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Atomic force microscopy study of the antimicrobial action of Sushi peptides on Gram negative bacteria

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Abstract

The antibacterial effect of the endotoxin-binding Sushi peptides against Gram-negative bacteria (GNB) is investigated in this study. Similar characteristics observed for Atomic force microscopy (AFM) images of peptide-treated *Escherichia coli* and *Pseudomonas aeruginosa* suggest that the Sushi peptides (S3) evoke comparable mechanism of action against different strains of GNB. The results also indicate that the Sushi peptides appear to act in three stages: damage of the bacterial outer membrane, permeabilization of the inner membrane and disintegration of both membranes. The AFM approach has provided vivid and detailed close-up images of the GNB undergoing various stages of antimicrobial peptide actions at the nanometer scale. The AFM results support our hypothesis that the S3 peptide perturbs the GNB membrane via the "carpet-model" and thus, provide important insights into their antimicrobial mechanisms.

Keywords: Gram negative bacteria; Antimicrobial Sushi peptide; Lipopolysaccharide; Membranolytic; Atomic force microscopy

1. Introduction

Bacterial resistance to traditional antibiotics has been on an increase [1,2]. As such, there is a need for a novel family of antimicrobial drugs. Antimicrobial peptides show good prospects for this family as their postulated mechanism of action is unlike that of traditional antibiotics [3,4]. Although many of such peptides have been worked on, their exact mechanisms of action are still under exploration [5–10]. Nevertheless, it is postulated that these peptides probably possess the same mechanism(s) of antimicrobial action and that they in particular act by targeting the bacterial membrane [11,12]. The cationic peptides first bind to negatively charged lipopolysaccharide (LPS) of Gram negative bacteria (GNB) [11–15]. This binding may cause membrane penetration in a variety of ways: (a) slight disturbance of phospholipid chain order and packing in the outer membrane (self-promoted uptake model) [13]; (b) transmembrane channel creation (barrel-stave or toroidal pore model) [16]; (c) damage of the bilayer via toroidal pores, disruption of lipid micelles and vesicles via the "carpet model" [12,17–19]; or (d) creation of micelle-like accumulation of peptides in the membrane (peptide-aggregate model) [3]. All these modes of action eventually kill the bacteria.

The horseshoe crab has a powerful innate immune system. During infection, the bacterial LPS activates Factor C to trigger a coagulation cascade in the horseshoe crab [20]. Factor C acts as a biosensor [2,20] which reacts to pg levels of LPS [4,20]. The LPS binding motif(s) of Factor C show extremely high affinity for LPS [21–23]. Close to the N-terminus of the multidomain Factor C molecule [24], are a number of repeating units of Sushi domains of ~60 amino acids each, which bind the LPS. The LPS binding region of Sushi 3 has been defined within a 34-mer peptide, hereafter referred to as S3. S3 has been shown to bind and kill GNB [25]. Akin to most antimicrobial peptides, the mechanism of

