The Potential of the Inhibitory Substance (E234) as a Possible Therapeutic Agent for Treating Staphylococcal Infections

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Background:
For more than 100 years, *S. aureus* has remained a versatile and dangerous pathogen in humans (Falagas *et al.*, 2006). The frequencies of both community-acquired and hospital-acquired staphylococcal infections have increased steadily, with little change in overall mortality. In addition, treatment of these infections has become more difficult because of the emergence of multidrug-resistant strains (van Griethuysen *et al.*, 2005).

In recent years, there has been much focus on a promising class of bacteriocins known as lantibiotics. The lantibiotics are antimicrobial peptides that are produced by some bacteria and are generally active against other bacteria belonging to the same or closely-related species (Hancock & Rozek, 2002). Gram-positive bacteria have attracted the focus of many researchers as class-I bacteriocin producers, i.e. the cationic peptide, lantibiotics. The most prominent representative of lantibiotics, nisin, has already a long history of use in the protection of foodstuffs (Mathiesen *et al.*, 2005). Lantibiotics have also been considered for application in humans; however, they have not yet been used in the setting of chemotherapy on the same scale as traditional antibiotics.

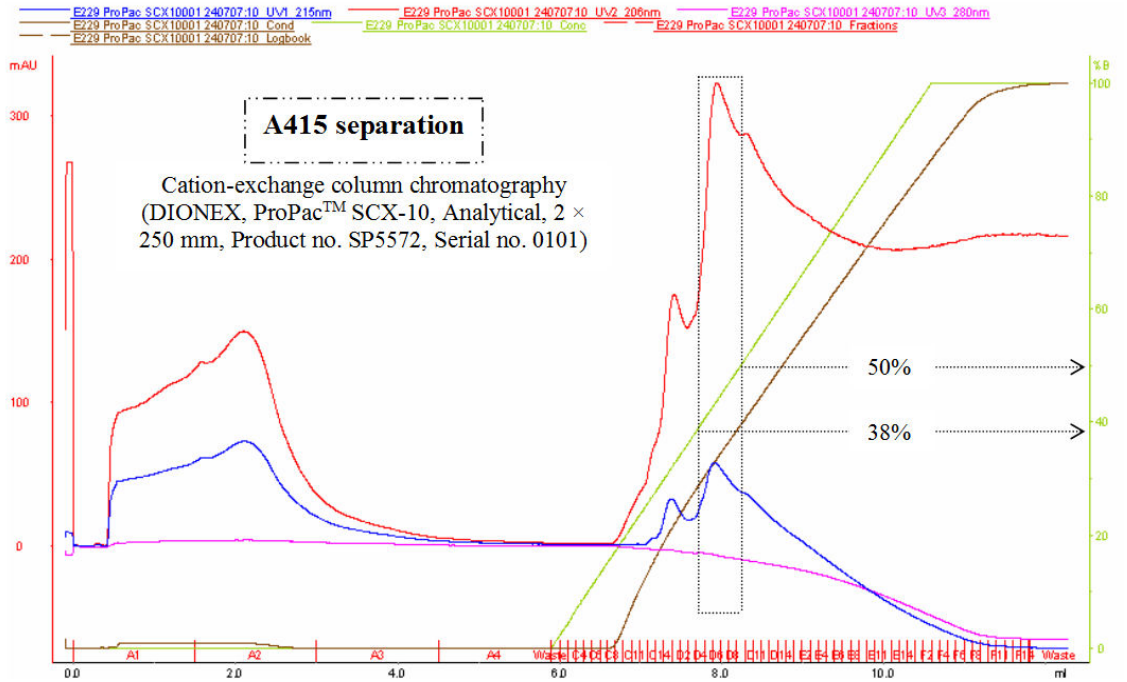
We here investigate the cationic peptide antibiotic (E234) as a future opportunity for treating staphylococcal infections.

Methods:
- *S. epidermidis* strain E234 was maintained on Columbia blood agar (CBA) (Oxoid) but was propagated in Trypticase soy broth (TSB) (Oxoid).
- The initial investigation of *S. epidermidis* strain E234 was carried out using simultaneous-antagonism assay (Tagg & Bannister, 1979) against strains of MRSA indicators (Gratia, 2000).
- Further characterization of the type of activity elaborated by E234 was through deferred antagonism (Tagg & Bannister, 1979); [at room temperature (RT) and 80^{o}C for 1h] (Figure 1).
- TSYcat80A media (30 g of Oxoid Trypticase soy powder per liter, 20 g of Bacto yeast extract per liter, 5 g of Bacto Calcium carbonate per liter, 10 g of Oxoid Biological agar, 5 ml tween-80) was used for the production of E234 by *S. epidermidis* strain E234.
- Partial purification of non cell-associated (free) inhibitors in broth supernatants was carried out using ammonium sulphate precipitation.
- Sep-Pak® cartridge was incorporated for active ammonium sulphate fractions to further separate contaminants.
- The active ammonium sulphate fractions were further purified using cation-exchange column chromatography (ProPac^{TM}).
- High-pressure liquid chromatography was the last step in the purification stage.
- Speed-Vac® was used to concentrate methanol fractions.
- MALDI TOF/TOF was used for molecular mass determination.
- Electron microscopy was used for ultra-structure diagnosis.
- All biological tasting of the active fractions were carried out using well-diffusion assay (du Toit & Rautenbach, 2000).
- Gene analysis of the lantibiotic operon was carried out using common designed primers (obtained from Dr. P. Wescombe, University of Otago - not published yet).
- The gene was cloned using T-tailed vector; and then sequenced.

