DL-I (25 residues), 11 residues (53% identity) from *S. simulans* DL (26 residues), five residues (80% identity) from *S. epidermidis* DL (25 residues) and seven residues (72% identity) from *S. aureus* strain W46M DL (26 residues). Further comparison between the different DL variants showed a number of conserved residues (Fig. 1).

The sequences of the *hld* gene from strain E229 and the encoded peptide have been submitted to GenBank and assigned accession number GU929338.

3.7. MIC determination of E229DL against EMRSA15-A208

The MIC of purified and synthetic E229DL for EMRSA15-A208 was approximately 16 and 512 μg/ml, respectively.

This value corresponded to an MIC of about 1 μM, indicating that the peptide was active in micromolar range. The synthetic form of the 26-residue DL from *S. aureus* was less active against EMRSA15-A208 having an MIC ≥6-fold higher than that of E229DL.

By use of spot-on-lawn assays, it was determined that all strains of staphylococci from various clinical specimens were sensitive to the synthesized and native forms of E229DL. All these strains were resistant to numerous antibiotics including the third generation cephalosporin, cefoxitin. In addition, both forms of E229DL were also active against a panel of Gram-positive bacteria, including some *Streptococcus* species and *Lactobacillus* species and showed a rather broad antimicrobial spectrum within this group, but were inactive against the small number of tested Gram-negative isolates in the collection (data not shown).

3.8. Determination of mutant frequency and mutation rate

The determination of both the mutant frequency and mutation rate in the core indicator strains showed that there were fewer than 1×10^{-9} mutants obtained for all isolates examined following growth in 16 μg/ml E229DL (Table 3).

3.9. Proposed mechanism of action for E229DL

The analysis of the mechanism of action using electron microscopy indicated that the most significantly affected part of the tested sensitive bacteria was the cell wall. Electron micrographs revealed breaks in the walls of several cells and ghosts of lyzed cells, in addition to larger cells (Fig. 2).

3.10. Hemolytic activity of E229DL against human erythrocytes

E229DL demonstrated dose-dependent hemolytic activity against human erythrocytes. Compared to the native purified E229DL, the synthesized E229DL form appeared to be far less potent for erythrocytes, since lysis was consistently reduced when

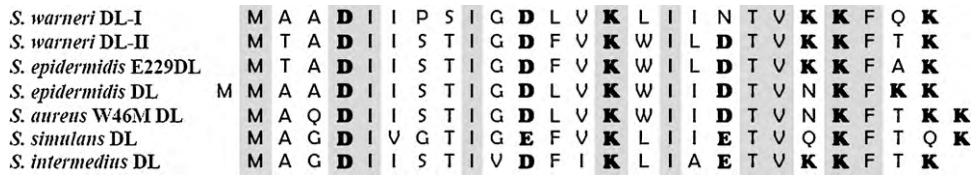

Fig. 1. The amino acid sequence of E229DL and other staphylococcal DL including the closest in homology, the *S. warneri*-II DL-II. Charged residues are in bold. Conserved region are grey-shaded.

Table 3
Frequency of spontaneous mutation of the core indicators after exposure to 16 μg/ml E229DL.

Indicator strain	No. of independent cultures	No. of cells per culture	Resistant bacteria			Frequency of mutants	Mutation rate per cell division
			Zero fraction[a]	Mean	Median		
EMRSA15-A208	15	5.0×10^8	13/15	0.26	0	0.05×10^{-8}	0.02×10^{-8}
MRSA-A269	15	4.8×10^8	14/15	0.20	0	0.04×10^{-8}	0.01×10^{-8}
A222	15	4.5×10^8	13/15	0.33	0	0.07×10^{-8}	0.03×10^{-8}
MRSA-A285	15	4.6×10^8	12/15	0.37	0	0.08×10^{-8}	0.04×10^{-8}
I_1	15	3.9×10^8	15/15	0	0	ND[b]	ND[b]

[a] Proportion of cultures without mutants = number of cultures without mutants/number of independent cultures.
[b] ND, not detectable.

