Mucroporin, the first cationic host defence peptide from the venom of *Lychas mucronatus*

**Running title:** a new template for antimicrobial peptide design

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ABSTRACT: The misuse of antibiotics has led our age to a dangerous edge, as antibiotic-resistant pathogens appears evolve quickly than antibiotics are invented. Thus, new agents to treat bacterial infection are badly needed. Cationic host defence peptides are on the first line of a host defence system, and are thought to be good candidates for treating bacterial infection. Here, a novel cationic host defence peptide, mucroporin, was cloned and characterized from the venom of Lychas mucronatus. The MIC for Staphylococcus aureus was 25\(\mu\)g/mL, including antibiotic-resistant pathogens. Based on the molecular template of mucroporin, mucroporin-M1 was designed by amino acid substitution. The MIC for Staphylococcus aureus was 5\(\mu\)g/mL, including antibiotic-resistant pathogens MRSA, MRCNS, PRSA and PRSE. Moreover, mucroporin-M1 also inhibited Gram-negative bacteria. The modes of action of mucroporin and mucroporin-M1 were both rapid killing by disrupting the cell membrane of bacteria, and the number of surviving bacteria was reduced by about 4-5 orders of magnitude immediately after peptide delivery. These results revealed that mucroporin could be considered a potential anti-infective drug, especially for treating antibiotic-resistant pathogens.

KEYWORDS: Cationic host defence peptide, Mucroporin, Scorpion venom, Rapid killing, Antibiotic-resistant, MRSA, Therapeutic
INTRODUCTION

When a new antibiotic comes into use, resistance will follow. During the development period of antibiotics discovery, when resistance to former antibiotics appeared, drug substitution was used to treat resistant pathogens. However, during the last decade, it seems that the frequency of antibiotic resistance, especially the evolution of multi-drug-resistant pathogens, happens faster than the invention of new antibiotics(44, 48). Among the existing antibiotic-resistant pathogens, MRSA (Methicillin-resistant Staphylococcus aureus) is considered the most lethal one. MRSA was first identified as a hospital-acquired pathogen in the 1960s, which was resistant to the entire class of β-lactam agents(20). Over the past 40 years, MRSA infections have become endemic in hospitals worldwide, and have recently appeared in communities(13, 20, 38). It was reported that there were roughly 94,000 invasive MRSA cases in the U.S., with roughly 19,000 deaths in 2005, which was higher than the number of death caused by AIDS in the same year(29). Facing the so-called “superbug”(29), we have only got one last weapon to defend ourselves; -vancomycin. Unfortunately, the over use of vancomycin in treating MRSA infection and the existence of vancomycin-resistant enterococcus has lead the emergence of vancomycin-intermediate Staphylococcus aureus(2, 4, 36). Therefore, new kinds of antimicrobial drugs are badly needed(26).

Cationic host defence peptides are produced by many organisms as part of their host defence system(23, 25, 34). These peptides are potent antimicrobial agents against Gram-positive and Gram-negative bacteria, fungi, parasites, and some viruses(9, 21), few of which have been reported to inhibit MRSA growth(42, 43). Studies indicated that the targets of cationic host defence peptides varied from the outer membrane to the signaling
pathway (8, 10, 27), which may be the reason why resistance to cationic host defence peptides is more difficult to attain than to the conventional antibiotics. The broad spectrum activity, low potential to induce resistance, and the huge family of over 1300 peptides (19) make cationic host defence peptides an attractive family of compounds that have the potential to be developed as therapeutic agents.

Cationic host defence peptides are usually very short, ranging from 10-50 amino acid residues with a net positive charge of 2-9 (27, 37, 49, 51). Despite their common physiological effects, cationic host defence peptides vary in both sequence and secondary structure (6, 22, 27, 49). Structurally, natural cationic host defence peptides can be classified as: (i) amphipathic $\alpha$-helix, (ii) $\beta$-sheet structures stabilized with two or three disulphide bonds, (iii) extended structures, and (iv) loop structures with one disulphide bond (40). Besides the main functions of antibacterial, antifungal, antiviral and antitumor, there is increasing evidence supporting the idea that cationic peptides have diverse functions in modulating immune responses, especially infection and inflammation (10, 24, 50). Several peptides are in clinical trial periods (1) and cationic host defence peptides presented the best option over the conventional antibiotics.