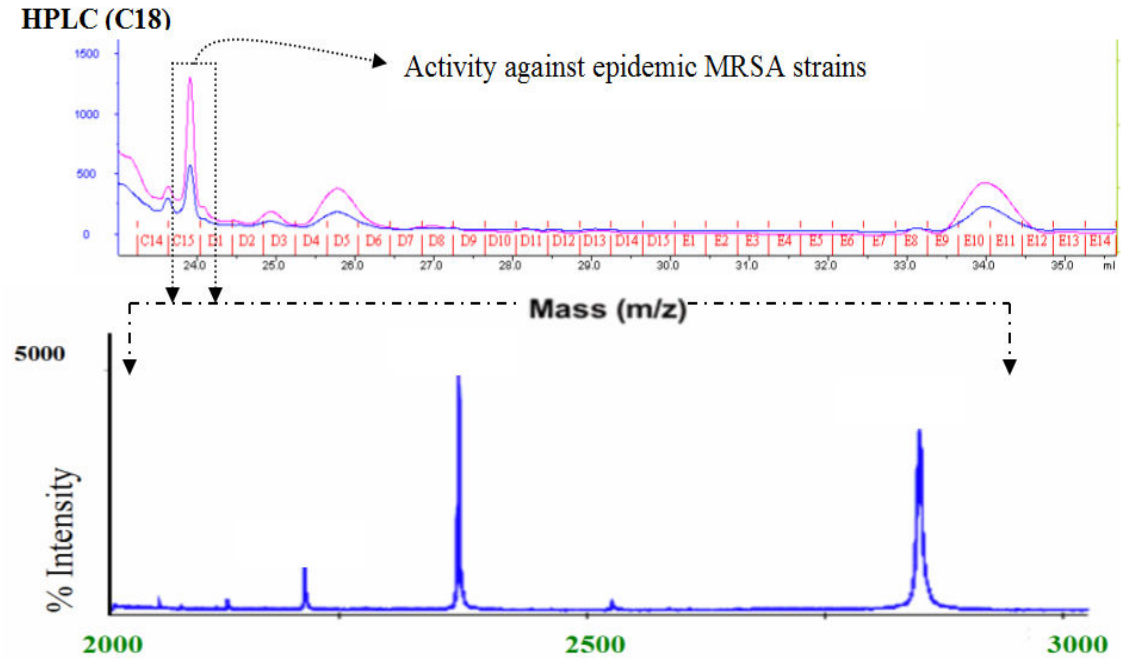
Figure 1 deferred antagonism assays of E234 against a number of staphylococcal indicator strains.

Results:

The characterization of the biological activity of E234 showed that it is a heat-stable agent and displaying specificity for the closely-related *Staphylococcus* spp (Graph 1). The high ammonium sulphate saturation (≥80%), needed for precipitating E234, indicated a small molecular mass inhibitory agent. Based on the electron microscopy diagnosis, E234 shows obvious damage to the protective cell wall of the sensitive indicators (Figure 4). The small size was confirmed using MALDI TOF/TOF, which showed 3 species and sized the mass of E234 in a window between 2100.5 and 2900.9 Da (Figure 3). The designed primers showed positivity confirming the presence of the lantibiotic gene.



Graph 1 Separation of ammonium sulphate active fractions of E234 using cation-exchange chromatography (ProPac™).



Graph 2 Separation of E234 ProPak™ active cuts by HPLC (C18) [top par]; analysis of HPLC active fractions by MALDI TOF/TOF [lower part].

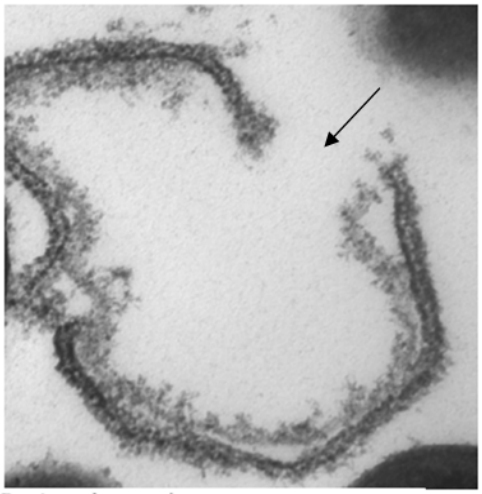


Figure 2: Thin section of EMRSA-15 strain A208 cell wall after incubation with E234 for 24 h at 37°C. A rupture wall is seen (arrow).

Conclusions:

- The biological activity of the highly-purified extract of E234, which is produced by *S. epidermidis* strain E234 and shows specific inhibitory activity against the resistant *S. aureus*, was characterized.
- The small molecular mass of E234 that was isolated (2100.5 - 2900.9 Da) maintained its inhibitory activity at high temperature (80°C/1h) against staphylococcal indicators, which suggests that E234 is a bacteriocin in nature, possibly of Class-I (lantibiotic). This suggests further purification using high resolution HPLC to eliminate, if any, unrelated species.
- The pH of the liquid phase (5.2) used in cationic-exchange separation and late elution of E234 from C18 reverse-phase column suggest that it is a hydrophobic in nature.

- The damage of the protective out-side layer observed in the electron microscopy diagnosis postulates that the reason for killing *S. aureus* is by targeting the negatively-charged lipid II (a bacteria-specific membrane component, which is essential in bacterial cell-wall synthesis) by the hydrophobic E234 agent, resulting in its lysis.
- The cloning of the amplified gene was successful and the obtained gene sequence will further be deduced back to its functioning peptide (data not shown), which then will be synthesised and re-tested for activity against staphylococcal indicators.
- These overall data explain the potency of E234 as a possible future therapeutic agent for treating highly drug-resistant staphylococcal infections.

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