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Investigation of morphological changes of HPS membrane caused by cecropin B through scanning electron microscopy and atomic force microscopy

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ABSTRACT

Background: Antimicrobial peptides (AMPs) have been identified as promising compounds for consideration as novel antimicrobial agents.
Objectives: This study analyzed the efficacy of cecropin B against *Haemophilus parasuis* isolates through scanning electron microscopy (SEM) and atomic force microscopy (AFM) experiments.
Results: Cecropin B exhibited broad inhibition activity against 15 standard *Haemophilus parasuis* (HPS) strains and 5 of the clinical isolates had minimum inhibition concentrations (MICs) ranging from 2 to 16 μg/mL. Microelectrophoresis and hexadecane adsorption assays indicated that the more hydrophobic and the higher the isoelectric point (IEP) of the strain, the more sensitive it was to cecropin B. Through SEM, multiple blisters of various shapes and dents on the cell surface were observed. Protrusions and leakage were detected by AFM.
Conclusions: Based on the results, cecropin B could inhibit HPS via a pore-forming mechanism by interacting with the cytoplasmic membrane of bacteria. Moreover, as cecropin B concentration increased, the bacteria membrane was more seriously damaged. Thus, cecropin B could be developed as an effective anti-HPS agent for use in clinical applications.

Keywords: Antimicrobial peptide; atomic force microscopy; scanning electron microscopy; Haemophilus parasuis

INTRODUCTION

Haemophilus parasuis (*H. parasuis*) is a Gram-negative bacterium of the *Haemophilus* genus within the *Pasteurellaceae* family. This organism is the etiological bacteria of Glässer's disease, which is characterized by porcine fibrinous polyserositis, meningitis, and arthritis syndrome, and it has become a serious problem in pig herds around the world [1]. Fifteen standard serovars of *H. parasuis* have been reported thus far based on immunodiffusion tests with heat-stable antigens [2]. Vaccines against *H. parasuis* have been developed; unfortunately, serovar diversity and non-typeable isolates have limited the cross-protective efficiency of the vaccines

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: He Q, Hu H; Data curation: Zhang B, Jiang C, Guo N; Formal analysis: Li Z, Guo X; Funding acquisition: He Q; Investigation: Guo X; Methodology: Hu H; Project administration: Zhang B; Resources: Wang Y; Software: Wang Y; Supervision: Liu B; Validation: Guo N; Visualization: Zhang B; Writing - original draft: Hu H, Jiang C; Writing - review & editing: He Q.

[3]. In addition, the overuse of antibiotics in veterinary medicine has encouraged the increase of resistance of HPS to antibiotics.

Antimicrobial peptides (AMPs) are useful components of the innate immune defense system in the natural kingdom, present in organisms from microorganisms to insects, plants, and mammals [4,5]. They have various biological functions, including antibacterial, antifungal, antiparasitic, antitumor, and antiviral [6-8]. AMPs mainly exert their antibacterial activity by destroying the bacteria membrane; as well, they have intracellular targets associated with gene and protein synthesis [9,10]. It is difficult for bacteria to become AMP-resistant due to the unique killing mechanism. AMPs could be developed as an alternative to conventional antibiotics to treat pathogenic microorganism infections [11].

Cecropin B, a 37-residue cationic antimicrobial peptide, was first identified in 1981 in the hemolymph of *Hylaphora cecrpina* [12]. It has the highest level of antibacterial activity in the cecropin family [13]. Previous research has shown that cecropin B can inhibit pathogenic microorganisms isolated from fish, plants, and mammals. Our previous research indicated that cecropin B had relatively good antibacterial activity against Gram-negative bacteria like *H. parasuis* SH0165, and it has great potential for application in future antimicrobial drug development [14]. Furthermore, cecropin B has been used in numerous biotechnological areas, from functional biomaterials and food preservatives to the production of disease-resistant crops [15-17]. Two mechanisms have been suggested to explain the antimicrobial characteristics of cecropins: a transmembrane pore formation mechanism and a "carpet-like" mechanism. A detailed study of dye release kinetics has shown that, after a certain cecropin treatment period, a pore would form, and the vesicle contents would leak out [18]. In addition, a fluorescence microscopy study of cecropins interacting with live *Escherichia coli* indicates that its membrane permeability is similar to a 100 nm diameter pore or several small pores [19].

On the other hand, the so-called carpet model suggests that cecropins can bind with the phospholipid membrane of bacteria, cover the entire membrane layer, and cause membrane failure. Based on spectroscopic experiments and molecular dynamic simulation, it was shown that cecropin P1 did not embed in the hydrophobic core of the membrane, supporting the observation of a "carpet-like" mechanism [20]. Lyu et al. [21] showed that cecropin P1 aggregates with solubilized lipids at a higher concentration, indicating the presence of the carpet mechanism.