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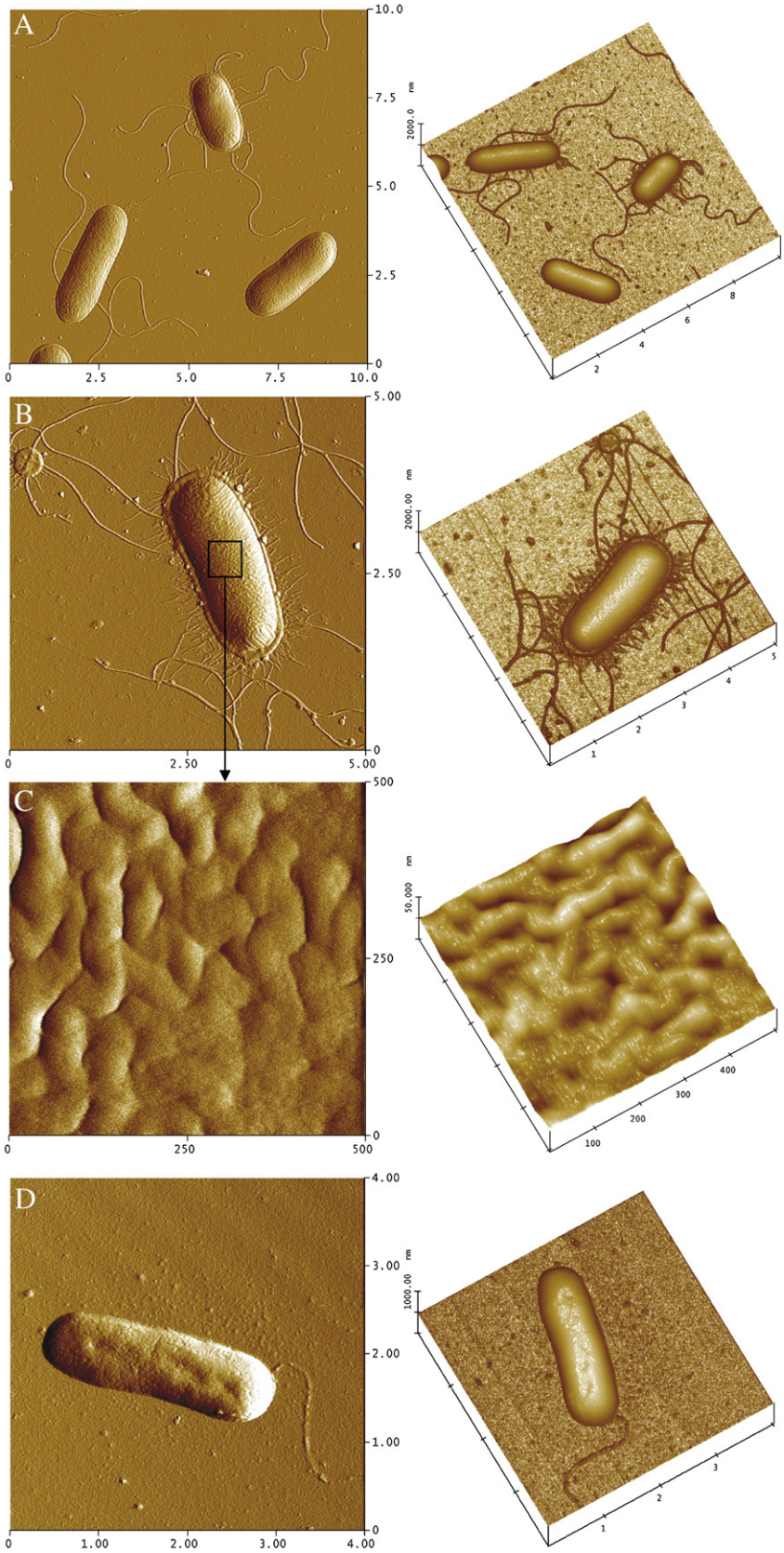

Fig. 1. Images of untreated *E. coli* and *P. aeruginosa* (the left panels show deflection images and the right panels are 3D reconstructions based on height data). (A) 10 × 10 μm scan size image of untreated *E. coli*. (B) 5 × 5 μm scan size image of single untreated *E. coli*. (C) 500 × 500 nm closeup image showing the rough surface of the *E. coli*. (D) 4 × 4 μm scan size image of an untreated *P. aeruginosa*.

action of S3 is postulated to be influenced by LPS [22]. An earlier study proposed that the capability of the S3 dimer to attach to LPS micelles enables it to preferentially perturb the bacterial membrane [4,26] and disrupt the fluidic integrity of the bacterial membrane by the 'carpet model' [26]. Being non-cytotoxic [22] and non-hemolytic [25] to the host, S3 is an excellent antimicrobial peptide candidate.

Atomic force microscope (AFM) is a powerful imaging tool which is capable of achieving high resolution images of biological samples even under physiological conditions such as in fluid and at physiological temperature. The usefulness of AFM to study antimicrobial peptide mechanisms has been proven through a series of experiments either on model membrane or intact cells [27–31].

Based on the above understanding which used LPS micelles and LPS vesicles in vitro, we further examined the antimicrobial mechanism of action of S3 against the whole bacteria. AFM is employed to image the peptide-treated bacteria. The AFM images obtained have provided significant insights into how S3 perturbs GNB.

2. Materials and methods

2.1. Peptides

Peptides used in this study were synthesized and purified to >95% purity by Genemed Synthesis Inc. (San Francisco, CA). The native S3 peptide, N-HAEHKVKIGVEQKYGQFPQGTEVTYTCSGNYFLM-C, with a molecular weight of 3,892, corresponds to residues 268–301 of the Factor C Sushi 3 domain (GenBankTM/EBI accession number S77063).

2.2. Bacteria

Escherichia coli ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were grown overnight at 37 °C in nutrient broth (Oxoid).