Cationic host defence peptides have also been found in scorpion hemolymph (14, 18) and venom, including hadrurin (46), scorpine (15), opistoporins, parabutoporin (39), ISCTs (17), pandinins (16), BmKn2 and BmKb1 (53). The functions of these scorpion derived peptides vary from cytotoxic (17), inhibiting bacteria (15, 46) to inhibiting fungus (18, 39).

In our previous study, we characterized two cationic host defence peptides, BmKn2 and BmKb1, from the venom of Buthus martensii Karsch (53). Here, we describe a novel
cationic defence peptide mucroporin, which is the first cationic host defence peptide characterized from the scorpion *Lychas mucronatus*. We found that mucroporin can effectively inhibit standard bacteria, especially Gram-positive bacteria. The optimizedly design of mucroporin-M1 by amino acid substitution resulted in the inhibition of both Gram-positive and Gram-negative bacteria at low concentrations. We chose *Staphylococcus aureus* as a model bacteria strain to further explore the mechanism of mucroporin and mucroporin-M1’s bioactivity. Some evidence showed that the cell membrane of *Staphylococcus aureus* was broken immediately after the treatment of mucroporin or mucroporin-M1. The assay revealed that the inhibitory effect of mucroporin and mucroporin-M1 was exerted by the action mode of rapid killing. Moreover, the *in vitro* treatment of clinically isolated pathogens showed that mucroporin-M1 is highly capable of inhibiting antibiotic-resistant pathogens, including MRSA, MRCNS, etc. Mucroporin and its analogue present potential anti-infective drugs or lead compounds, especially for treating antibiotic-resistant pathogens.
MATERIALS AND METHODS

cDNA library construction. Scorpion *Lychas mucronatus* was collected in Hainan province, China. Their glands were collected 2 days after electrical extraction of their venom. Total RNA was prepared from the glands using Trizol reagent (Invitrogen) method. Poly(A)-mRNA was purified by a Poly(A) Tract mRNA Isolation System (Promega). cDNA library was constructed according to Superscript Plasmid System cDNA library construction kit (Gibco/BRL). cDNAs were cloned into pSPORT1 plasmids and transformed into *Escherichia coli* DH5α cells. Randomly chosen cDNA clones were sequenced to obtain a reliable representation of the toxin content in the venom gland.

Screening of the cDNA library with PCR strategy. A specific primer was designed and synthesized to screen mucroporin gene (homologue of BmKb1/BmKb2) from the venomous gland cDNA library of *Lychas mucronatus* by PCR method as described(52). The specific forward primer and reverse primer were 5’-TCGACCCACGCGTCCG-3’ and 5’-GCGTTTCCTTCGGCC-3’, corresponding to the digestion sites of the vector and the conserved processing region of the propeptide, respectively.

cDNA sequencing and computer analysis. The plasmids characterized as positive clones were determined by using ABI PRISM™ 377XL DNA sequencer with a universal T₇ promoter primer. Sequence analysis was carried with BLASTX, DNAMAN and GENRUNR. All homologue sequences of mucroporin were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/) by BlastP method. Multiple sequence alignments of mucroporin proteins were carried out using the CLUSTAL X version 1.83 (http://www2.ebi.ac.uk/clustalw/).
**Chemical synthesis.** The peptide was synthesized by GL Biochem (Shanghai) Ltd. China, with a purity of higher than 95%.

**Bacteria strains.** *Escherichia coli* (AB94012), *Pseudomonas aeruginosa* (AB93066), *Bacillus thuringensis* (AB92037), *Staphylococcus aureus* (AB94004), *Bacillus subtilis* (AB91021), *Micrococcus luteus* (AB93113) were purchased from CCTCC (China Center of Type Culture Collection).