In previous research, cecropin B exhibited relatively good activity against *H. parasuis* SH0165 [14]. In this study, the activity of cecropin B against reference strains and clinical isolates were tested. The hydrophobicity and electric charge of the surface of these reference strains were tested to analyze the relationship between bacteria membrane physical characteristics and membrane sensitivity to cecropin B. Scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used to observe HPS membrane alterations caused by cecropin B.

MATERIALS AND METHODS

Synthesis of peptide

The peptide cecropin B was synthesized by applying a previously reported method [14]. Briefly, the peptide was synthesized by an automated solid-phase peptide synthesizer (Wuhan

Bioyeargene Biotechnology Inc., China). The crude peptide was purified using a reverse-phase high-pressure liquid chromatography (RP-HPLC) system (Waters, USA) with a C_{18} column (Waters Xbridge, USA). Finally, the purity and accurate mass of the purified peptide were determined using HPLC and mass spectrometry. Arguslab 4.0.1 software (Planaria Software LLC, USA) and swissmodel.expasy.org were employed to draw the structure of cecropin B.

Antimicrobial activity

The antimicrobial activity of cecropin B was tested against 15 standard and 5 clinical strains of HPS (**Table 1**). Reference strains for *H. parasuis* serovars 1–15 were kindly donated by Dr. Blackall (Bacteriology Research Laboratory, Animal Research Institute, Yeerongpilly, Australia). The 5 clinical strains, obtained from the Animal Disease Center of Huazhong Agricultural University, were isolated between 2001 and 2017 from samples of clinically affected pigs in central and northern parts of China. *H. parasuis* SH0165 was isolated from the lung of a diseased piglet from Hebei province. HB427-2 and HB82 were isolated from the lung tissue samples of diseased pigs from Hubei province. ZJ427-4 and JS19428 were isolated from the diseased pig lung tissue samples from Zhejiang and Jiangsu provinces.

The minimum inhibitory concentrations (MICs) of cecropin B against the tested strains were determined using a standard broth micro-dilution method as previously reported [14]. Briefly, the bacteria were grown in tryptic soy broth (BD Difco, USA) supplemented with 5% bovine serum, and 10 μg/mL nicotinamide adenine dinucleotide until the bacteria reached the mid-log phase (inoculated from a 16–24 h overnight culture at 37°C). The cultures were resuspended in medium to a final concentration of 1×10^5 CFU/mL. Serial two-fold dilutions of the initial concentration of the peptide (128 μg/mL) were performed to obtain a final concentration of 2.5 μg/mL. Diluted peptide (10 μL) was inoculated into each well containing 90 μL of a bacterial suspension. The MICs were defined as the lowest concentrations that completely inhibited bacterial growth.

Table 1. Antimicrobial activity of cecropin B

No.	Strain	Serotype	Virulence	MIC vales (μg/mL)
1	N4	1	H	8
2	SW140	2	L+	2
3	SW114	3	A	4
4	SW124	4	L+	4
5	Nagasaki	5	H	8
6	131	6	A	4
7	174	7	A	2
8	C5	8	L-	4
9	D74	9	A	4
10	H367	10	H	8
11	H465	11	A	8
12	H425	12	H	4
13	IA-84-17975	13	H	8
14	IA-84-22113	14	H	4
15	SD-84-15995	15	L+	8
16	SH 0165	5	-	2
17	HB427-2	1	-	8
18	HB82	4	-	16
19	ZJ427-4	13	-	4
20	JS19428	14	-	8

H, highly virulent, death of pig with 96 h post-inoculation; L+, Polyserositis and arthritis at necropsy; L-, Mild clinical symptoms; A, Avirulent, no clinical symptoms at necropsy as described by Kielstein and Rapp-Gabrielson (1992).

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Kill-curve studies

For the 10^8 CFU/mL kill-curve assay, 51, 10, and 0.2 μL of 2.5 mg/mL cecropin B (final concentrations, 512, 100, and 2 μg/mL, respectively) were mixed with 100 μL of 10^9 CFU/mL *H. parasuis* SH0165 at the mid-logarithmic phase, and fresh TSB medium was added to obtain 250 μL. The mixture was then incubated anaerobically at 37°C. For the 10^5 CFU/mL kill-curve assay, 0.8, 0.4,and 0.2 of 2.5 mg/mL cecropin B (final concentrations, 8, 4, and 2 μg/mL, respectively) were mixed with 100 μL of 10^6 CFU/mL *H. parasuis* SH0165 at the mid-logarithmic phase and fresh TSB medium added to obtain 250 μL. The mixture was then incubated anaerobically at 37°C. Untreated bacteria were used as a negative control. At different indicated times (5, 10, 20, 30, 60, 90, and 120 min), 10 μL bacterial suspensions were collected and diluted in 490 μL TSB medium. The samples were spread onto TSA plates with appropriate dilution, incubated at 37°C for 24 h, and the bacteria colonies counted. The experiments were repeated three times independently.