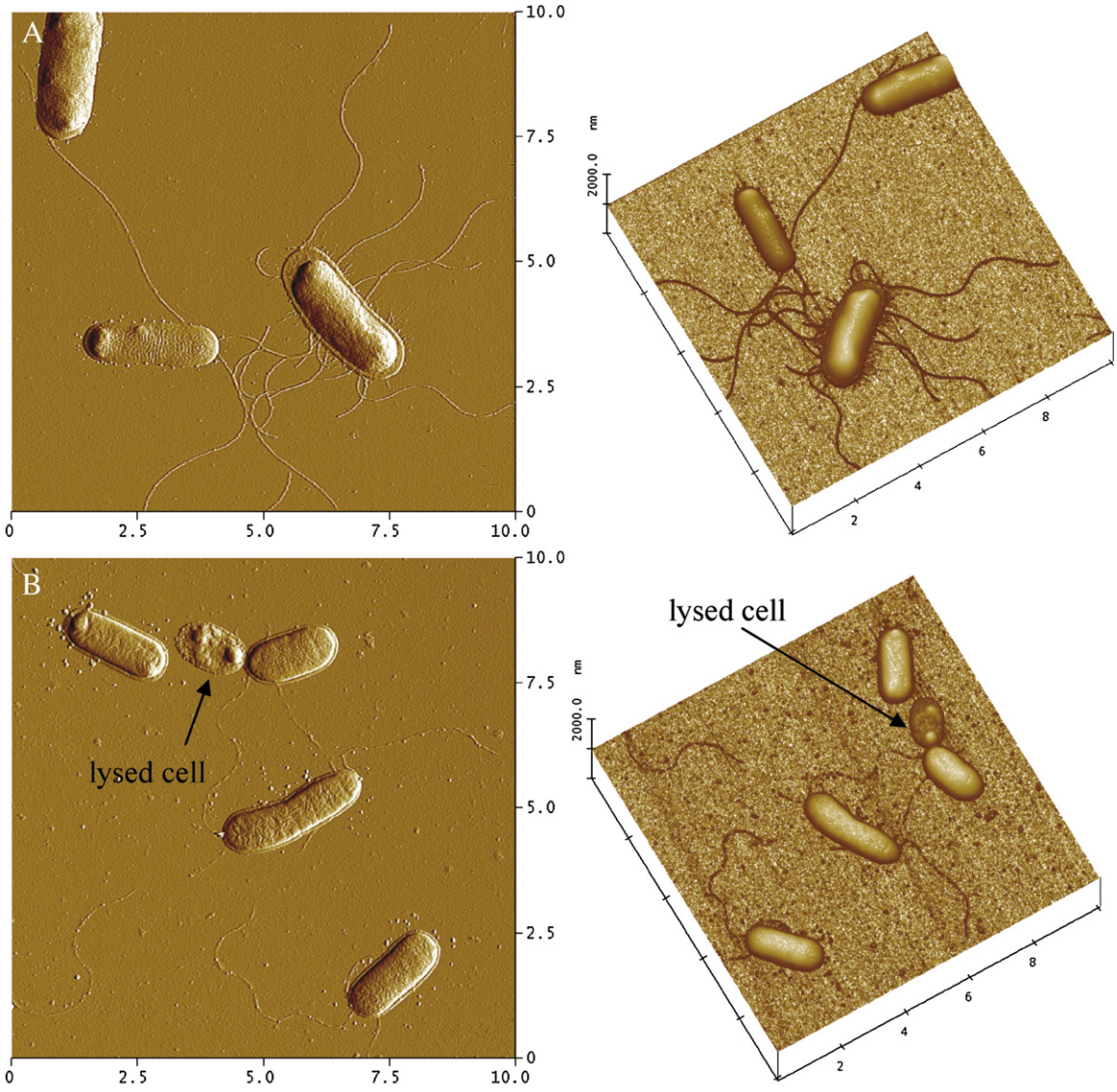

Fig. 2. Gram negative bacteria which have undergone 26 h incubation in distilled water (the left panels show deflection images and the right panels are 3D reconstructions). (A) *E. coli*. (B) *P. aeruginosa*. The arrow indicates a lysed cell in panel B.

2.3. Sample Preparation

E. coli and *P. aeruginosa* were resuspended in deionized distilled water and equal volumes of S3 and bacteria were incubated in depyrogenised borosilicate tubes for durations of 1, 5, 15, 30 and 60 min at room temperature. The initial bacterial populations were kept constant at 10^6 cfu/ml, while varying concentrations of S3 were used, namely 0.25, 0.5, 1.0, and 5.0 μM, in the reaction mixtures. After incubation, the peptide-treated bacterial suspensions were applied onto poly-L-lysine pretreated glass slides and air dried in a vacuum desiccator. Control untreated bacteria were similarly prepared within 2 h of being resuspended in distilled water. Overnight cultures in the distilled water were also tested to exclude the possible osmotic effect by water.