Antibiotic-resistant strains were obtained from the 302nd military hospital of Beijing, China, including penicillin-resistant *Staphylococcus aureus* (PRSA) P1383, penicillin-resistant *Staphylococcus epidermidis* (PRSE) P1389, methicillin-resistant *Staphylococcus aureus* (MRSA) P1381, P1386 and P1374, and methicillin-resistant coagulase-negative *Staphylococcus* (MRCNS) P1369. Methicillin-resistant coagulase-negative *Staphylococcus* (MRCNS) 1538 was obtained from the Hubei maternal and child health hospital, Hubei, China.

Antibiotics-sensitive clinical isolates were also obtained from the 302nd military hospital of Beijing, China, including penicillin-sensitive *Staphylococcus aureus* (PSSA) P969, and penicillin-sensitive *Staphylococcus epidermidis* (PSSE) P1111 and P1368.

**MIC determination.** Overnight-cultured bacteria were diluted with Luria-Bertani (LB) medium to about $10^4$-$10^6$ CFU/mL. This bacterial suspension and serial diluted peptides were added to 96-well plates at ratio of 4:1 in a final volume of 100 µL. The microplates were incubated at 37°C with continuous shaking. After 12-16 hours, the optical density $OD_{630}$ was measured with a microplate reader.

Overnight-cultured clinically isolated strains were diluted with LB to 0.5 McFarland. This bacterial suspension and serial diluted peptides were added to 96 well plates at a
ratio of 4:1 in a final volume of 100 µL. The microplates were incubated at 37°C with continuous shaking for 12-16 hours. The optical density OD$_{630}$ was measured with microplate reader.

Each concentration reading was repeated 3 times each. The MIC was determined at the concentration at which there was no optical density. All of the above experiments were repeated at least twice.

**Bactericidal assay.** Overnight-cultured *Staphylococcus aureus* was transferred to LB, and was cultured to exponential phase (optical density at OD$_{600}$ of approximately 0.6). 300 µL peptides solution was added into 1200 µL bacterial suspension and the mixture was incubated at 37°C with continuous shaking. At each time point, 200 µL treated bacterial suspension was transferred to a sterilized 1.5 mL tube. After centrifuging at 1000 g for 5 min, the supernatant was removed and the pellet was resuspended with 200 µL LB. This bacterial suspension was placed on agar plates, and was incubated at 37°C until the viable colonies can be counted.

**Scanning electron microscopy.** Overnight-cultured *Staphylococcus aureus* was transferred to LB, and was cultured to the exponential phase. 300 µL peptides solution was added into 1200 µL bacterial suspension and the mixture was incubated at 37°C with continuous shaking. Two minutes after incubation, the bacterial suspension was centrifuged at 1000 g for 5 min, and the pellet was washed with 0.1M PBS several times, and was then fixed overnight with 2.5% Glutaraldehyde in 0.1M PBS at 4°C. After fixation, the bacteria were washed with PBS for a minimum of 15 min, and were then dehydrated using a series of graded ethyl alcohols (50% for 15 min, 60% for 15 min, 70% for 15 min, 80% for 15 min, 90 for 15min, and 2 changes of 100% for 10 min, each).
After this, the samples were mounted on aluminum stubs with adhesive tabs and sputter coated for 3 minutes using a polaron. The samples were then ready to view on the HITACHI X650 scanning electron microscope.
RESULTS

Sequence analysis. After systemic screening of 500 clones from the venomous cDNA library of *Lychas mucronatus*, we got 8 positive clones. Sequencing of all these positive clones revealed a novel precursor, termed as mucroporin, with high similarities to BmKb1/BmKb2 and a number of antimicrobial peptides from other scorpion species (Fig.1). The mucroporin precursor consists of three parts: 5’UTR, ORF and 3’UTR. The 5’ and 3’UTRs of mucroporin have 10 bp and 120 bp, respectively. At the 3’UTR end of the cDNA, a single AATAAA polyadenylation signal is found 12 bp upstream of the poly (A) tail. An ORF of 222 bp encodes a precursor of 74 amino acid residues (Fig.2).

Mucroporin has a putative 22 residues followed by a presumed 17 residues mature peptide, and an uncommon acidic propeptide at the C-terminal. The 35 residue propeptide starts with a conserved posttranslational processing signal: Gly-Arg-Arg at positions 40-42. Conventionally, removal of propeptide with such processing signal would result in a mature peptide with C-terminal amidation as described(17).