Hexadecane adsorption assay

The hydrophobicity of the tested bacteria was measured according to the method reported by Pelletier et al. [22]. Briefly, bacteria during the stationary phase were harvested by centrifugation at 3,000×*g* for 10 min, washed twice, and resuspended to an optical density of 0.4 at 400 nm (A_0) in 0.1 M KNO_3 (pH 6.2). To 1.2 mL of the cell suspension was added 0.2 mL of hexadecane (Sigma-Aldrich, USA). After 10 min of pre-incubation at room temperature, the two-phase system was mixed on a vortex mixer for 2 min. To allow complete phase separation of the mixture, the aqueous phase was removed after 15 min, and its optical density at 400 nm (A_1) was measured. The ratio of microbial adhesion to hexadecane was expressed as a percentage and calculated as $(1\text{-}A_1/A_0) \times 100$.

Microelectrophoresis

Electrophoretic mobility (EM) was measured to determine the cell surface net charge of the bacteria. Immediately before measurement, cells in the stationary phase were harvested by centrifugation at 5,000 × *g* for 10 min, washed twice, and resuspended in KNO_3. The EM as a function of pH was first determined in 1 mM KNO_3 (ionic strength of 1 mM) at a concentration of approximately 10^7 cells/mL. The KNO_3 solution used as the reference medium is commonly used to avoid nonspecific absorption of ions on cell surfaces. The pH of the resuspending fluid was adjusted to 2, 3, 4, 5, 6, 7, and 8 by the addition of KOH or HNO_3. The EMs of the bacteria at the appropriate pH level were measured at room temperature on a Zetameter (Zetameter Inc., USA). The EMs, expressed in 10^{-8} $m^2 \cdot V^{-1} \cdot s^{-1}$, were derived from the velocities of the bacteria in suspension under an applied electric field of 100 V.

Scanning electron microscopy

Mid-logarithmic phase *H. parasuis* SH0165 cells (~2×10^8 CFU/mL) were treated with cecropin B (2, 100, and 512 μg/mL) for 20 or 120 min. Sample processing involved washing, fixing, and drying the bacteria at 4°C. The collected cells were washed three times with phosphate buffer saline. The bacteria were dehydrated by a series of ethanol dehydration steps at 30% (10 min), 50% (10 min), 70% (15 min), 90% (20 min), and 100% (30 min). The samples were freeze-dried and sputter-coated with gold under vacuum and mounted on specimen holders to be analyzed on a JSM-6390/LV scanning electron microscope (Jeol Inc., Japan) under 20 kV and 1 nA.

Atomic force microscopy

Mid-logarithmic phase *H. parasuis* SH0165 cells were incubated with cecropin B at concentrations of 2, 4, 32, and 128 μg/mL for 1 h. After incubation, the peptide-treated

bacterial suspensions were washed with and resuspended in water and then diluted 100-fold. About 10 μL of the diluted sample was dropped onto poly-L-lysine pretreated glass slides (Sigma-Aldrich) and air-dried. Control untreated bacteria were similarly prepared. Imaging was performed in tapping mode on an Agilent 5500 AFM (Agilent Technologies, USA). The areas for imaging were chosen randomly from the total sample area. Images were collected and then analyzed by using Picoscan software version 5.3.1 (Agilent Technologies)

RESULTS

Antibacterial activity of cecropin B

In our previous study, cecropin B exhibited antibacterial activity against different kinds of bacteria isolated from swine, and *H. parasuis* SH0165 was one of the strains most sensitive to cecropin B [14]. The structure of cecropin B was composed of two α-helix, N-terminal amphiphilic helix (F5-A22) and C-terminal hydrophobic helix (A25-L35) linked by a hinge area (G23-P24) (**Fig. 1**). To assess the bactericidal activity of cecropin B against HPS, 15 reference strains and 5 clinical isolates were used to test the activity of cecropin B (**Table 1**). Serovars 1, 5, 10, 12, 13, and 14 were known as highly virulent, causing pig death in 4 days. The other serovars were known as moderately virulent or avirulent without causing death. According to the MIC results, cecropin B could inhibit the growth of all tested strains, with the MIC values ranging from 2 to 16 μg/mL (**Table 1**).

Kill-curve studies were performed using cecropin B on a logarithmic phase *H. parasuis* SH0165 culture. Cecropin B showed dose-dependent killing activity against the bacteria. Twenty minutes were sufficient for cecropin B to kill all bacteria (**Fig. 2A**). Moreover, all bacteria were eliminated after treatment with cecropin B for 10 min at 8 μg/mL (**Fig. 2A**). As bacterial density must be high for SEM imaging, a higher initial bacterial concentration of 10^8 CFU/mL was applied to assess the killing kinetic (**Fig. 2B**). Bacteria were killed within 10 min at 100 μg/mL and above. An approximately one log unit decrease in viable *H. parasuis* SH0165 could be detected after a 120 min treatment with 2 μg/mL cecropin B (**Fig. 2B**).