2.4. AFM analysis

Imaging was performed with a Nanoscope III Dimension 3100 AFM (Digital Instruments, Santa Barbara, CA). Standard silicon AFM cantilevers, OTR8, with spring constant of 0.15 N/m, and a nominal tip radius of <20 nm were used to image both the controls and peptide-treated bacteria. The samples were imaged in air using the contact mode with settings of 512 pixels/line and 1 Hz scan rate. Some of the images were first order flattened and contrast enhanced using Nanoscope 5.13 for better demonstration on details.

3. Results and discussion

3.1. AFM images of control E. coli and P. aeruginosa

The AFM images of freshly prepared untreated *E. coli* showed characteristic rod shape with the distinctive peritrichous flagella and pili structures (Fig. 1A, B). The cell surface displayed roughness similar to that observed by Ricci et al. [30] (Fig. 1C).

The AFM images of freshly prepared *P. aeruginosa* also showed rod shape cell with polar-monotrichous flagellum on the cell. The surface is comparatively smooth compared with *E. coli* (Fig. 1D).

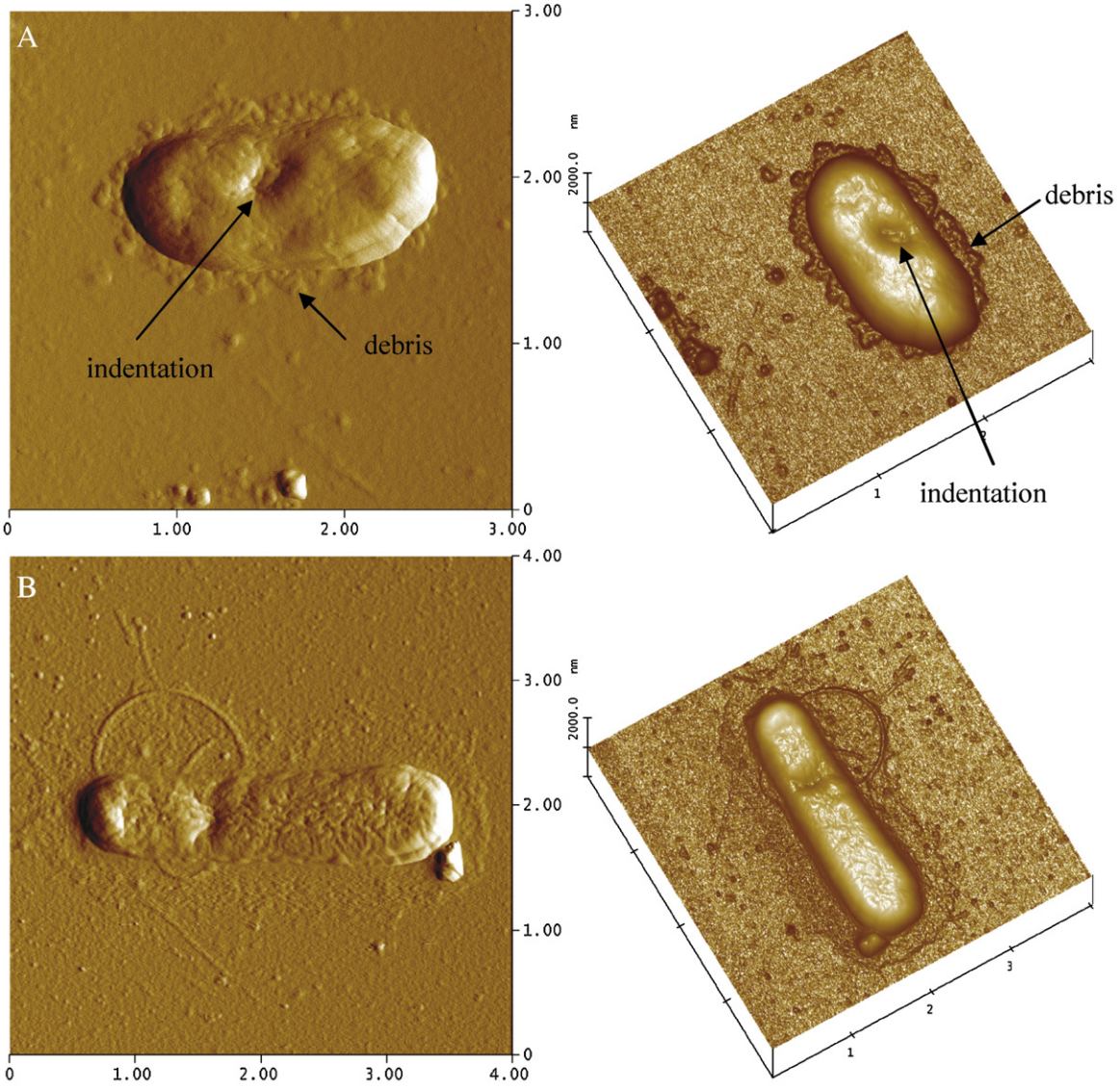

Fig. 3. Indentations and outer membrane debris (arrows) found on and around GNBs treated with low concentration peptide (the left panels show deflection images and the right panels are 3D reconstructions). (A) *E. coli* treated with 0.25 μM of S3 for 15 min. (B) *P. aeruginosa*: treated with 0.25 μM of S3 for 15 min.