Mucroporin-M1 (LFRLIKSLIKRLVSAFK) was designed based on the protein sequence of mucroporin for the purpose of enhancing the net positive charge of the hydrophilic side. Mucroporin and mucroporin-M1 was synthesized by GL Biochem (Shanghai) Ltd, China, with reliable quality. The molecule weight measured with MS spectrum (1731.22 and 2031.57, respectively) matched with the calculated molecule weight of the amidated mucroporin and mucroporin-M1 (1731.13 and 2031.58, respectively) very well.

Minimal inhibition concentration. The effect of mucroporin and mucroporin-M1 on bacteria was studied by the microdilution method. As shown in table 1, it can be seen that
mucroporin was more effective on Gram-positive bacteria than on Gram-negative bacteria. Totally, the MIC of mucroporin was 25 µg/mL for *Staphylococcus aureus* AB94004, 25 µg/mL for *Bacillus thuringensis* AB92037, and 50 µg/mL for *Bacillus subtilis* AB91021. The MIC for mucroporin-M1 was 5 µg/mL for *Staphylococcus aureus* AB94004, 25 µg/mL for *Bacillus thuringensis* AB92037, and 25 µg/mL for *Bacillus subtilis* AB91021. In addition, *Escherichia coli* AB94012 and *Pseudomonas aeruginosa* AB93066 were both insensitive to 100 µg/mL of mucroporin, while the MIC of mucroporin-M1 was 12.5 µg/mL for *Escherichia coli* AB94012 and 100 µg/mL for *Pseudomonas aeruginosa* AB93066. As shown in Fig.3A, we investigated the growth of *Staphylococcus aureus* AB94004 8 hours after peptide treatment. It was found that *Staphylococcus aureus* AB94004 treated by mucroporin reproduced faster than that of *Staphylococcus aureus* AB94004 by mucroporin-M1 at the same concentration. These results indicated that the modification of the mucroporin sequence not only enhanced its *in vitro* activity, but also expanded its antibacterial spectrum.

The antibiotics-resistant pathogens were clinical isolates, all of which were tested with traditional antibiotics to determine the resistance before experiments. As shown in table 1, mucroporin and mucroporin-M1 can both inhibit MRCNS 1538 growth. Comparing the MICs of penicillin to 1538 and *Staphylococcus aureus* AB94004, we found that MRCNS 1538 was resistant to penicillin. However, the MIC of mucroporin for 1538 was 5-fold greater than that of mucroporin-M1. This result further demonstrated that the modification of the mucroporin amino acid residues enhanced its *in vitro* activity.

As shown in table 2, mucroporin-M1 was as effective to antibiotics-resistant pathogens (MRSA, MRCNS, PRSA and PRSE) as to antibiotic-sensitive pathogens (PSSA and
The MICs of mucroporin-M1 for penicillin-resistant strains P1383 (PRSA) and P1389 (PRSE) were 10 µg/mL and 8 µg/mL, while the MICs of mucroporin-M1 were 40 µg/mL for penicillin-sensitive strains P969 (PSSA), 20 µg/mL for P1111 and 8 µg/mL for P1368 (PSSE). In addition, the MICs of mucroporin-M1 for methicillin-resistant strains were 20 µg/mL for P1381 (MRSA), 25 µg/mL for P1386 (MRSA), 8 µg/mL for P1374 (MRSA), and 8 µg/mL for P1369 (MRCNS), which was at the same level as for methicillin-sensitive strains. The MICs of penicillin G salt, cefotaxime and vancomycin to penicillin-sensitive strains, penicillin-resistant strains and methicillin-resistant strains were all determined to define susceptibility. Furthermore, as shown in Fig.3B and 3C, 400 µg/mL penicillin and 40 µg/mL cefotaxime can’t effectively inhibit P1386 strain (MRSA) growth during the first 8 hours after drug delivery, whereas its growth was totally inhibited by 40 µg/mL dose of vancomycin and mucroporin-M1. Comparatively, 8 µg/mL dose of each antibiotics and mucroporin-M1 effectively inhibited P969 (PSSA) growth. The above described results indicated that mucroporin-M1 can effectively inhibit antibiotics-resistant pathogens, including MRSA.