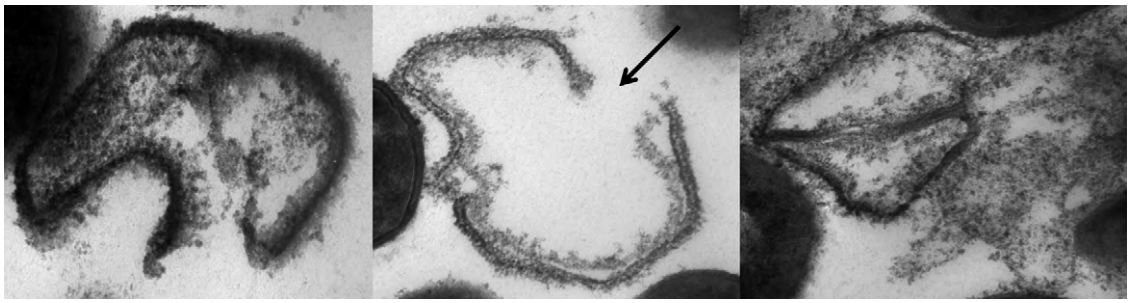

Fig. 2. Thin section of EMRSA15-A208 cells after incubation with a pure E229DL peptide at 37 °C for 24 h. (Left) A ruptured wall, resulting in enlargement of the affected cell, is compared to a normal cell. (Centre) A rupture wall is seen (arrow). (Right) The release of the internal materials through the ruptured cell wall is seen in this field.

comparing the same concentrations of peptides. Partial hemolysis was achieved with a concentration of double the MIC of both the purified (16 μg/ml) and synthesized (512 μg/ml) peptide for EMRSA15-A208.

In addition, strain E229 was negative in the traditional CAMP-like assay for detection of enhanced hemolysis.

3.11. Cytopathic effects of E229DL against Vero cell-line

Only 10% of Vero cells displayed CPE at ≥4× MIC for EMRSA15-A208 (≥65 μg/ml of the native and 2048 μg/ml of the synthetic peptide). The CPE observed included cell rounding, swelling, shrinking and nucleus decomposition.

The synthesized 26-residue DL of *S. aureus* (the positive control) showed CPE on Vero cells at a lower concentration of approximately 4 μg/ml.

4. Discussion

In the present study, the agent responsible for the production of the antibacterial activity elicited by *S. epidermidis* strain E229 was shown to be a 25-residue DL that shares a high degree of homology to DL-II of *S. warneri* [35,38].

The initial investigation of strain E229 using the deferred antagonism test showed a wide range of susceptible organisms with relatively high activity directed against the staphylococcal indicators. Another characteristic was stability of the agent to high temperatures. Characteristics such as these are common to bacteriocin-like substances of Gram-positive bacteria [18,40]. In addition, the total and partial loss of activity when treated with ficin and trypsin and stability upon treatment with β-amylase and lipase was consistent with the active compound being a peptide without essential polysaccharide or lipid moieties [32]. By and large, all of these characteristics provided preliminary support for the proteinaceous nature of the inhibitor, which we subsequently showed to be a DL.

Several reports in recent decades have indicated that DL possess limited antibacterial activity [14,17] and are toxic to various human and animal cells [13–15], and mitogenic for human lymphocytes [10]. However, whether each of these observations can be definitively attributed to the DL content of the test preparations is sometimes not clear [20]. There are no very recent studies of the toxicity of the different variants of DLs from CNS. In the present study, there was little CPE evident at the MIC level for EMRSA15-A208 of E229DL against Vero cells and human erythrocytes *in vitro*, and this indicates that further research in this area is warranted. In other words, the present study at least indicates that the reported toxicity of DL from *S. aureus* strains may not be representative of the activities of DL produced by CNS.

It is noteworthy that there was a marked difference in the MIC of the native (16 μg/ml) and the synthetic (512 μg/ml) forms of E229DL. The basis of this difference requires further investigation, as it is currently unexplained. Our own circular dichroism measurements with related peptides suggest that they do not aggregate when rehydrated at the stock concentrations used (22.5269 μM), so this should not have affected activity. The native form of E229DL appears to carry a formyl-Methionine. The synthetic peptide was not formylated, but it has previously been reported that such

modifications in similar peptides do not have a marked affect on antimicrobial activity or propensity to form helices [8]. In the same paper, it was shown that net charge and hydrophobic properties are important in determining antimicrobial activity of these peptides, properties that have also been closely linked to hemolytic activity in cationic peptides [31]. Given their identical sequences, hydrophobicity and charge should be the same for native and synthetic peptides, though synthetic E229DL carried an acidified C-terminus and synthesis with an amidated C-terminus would have increased the net charge on the peptide. Previous work has shown that addition of acid or amide termini to short cationic peptides can either have no affect on antibacterial activity, or can lead to significantly different activity, depending on the synthesized peptide [31]. Thus, amidation of the C-terminus may have increased the antimicrobial activity, though it would have been likely to compromise the limited hemolytic activity seen in the native peptide. Given the conflicting data in the field, it is not currently possible to predict the effect of such modifications on the antibacterial activity of short cationic peptides, but further work is warranted in this area.