To check the osmotic effect of distilled water on the morphology of bacteria, we incubated both *E. coli* and *P. aeruginosa* in distilled water for 2 h (within the range of duration of peptide treatment), 26 h and 48 h. Results indicated that only slight dehydration and indentation were observed on the cells (Fig. 2). Furthermore, some lysed cells were observed after 26 and 48 h incubation but their morphologies were distinctly different from those treated by S3 peptide (Fig. 2B).

3.2. AFM images of low concentration S3 treated E. coli and P. aeruginosa

At the low concentrations of S3 (0.25, 0.5, 1 μM), the initial morphological changes observed are that of indentations appearing on the surface of some cells as well as some micelle-like structures or outer membrane residues found around the cells (Fig. 3). This indicates the disruption of the outer membrane of the bacteria, probably due to direct interaction and binding of S3 to the LPS. This perturbation may have damaged the outer membrane, thus exposing the peptidoglycan wall beneath it. This observation is consistent with that made by Da Silva et al. [31], where the *E. coli* was treated with the antimicrobial peptide PGLa. It supports the hypothesis that S3 preferentially reacts with LPS micelles aggregated on GNB outer membrane and perturbs the membrane by the "carpet-model" [26].

The next stage of morphological change arising from low concentration treatment is shown in Fig. 4. Some fluid and debris can be observed around the apical end of the cells. As can be seen from Fig. 4B, the outer membrane collapsed while the inner part of the cell still remained intact. It is postulated that the debris originates from the bacterial periplasm. S3 first binds to negatively charged LPS of GNB to enable its initial penetration