**Killing assay.** To study the functional mechanisms of mucroporin and mucroporin-M1, we performed the killing assay for mucroporin and mucroporin-M1 on the selected model strain *Staphylococcus aureus*. The 1-, 2-, or 5-fold of the MIC of mucroporin or mucroporin-M1 for *Staphylococcus aureus* was directly added into exponential phase bacteria, and then the survival rate was determined by counting the viable colony on the plates. The results revealed that the viable colony was decreased dramatically in a very short time, and the reaction started as soon as the bacteria mixed with these peptides. The killing curves (Fig.4) show that the killing rate corresponded with the increasing peptide
concentration. In contrast, the 5-fold greater MIC of ampicillin sodium (40 µg/mL) didn’t make a dramatic decrease in survival rate, which was consistent with its molecular mechanism of inhibiting bacterial wall synthesis. This result suggested that mucroporin and mucroporin-M1 exerted killing effects on microorganisms, which was probably the reason it can effectively inhibit the growth of microorganisms.

**Scanning electron microscope.** *Staphylococcus aureus* cells treated with mucroporin or mucroporin-M1 were observed with a scanning electron microscope to present single bacteria changes (Fig.5). As shown in Fig.5B and 5C, *Staphylococcus aureus* cells were found broken and the cell contents were assembled as small particles, which was direct evidence supporting the idea that the bacterial membrane was broken after treatment with mucroporin or mucroporin-M1.

The results of the *in vitro* bactericidal activity and scanning electron microscopy provided the solid evidence that the bacterial membrane was broken immediately after mucroporin or mucroporin-M1 treatment and the inhibitory effect of mucroporin and mucroporin-M1 on bacteria was through the action mode of rapid killing.
DISCUSSION

Toxins are specific poisonous substances developed by venomous animals for predation and defense, composed of various proteins/polypeptides. Recently the output of data by cDNA library sequencing and proteomics profiling research(28) have revealed large numbers of peptide toxins from animal venoms, including ion channels modulators, bradykinin potentiating peptides, cationic host defence peptides, etc. Some of them have been developed and have shown favorable in vivo activity(33), which suggests that the large number of venom peptides is a rich source of potential therapeutic drugs.

Scorpion toxins have been recognized as potential therapeutic drugs for many years, especially as ion channel modulators. For example, margatoxin (MgTX), from the scorpion Centruroides margaritatus, was the first peptide to be tested in vivo and is a potent blocker of the voltage-gated potassium channels Kv1.1, Kv1.2, and Kv1.3(5). MgTX depolarizes the T cells of both pigs and humans in vitro, and also inhibits the delayed-type hypersensitivity reaction to tuberculin in the mini-pigs, as determined by both the size of induration and the extent of T-cell infiltration(31). Another toxin, chlorotoxin (CTX), derived from the venom of scorpion Leirurus quengestriatus, displays an extraordinary feature that specifically targets glioma cells through MMP-2, the primary receptor highly expressed on the glioma cell membrane. Radioactive $^{131}$I-chlorotoxin analogue has cytolytic activity and therefore the potential to selectively affect tumors and gliomas of neuroectodermal origin(35, 45). On the basis of these findings, TransMolecular Inc. is running trials with $^{131}$I-TM-601 ($^{131}$I-chlorotoxin) as an investigational new drug for the treatment of gliomas.

So far, several cationic host defence peptides of scorpions have been isolated and
characterized(16-18, 39, 46). In our present study, the first cationic host defence peptide from *Lychas mucronatus* was characterized. It has been shown that mucroporin exerted an inhibitory effect, especially on Gram-positive bacteria strains, including clinically isolated pathogens. However, the activity of mucroporin was not very high, that’s why we designed the sequence for mucroporin-M1. The principal design aim was to replace the amino acid residues at the hydrophilic site with positively charged residues. As a result, the antibacterial activity was improved, including the activity against Gram-positive, Gram-negative bacteria and clinically isolated antibiotic-resistant pathogens (MRSA, MRCNS, etc). This result showed us that mucroporin was an ideal template for anti-infective drug design.