A

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                + +  ++ +          +            +
Cecropin B  KWKVFKKIEKMGRNIRNGIVKA GP AIAVLGEAKAL-NH2
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B

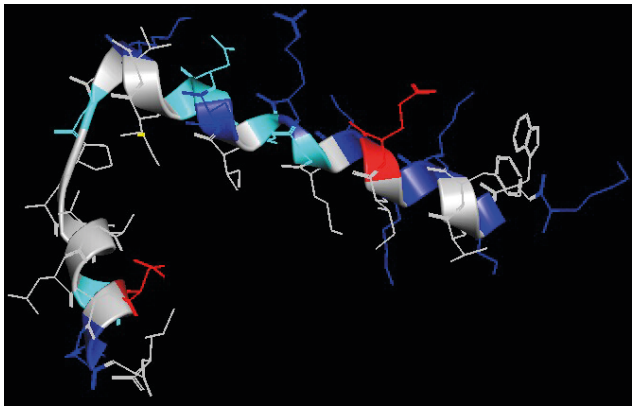

Fig. 1. The amino acid sequence (A) and structure (B) of cecropin B. The positively charged amino acids are marked by "+". The structure of cecropin B was drawn by using Arguslab software.

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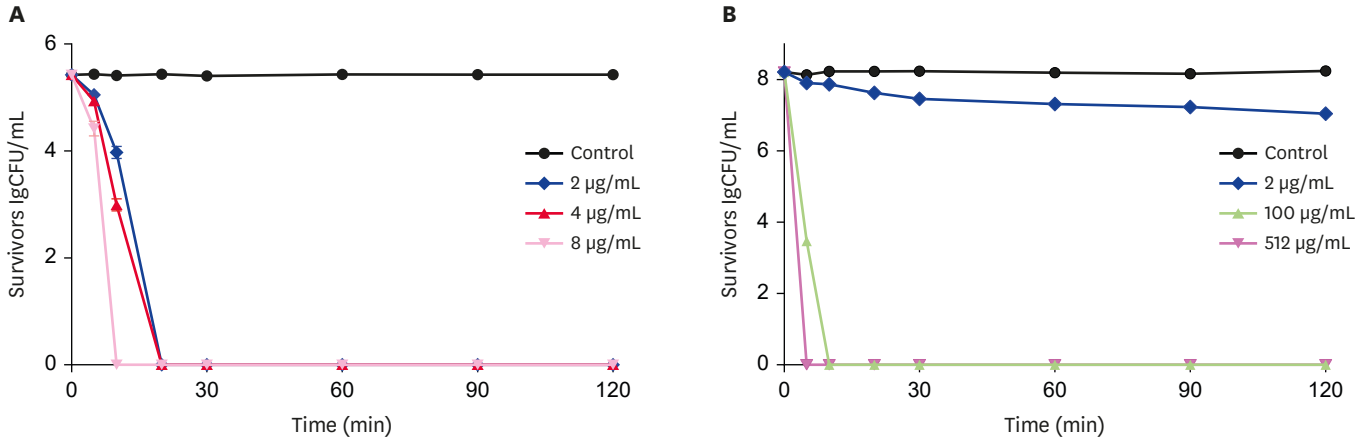

Fig. 2. Killing kinetics of cecropin B against *Haemophilus parasuis* SH0165. Bacteria were treated with cecropin B at different initial concentrations. Samples were taken after various incubation times and viable bacteria were determined by performing colony count assays. Data represent mean ± SD (n = 3 for all observations).

Physicochemical properties of HPS membrane

Hexadecane was employed to measure the cell surface hydrophobicity and hydrophilicity at the high ionic strength of 0.1 M KNO_3. In general, the percentages of strains that adhered to the apolar solvent ranged from 0.7 to 27.4%, indicating a hydrophilic surface. According to the result, HPS 6 (27.4%) was the most hydrophobic strain, and HPS 15 (0.7%) was the most hydrophilic one (**Table 2**).

In addition, net charges of the membranes of all tested bacteria were examined by performing microelectrophoresis to determine the EM values of the bacteria. The EM was tested in 1 mM KNO_3 at pH levels ranging from 2 to 8 (**Fig. 3**). The results showed that all of the tested strains were negatively charged at high pH values and became positively charged as the pH value decreased. We also observed that the isoelectric points (IEPs) for all of the reference strains ranging were comparable, ranging 2.8 (HPS 12) to 4.14 (HPS 4) (**Table 3**).

When hydrophobicity was plotted against IEP of isolates with different MICs (**Fig. 4**), it appeared that most of the strains followed the trend that the more hydrophobic or higher IEP the isolate was, the more cecropin B sensitive the strain would be (exceptions, HPS 3 and HPS 12).