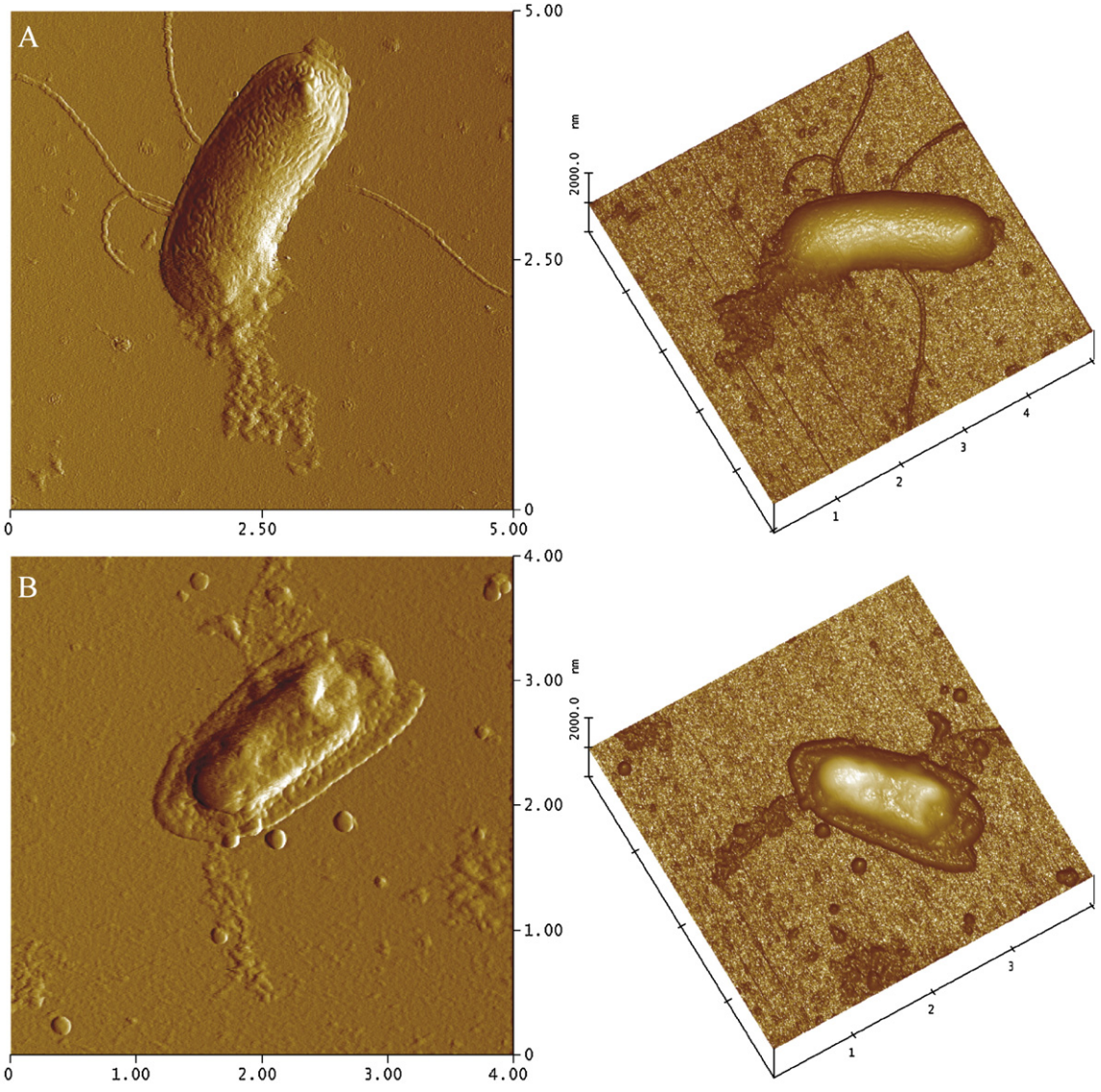

Fig. 4. Small amount of leakage of periplasmic fluid found at the apical end of GNB treated with lower concentration of peptide (the left panels are deflection images and the right panels are 3D reconstructions). (A) *E. coli* treated with 0.5 μM of S3 for 30 min. (B) *P. aeruginosa* treated with 0.25 μM of S3 for 30 min.

into the bacterial outer membrane [11–13] and hence, releasing the periplasmic material. Also, leakage of fluid was found to be at the poles of the bacteria. Thus, it is possible that the apical ends are where the bacteria are being targeted first, or where the damage is first concentrated. This is logical since the domains of cardiolipin (a negatively charged phospholipid) reside at the apical ends of *E. coli* inner membrane (other than the septal regions) [32]. S3 is highly cationic and would have greater affinity for negatively charged phospholipids [4,26,33]. Hence, it is probable that the peptide concentrated at the apical ends, which initiated the cell leakage.

3.3. AFM images of higher concentration of S3 treated E. coli and P. aeruginosa

Fig. 5 shows AFM images of the bacteria exposed to a higher peptide concentration of 5μM for a short period of time. The initial reaction is the leakage of large amount of fluid from the partially disintegrated cells. It is probably the cytoplasmic fluid that had leaked out from the inner membrane of the cell. This indicates that the peptides had caused damage to the bacterial inner membrane.

When exposed to high S3 peptide concentration of 5 μM for a longer period of time, Fig. 6 shows copious amount of cytoplasmic fluid exuded from the bacteria. These bacteria appear either severely damaged or their membrane fully collapsed. This indicates drastic permeabilization of the inner membrane. It is suspected that S3 binds to the LPS on the outer cell wall but at higher concentration, there appears to be a speeding up of events as the peptides are known to show co-operativity of binding with their target ligand to result in such aggregation of the bacteria [4,26].

In summary, the action of S3 can be classified into three stages. In the initial stage, S3 damaged the bacterial outer