Dhople and Nagaraj reported that the native form of the 26-residue DL of *S. aureus* does not exhibit antibacterial activity [8]. This was not the case with the native 25-residue E229DL. In addition, Dhople and Nagaraj reported that the *in vitro* synthesis of derivatives of the DL of *S. aureus* changed the nature of its activity. For instance, when proline and aspartic acid residues were substituted with positively charged lysine, antibacterial activity was exhibited and erythrocyte hemolysis was decreased. In addition, they mentioned that the hemolytic activity was abolished when proline was introduced instead of valine in position 13. In contrast, the current study reports the presence of antibacterial activity in the native form of the DL and the lack of hemolysis, at least at the MIC for EMRSA15-A208. Compared to the sequence of DL from *S. aureus* that was investigated by Dhople and Nagaraj, E229DL showed a number of different amino acids, but alanine, encoded in position 24, remained a signature specific for E229DL and might be the key determinant of its antibacterial activity and diminished hemolysis (Fig. 1).

It has been reported that DLs are cytolytic for a wide range of membranes including bacterial protoplasts and spheroplasts [20]. However, the exact mechanism of action has remained undetermined [21]. In the current study, firstly, E229DL was seen to be hydrophobic, which was shown by its delayed elution from C_{18} resins and GRAVY score of 0.440. Secondly, the charged residues comprised three aspartic acids and four lysines, forming 12% and 16%, respectively of the peptide. The cationic residues were located predominantly in the C-terminus of the peptide. Thirdly, electron microscopy of cells exposed to highly purified E229DL revealed that the damaged part in EMRSA15-A208 was the cell wall, indicating the possibility of pore formation and cell rupture. Other short, cationic, hydrophobic peptides have been shown to bind to the negatively charged lipid-II of the cell membrane of EMRSA15-A208, resulting in pore formation in the cell wall [2,3,8,28,39]. Whether this is the case for E229DL requires further investigation. Evidence from previous studies suggests that the periodic distribution of the charged residues, at least in the case of *S. aureus* DL, results in an alpha-helical structure with a strongly surface-active amphipathic rod with hydrophobic and hydrophilic faces since it is inactivated by phospholipids [6,12,20,39].

The strong homology between E229DL and the DL-II in *S. warneri* implies that E229DL could also be classified as DL-II. On this basis, a comparison of the DL structural gene in strain E229 with the *hld-II* genes in staphylococci, in general, and *S. warneri* in particular indicated that the codon responsible for the production of the alanine residue at position 24 possibly emerged as a result of a point mutation change from adenosine (tyrosine codon) to guanine (alanine codon). DL-I from *S. warneri* has been shown to have activity against *Legionella* species, while lacking activity against staphylococci [38]. In the current study, E229DL was shown to elicit weak activity against two strains of *L. pneumophila*. The only previous report of DL with activity against staphylococci referred to the analysis of synthetic peptides derived from the sequence of *S. aureus* DL [8], but this study did not examine inhibition of *Legionella*. Consequently, no direct comparison of the activity of E229DL with these derivative peptides can be made. It appears that an extensive examination of the activity spectra of some of the key peptides previously described as DL may illuminate sequence motifs that correspond to specific activities.

Investigations were conducted to estimate the rate of mutation per generation (cell division) and the frequency of spontaneous mutants present in a given population [22]. The findings presented here showed a very low rate of generation of resistance and a low frequency of naturally resistant strains.

This study introduces the concept that DL can display relatively broad-spectrum antibacterial activity, with a focus on inhibition of staphylococci. We describe the purification to homogeneity of the active agent from *S. epidermidis* strain E229 and demonstrate that at concentrations in excess of the antibacterial MICs for EMRSA15-A208, this peptide is non-hemolytic and not cytotoxic.

Conflict of interest

All authors have nothing to declare.

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