Table 2. HPS adhesion to hexadecane

Strains	% of adhesion (± SD) to hexadecane
HPS1	1.7 ± 2.9
HPS2	8.4 ± 4.9
HPS3	2.7 ± 1.4
HPS4	1.0 ± 0.8
HPS5	1.3 ± 0.1
HPS6	27.4 ± 2.0
HPS7	8.4 ± 1.5
HPS8	2.1 ± 3.1
HPS9	5.3 ± 4.0
HPS10	5.4 ± 3.0
HPS11	2.2 ± 0.1
HPS12	1.8 ± 0.7
HPS13	7.5 ± 2.6
HPS14	8.1 ± 4.1
HPS15	0.7 ± 2.0

Mean ± SD of two measures of at least three separate experiments.
HPS, *Haemophilus parasuis*.

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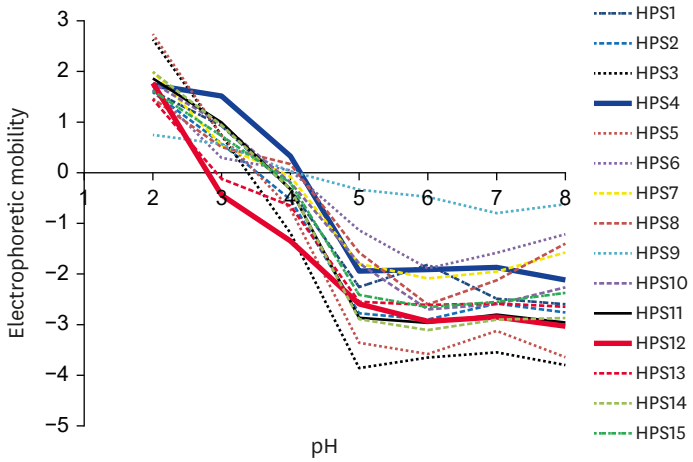

Fig. 3. Electrophoretic mobility of *Haemophilus parasuis* strains as a function of pH in 1 mM KNO_3. The lowest IEP of HPS 12 is plotted in red, and the highest IEP of HPS 4 is plotted in blue. The others are plotted in different colors as indicated.

Table 3. IEP of HPS strains in 1mM KNO_3

Strains	IEP
HPS1	3.74
HPS2	3.52
HPS3	3.38
HPS4	4.14
HPS5	3.5
HPS6	4.03
HPS7	3.88
HPS8	4.09
HPS9	4.08
HPS10	3.83
HPS11	3.75
HPS12	2.8
HPS13	2.92
HPS14	3.74
HPS15	3.78

IEP, isoelectric point; HPS, *Haemophilus parasuis*.

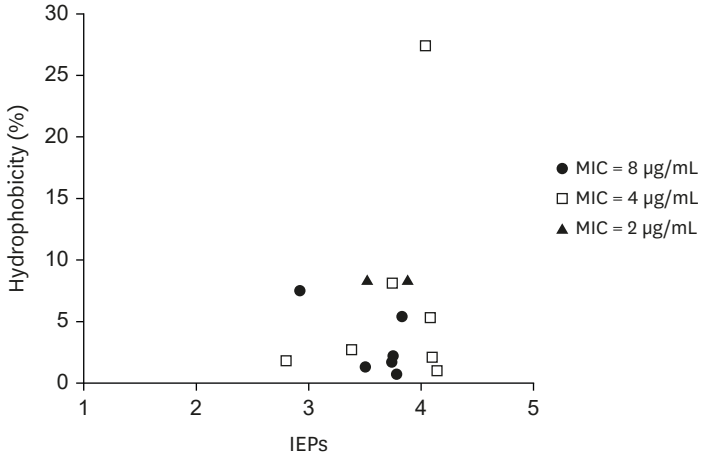

Fig. 4. The IEPs of the *Haemophilus parasuis* strains (**Table 3**) are plotted against the hydrophobicity of the bacteria (**Table 2**). The X-axis represented the IEPs of the strains, and the Y-axis represented the hydrophobicity of the bacteria. •, □, ▲ indicate isolates with MIC = 8, 4, or 2 μg/mL, respectively.
IEP, isoelectric point; MIC, minimum inhibition concentration.

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SEM of cecropin B-treated *H. parasuis* SH0165

In this study, SEM was applied to better elucidate the membrane damages caused by cecropin B. Different doses (2, 100, and 512 μg/mL) of cecropin B were incubated with the tested bacteria for different times (20 min or 120 min), separately. Untreated bacteria had a normal smooth membrance surface (**Fig. 5A and B**). In contrast, numerous blisters or blebs on the cell surface could be observed after incubating with 2 μg/mL cecropin B (**Fig. 5C and D**). As cecropin B concentration and incubation time increased, the membrane damage caused by cecropin B increased. Cell lysis and holes in cell membranes were observed (**Fig. 5E-H**). Cell debris was also prevalent, indicating that cell damage had occurred.

Atomic force microscopy of cecropin B-treated *H. parasuis* SH0165

It was observed that the membrane structure of untreated bacteria was reasonably structured with no visible ruptures or pores (**Fig. 6A**). For MIC (2 μg/mL)-treated samples, the bacteria's surface became rough and membrane blebbing was observed (**Fig. 6B**). This was consistent with the membrane alterations observed in the SEM micrographs (**Fig. 5**). Exposure of bacterial cells to higher concentrations led to more significant membrane damage; striations became more obvious, and vesiculation, as well as deep lesions, were observed (**Fig. 6C-E**). Some leaked contents or debris could also be observed adjacent to partly disintegrated bacteria (**Fig. 6**).

