Discovery of an unusual antimicrobial peptide, NI04, active against antibiotic-resistant Gram-positive bacterial pathogens

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Introduction

Rapid Spread of antibiotic resistance (AMR) among pathogenic bacteria, combined with diminished new antibiotic discovery, is an increasing threat to human health (Livermore 2011). Bacterially derived antimicrobial peptides (bacteriocins) can be the solution. This study embraces natural antimicrobial product screening and combines it with modern HPLC, mass spectrometry (MS), genomics and bioinformatics techniques that negate lengthy de-replication and reverse genetic methods, in order to identify and characterise an effective bacterially derived novel inhibitor (peptide NI04) of Gram positive AMR bacteria.

Peptide NI04, isolated from *Bacillus pumilus*, has been demonstrated to inhibit Gram positive AMR pathogens MRSA and VRE effectively and has interesting homology with a known virulence factor in *Mycobacterium tuberculosis* and *Staphylococcus aureus*.

Methods

Purification and initial characterisation: Peptide NI04 was purified using solid phase extraction followed by RP-HPLC and its physicochemical properties were confirmed using enzyme and heat stability tests. Spectrum of activity and MIC were determined using a wide range of Gram positive pathogens, including antimicrobial resistant species following CSLI guidelines (http://clsi.org/).

***In vitro* toxicity studies:** Toxicity of peptide NI04 was determined towards human keratinocyte and monkey Vero cells using trypan blue exclusion and neutral red uptake assays Repetto et al. 2008 and haemolytic activity tested using mouse blood erythrocytes.

De-replication and *De novo* peptide sequencing: The mass of the peptide was initially determined using ESI-MS. The draft genome sequence of the producer strain was obtained and the peptide sequence and underlying gene responsible for production was determined through a de novo peptide sequencing approach, using tags from tandem MS fragmentation of the tripsinised peptide using an Orbitrap MS system.

Draft genome sequence determination: The draft genome sequence of the *B. pumilus* producer was determined using the Illumina HiSeq platform. Raw data were assembled using CLC main workbench and the genome sequence annotated using Prokka annotation tool with B. pumillus SAFR-32 strain as a reference.

Results

- Minimum inhibitory concentration (MIC) assays have shown peptide NI04 to be highly effective against MRSA (40ug/ml) and VRE (20ug/ml)(Table 1).
- Enzyme and heat stability studies confirmed highly heat stable proteinatious structure of pumicin NI04 (Table 2).
- No haemolytic activity and no toxicity against eukaryotic cells (Table 2).

Table1: MIC of peptide NI04 against a selection of clinically important bacterial species.

Gram positive indicator strains	Activity of NI04
M. luteus	**10 ug/ml**
S. aureus 308	**20 ug/ml**
S. aureus 1195	**20 ug/ml**
MRSA	**40 ug/ml**
VREnterococci	**20 ug/ml**
S. pneumoniae	**20 ug/ml**

- The ESI-MS analysis of the HPLC purified peptide revealed peptide NI04 to have a mass of 10722.993Da.
- The draft genome sequence of 4,421,862bp was recovered with an N50 value of 55,948 from a total of 5,996,404 sequence reads.
- Protein sequence for peptide NI04 was determined by protein sequence tags that matched to the draft genome sequence (Figure 1).
- HHPRED and PSI-BLAST showed peptide NI04 has strong similarities to ESX-A peptide belonging to the ESAT-6/WGX100 peptide superfamily, which is associated with virulence in S. aureus and M. tuberculosis (Burts et al. 2005; De Jonge et al. 2007).

Table 2: Physicochemical and preliminary toxicity assesment of peptide NI04

Test	Peptide NI04
Cell toxicity (Keratinocyte/Vero cells)	**Non toxic up to 18x the MIC**
Haemototoxicity (mice blood)	**Non toxic up to 18x the MIC**
Heat stability at 80^{0}c for 30mins	**Yes**
Enzyme stability	**Protease Inhibition only**

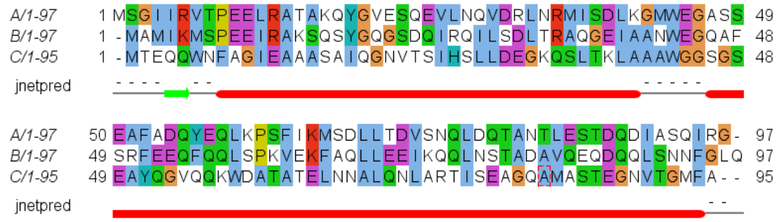

Figure 1: Alignment of pumicin NI04 (A) against ESX-A peptides known to be associated with virulence of *S. aureus* (B) and *M. tuberculosis* (C)**.**

Conclusion

These findings reveal an interesting observation as, to the best of our knowledge, there have been no antimicrobial peptides identified from this family and peptide NI04 does not fit with any existing bacteriocin classification scheme, as it is larger than 10kDa while still being heat stable. We believe that further study of this peptide may reveal interesting new facts about bacterially derived antimicrobial peptides.

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