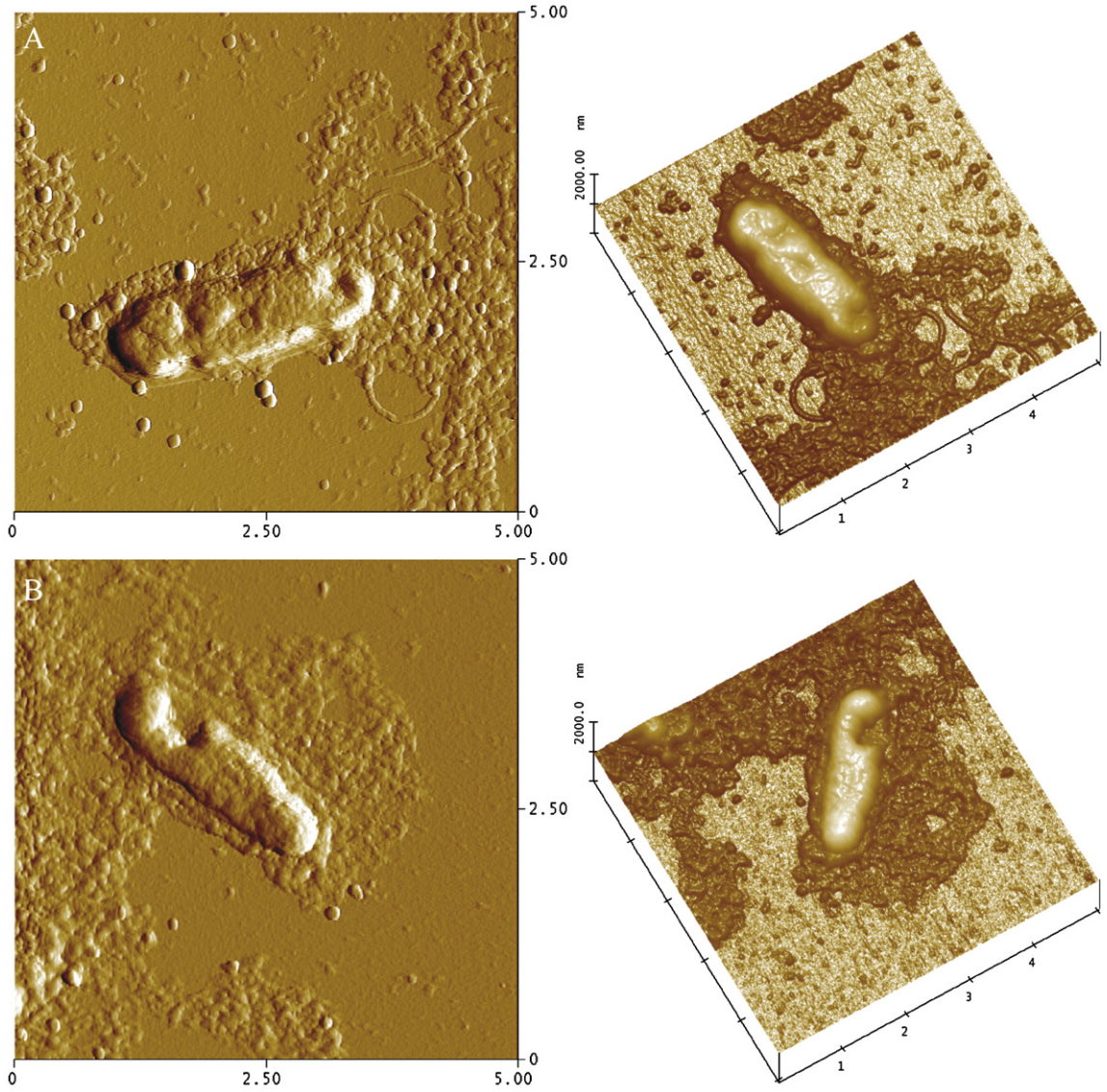

Fig. 5. Extensive leakage of cytoplasmic fluid from the partially disintegrated GNBs when exposed to 5 μM of S3 for a short period of time (the left panels show deflection images and the right panels are 3D reconstructions). (A) *E. coli* treated with 5 μM of S3 for 5 min. (B) *P. aeruginosa* treated with 5 μM of S3 for 1 min.