DISCUSSION

In this study, the activity of cecropin B against the 15 reference strains of different serovars and 5 clinical isolates were tested (**Table 1**). Cecropin B had inhibitory activity against all tested isolates, with MIC values ranging from 2 to 16 μg/mL (**Table 1**). Giacometti et al. [23] also reported that AMPs (i.e., buforin II, cecropin P1, indolicidin, and magainin II) had comparable activity levels against bacteria of different serotypes. This indicates that the antimicrobial activity of cecropin B is not against any specific strain but is against almost all isolates, regardless of serotype. This is somewhat meaningful clinically as there are many different serotypes of HPS prevalent across China, and current vaccines cannot offer sufficient protection [3]. It is reasonable to assume that cecropin B, developed as a template anti-HPS drug, could be efficient against all the serotypes.

It was reported that the bactericidal activity of cecropin B depends on its helical structure. The whole sequence of cecropin B contains 35 amino acids, and its C-terminal is amidated (**Fig. 1**). According to previous reports, C-terminal amidation can enhance the antibacterial activity of AMPs [24]. The N-terminal helix is larger than the C-terminal part, and there are several positively charged amino acids distributed around the N-terminal (**Fig. 1**). Both of these characteristics may contribute to the binding of cecropin B to bacterial membranes [25,26].

The binding process is a critical mechanistic step in the killing process of AMPs [27]. The binding process is achieved through both a charge interaction between positively charged amino acids and anionic membrane surfaces and a hydrophobic interaction between hydrophobic amino acids and the membrane bilayer. In this study, different cecropin B sensitivity existed among the HPS strains; although cecropin B could inhibit all of them (**Table 1**). It is speculated that this variance might result from the different hydrophobic and electrostatic properties of the tested strains.