Vancomycin is considered the most effective drug for the treatment of MRSA infection, but vancomycin-resistant *Staphylococcus aureus* strains have also been identified(2, 3). Thus, the treatment of MRSA infection will be a difficult problem in the near future. Cationic host defence peptides may give us a solution. The results showed that the *in vitro* effect of mucroporin-M1 on MRSA and MRCNS were at the same level as vancomycin. The clinical trials of cationic host defence peptides have been ongoing for many years(11, 12, 47), including Phase IIIa trial by utilizing these peptides in topical treatment(32, 40). However, none of them has obtained FDA approval for their various clinical indications so far(21, 41). Designed peptides with high effectiveness and low toxicity are our future goal.

In conclusion, we showed that mucroporin and mucroporin-M1, a native cationic host defence peptide and its analogue, have shown specific effects on inhibiting bacteria. These peptides kill *Staphylococcus aureus* very fast. Mucroporin-M1 can effectively
inhibit the hospital acquired MRSA, MRCNS, PRSA and PRSE. The antibacterial activity suggests us that mucroporin is a good template for anti-infective drug design.
Acknowledgments

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REFERENCES


16. **Corzo, G., P. Escoubas, E. Villegas, K. J. Barnham, W. He, R. S. Norton,**


Table 1. Antibacterial effects of mucroporin and mucroporin-M1

<table>
<thead>
<tr>
<th>Strains</th>
<th>Minimal inhibition concentration (µg/mL)</th>
<th>Mucroporin</th>
<th>Mucroporin-M1</th>
<th>Penicillin</th>
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<tr>
<td>1538*</td>
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<td>25</td>
<td>5</td>
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* 1538 was MRCNS, clinical isolated from Hubei maternal and child health hospital.
Table 2. The MICs of mucroporin-M1 to clinical isolates

<table>
<thead>
<tr>
<th>Strains</th>
<th>Minimal inhibition concentration (µg/mL)</th>
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<th>Cefotaxime</th>
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<td>Methicillin resistant</td>
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<td></td>
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<td>P1369 (MRCNS)</td>
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<td>Penicillin sensitive</td>
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<td>P969 (PSSA)</td>
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<td></td>
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<td>P1368 (PSSE)</td>
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</table>

All strains were clinical isolates obtained from the 302nd military hospital, Beijing, China.
Figure legends:

Fig.1. cDNA and protein sequences of mucroporin (Accession number EU669864). The deduced amino acid residues are shown below the corresponding nucleotide sequences. The signal peptide is in italics and the propeptide is underlined. The potential polyadenylation signal AATAAA is double underlined.

Fig.2. Protein sequence alignments of mucroporin with AP4_TITCO, AP5_TITCO, AP6_TITCO, caerin-1, caerin-2, BmKb1 and BmKb2. Percentage of sequence similarity relative to mucroporin is represented by % sim. The signal peptide is italics. The propeptide is underlined.

Fig.3. Time growth curves of *Staphylococcus aureus* treated by mucroporin or mucroporin-M1. The growth of *Staphylococcus aureus* was measured at 630nm. (A) Comparison of mucroporin and mucroporin-M1 activities against *Staphylococcus aureus* AB94004. (B) Comparison of mucroporin-M1 and antibiotics activities against clinical isolated MRSA P1386. (C) Comparison of mucroporin-M1 and antibiotics activities against clinical isolated PSSA P969.

Fig.4. Killing assay. Killing assay was conducted with determining the count of the surviving bacteria without the supernatant. Set point in the picture meant the initial bacterial count: the 0 minute was defined as the time of the first sample collection, which was immediately after mixing bacteria and mucroporin or mucroporin-M1, and the other samples were collected at 5 min, 15 min, 30 min and 60 min after 0 min. All the counts were the average of three dishes. The experiment was repeated and showed the same trend. (A) Mucroporin at different concentrations. (B) Mucroporin-M1 at different concentrations.
Fig. 5. The scanning electron microscopy images of *Staphylococcus aureus* treated by mucroporin or mucroporin-M1. (A) Negative control. (B) Ten minutes after mucroporin treatment. (C) Ten minutes after mucroporin-M1 treatment.
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<th>Protein</th>
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<th>Identity (%)</th>
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