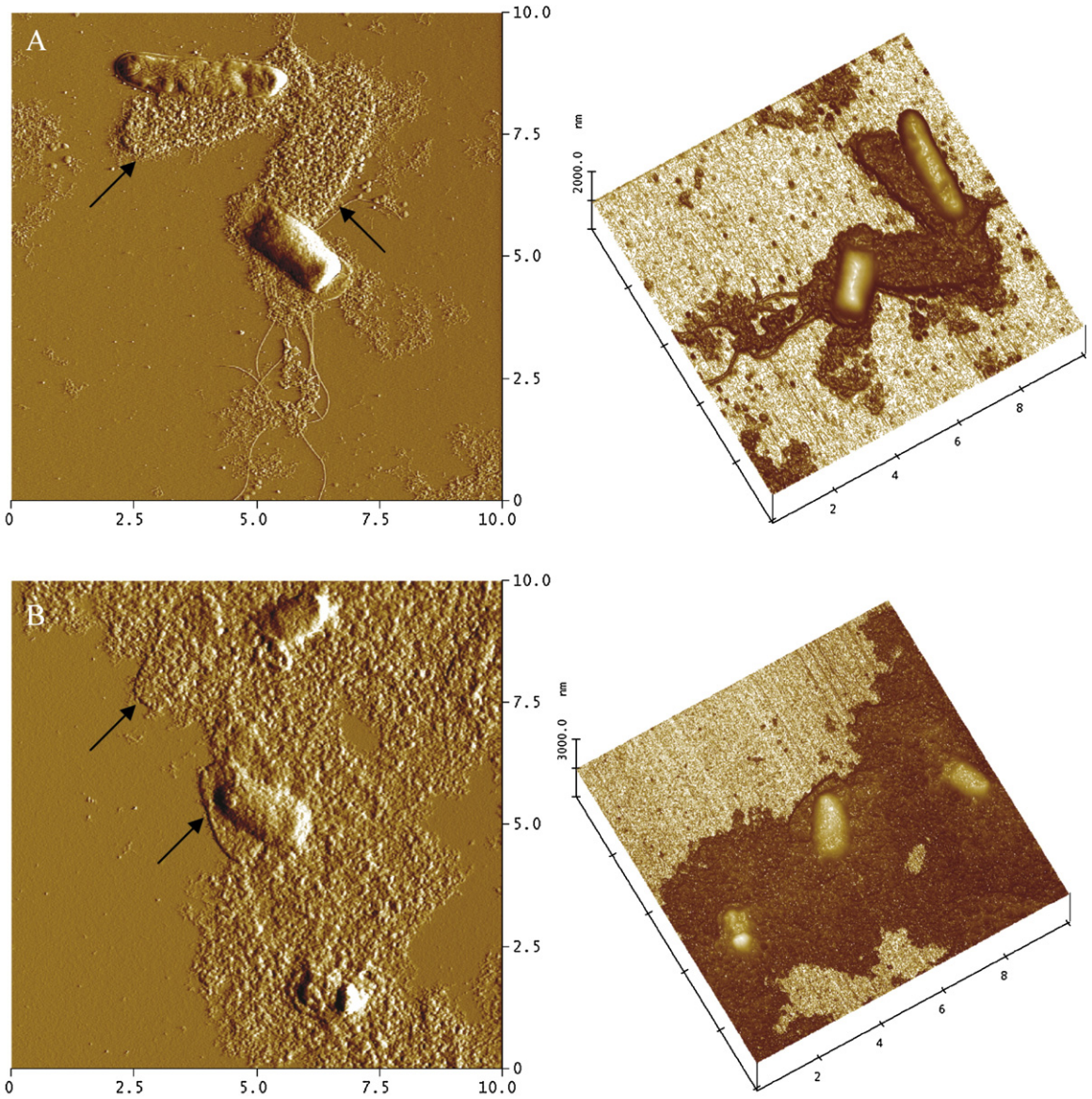

Fig. 6. Severely damaged GNBs with cell debris as well as large amount of exuded cytoplasmic fluid from bacteria treated with 5 μM S3 peptide (the left panels are deflection images and the right panels are 3D reconstructions). (A) *E. coli* treated with 5 μM of S3 for 1 h. (B) *P. aeruginosa* treated with 5 μM of S3 for 30 min. Arrows indicate the outlines of lysed cells.

membrane, which is mainly composed of LPS, causing the formation of LPS micelles. This supports the "carpet-like" mechanism, which had been proposed to account for the action of S3 in an earlier study [26]. The indentations on the cells as well as the debris and small amount of fluid found around the damaged cells suggest that the outer membrane and peptidoglycan wall structures were affected by the action of S3. In the second stage, permeabilization of the inner membrane was initiated, leading to the leakage of cytoplasmic fluid. Further membrane damage resulted in large losses of cytoplasmic fluid. Being devoid of intracellular fluid and organelles, the cell architectural support was compromised and hence the bacteria collapsed. In the third stage, the cell membranes were disintegrated, leaving behind massive amounts of membrane residues or debris. Only bacteria exposed to high concentrations of S3 showed the characteristics of the last stage.

Here, the samples were air dried and then imaged using AFM at different time points of treatment with S3 peptide. However, using this endpoint protocol, the dynamic morphological changes induced by the peptide were lacking. This may only be resolved by visualising the membrane perturbation using real-time AFM. Nevertheless, the AFM approach here is still able to provide substantially vivid and detailed close-up images of the GNBs undergoing the various stages of antimicrobial peptide actions, down to the nanometer scale and hence, gives further important insights into the antimicrobial mechanisms involved.

4. Conclusions

For the first time, an antimicrobial LPS-binding peptide, S3, has been studied on two different species of Gram negative

bacteria, *E. coli* and *P. aeruginosa*. The AFM results obtained suggest that the mechanism of antimicrobial actions of S3 is comparable for both *E. coli* and *P. aeruginosa* and can be classified into three stages: damage of the bacterial outer membrane, permeabilization of the inner membrane and disintegration of both membranes and spillage of cytoplasm. The morphological effects seen in both of the bacteria are vivid and dramatic. The results of this study are consistent with and validates earlier studies where different biochemical and biophysical approaches were used.

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