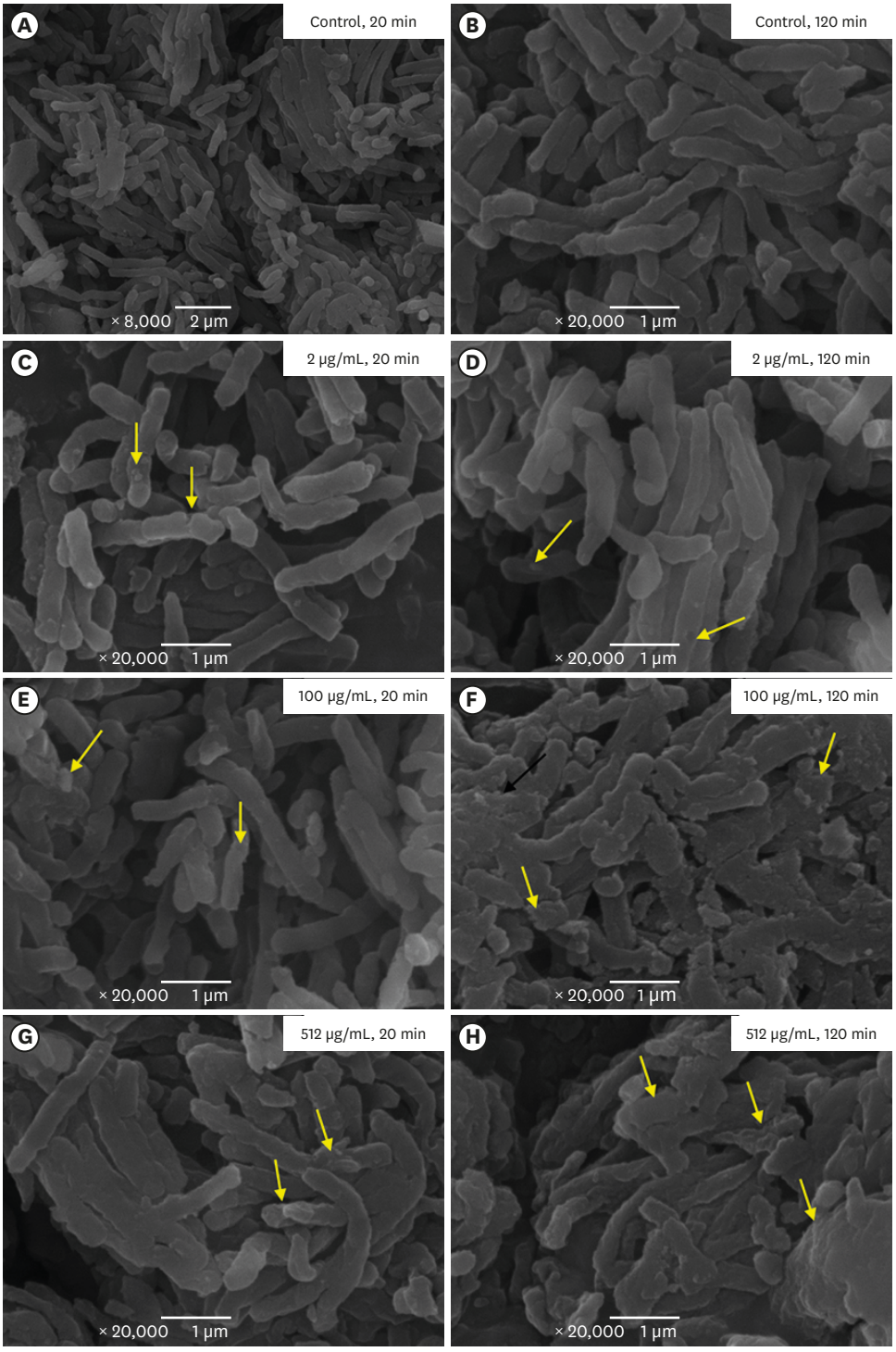

Fig. 5. The Scanning electron microscopy images of HPS SH0165 treated with cecropin B at different concentrations and for different times. HPS incubated with the buffer but without cecropin B for 20 min (A), 120 min (B), with cecropin B at 2 μg/mL for 20 min (C), 120 min (D), at 100 μg/mL for 20 min (E), 120 min (F), at 512 μg/mL for 20 min (G), 120 min (H). Arrows indicate blisters, dents on the membrane, or membrane disintegration.
HPS, *Haemophilus parasuis*.

Assessments via the microbial adhesion to hexadecane method and by performing microelectrophoresis detected the hydrophobic and electrostatic characteristics of the tested strains [22]. Hexadecane is an apolar solvent, and microbial adhesion to hexadecane

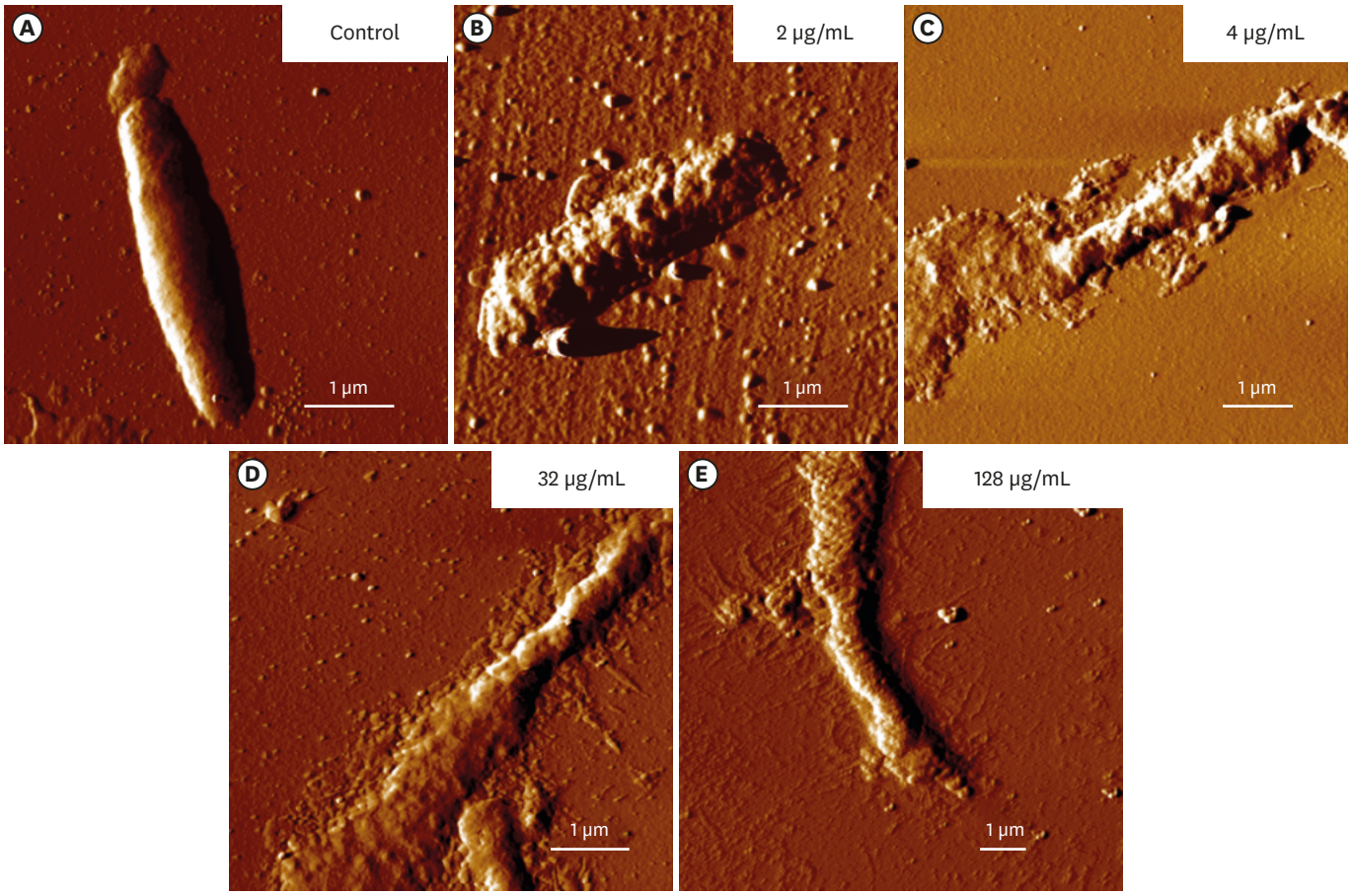

Fig. 6. The atomic force microscope images of *Haemophilus parasuis* SH0165 treated without cecropin B, image size 5 × 5 μm^2 (A); with cecropin B at 2 μg/mL, image size 5 × 5 μm^2 (B); with cecropin B at 4 μg/mL, image size 6.25 × 6.25 μm^2 (C); with cecropin B at 32 μg/mL, image size 5.75 × 5.75 μm^2 (D); with cecropin B at 128 μg/mL, image size 10 × 10 μm^2 (E). All images were performed in AFM-tapping mode. Arrows indicate ruptures, membrane protrusions, or cytoplasmic leakage.

could indicate the hydrophobicity of the bacteria's surface. The results showed that a low percentage (ranging from 0.7 to 27.4%) of bacteria adhered to this apolar solvent with HPS 6 being the most hydrophobic strain and HPS 15 being the most hydrophilic one (**Table 2**). Similar results for strains of *Lactobacillus casei subsp. casei* and *Lactobacillus rhamnosus* have been reported [22]. In addition, the surface net charges of all the reference isolates were examined by microelectrophoresis, which measures the EM of microorganisms in the stationary phase. The EM was determined in 1 mM KNO_3 at pHs ranging from 2 to 8 (**Fig. 3**). The results revealed that all the isolates were negatively charged at pH 7.4, indicating that, under physiological conditions (PH = 7.4), the HPS membrane is negatively charged. This supports the presence of a charge interaction between positive AMPs and negative membranes. It was also observed that the IEPs ranged from 2.8 (HPS 12) to 4.14 (HPS 4) (**Table 3**). We tried to establish a correlation between MICs and hydrophobicity or IEPs (**Fig. 4**). The trend shown in **Fig. 4** indicated that the sensitivity of HPS is affected by both of those physicochemical properties, not by just one of them. There might also exist membrane composition factors other than charge and hydrophobicity of *H. parasuis* that could affect the interaction with cecropin B. For example, there are reports that cecropin B might interact with lipopolysaccharide [28,29].

In this study, SEM was applied to elucidate better the morphological changes caused by cecropin B. Untreated bacteria had smooth and complete membranes (**Fig. 5A and B**).

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However, multiple blisters of various shapes and dents on the cell surface could be observed after incubating with cecropin B at 2 μg/mL (**Fig. 5**). Previous TEM observations of cecropin B-treated HPS showed vacuoles inside the cells, indicating leakage of cellular contents [14]. According to these results, it was reasonable to assume that leaked cytoplasmic contents had caused the observed blisters. Xia et al. [30] also observed disrupted cytoplasmic membranes and leaked cytoplasmic contents of bacteria by using TEM. Chen et al. [31] reported that cecropin B could form large openings in the outer membrane of *E. coli*, which could assist cecropin B in approaching the cytoplasmic membrane. Thus, it is possible that after binding to the outer membrane (self-promoted uptake), cecropin B could penetrate the bacterium and aggregate on the inner membrane, forming pores on the inner membrane. As a result, cytoplasmic materials would flow into the periplasmic space, causing the formation of protrusions. This sequence is consistent with the pore-forming mechanism of AMPs. As treatment concentration and incubation time increased, the amount of membrane damage caused by cecropin B increased, resulting in cell lysis and holes in cell membranes (**Fig. 5**). In a previous study, the TEM images of HPS treated by cecropin B also showed the lysed cells [14]. In addition to the bacteria's membrane, intracellular bacterial components, such as DNA, could also be the targets. More experiments need to be conducted to address this hypothesis.

By using AFM, it was observed that untreated bacteria had a rod-like structure without flagella, which was consistent with previous reports on the morphology of HPS. The membrane structure of the control bacteria was relatively smooth, with no ruptures or pores observed (**Fig. 6**). For MIC (2 μg/mL)-treated samples, membranes appeared rough, and membrane protrusions were detected (**Fig. 6**). This was consistent with the membrane alterations observed using SEM (**Fig. 5**). As the cecropin B concentration increased, obvious membrane leakage was observed (**Fig. 6**). The AFM results support the hypothesis advanced above, i.e., at a low concentration, cecropin B possibly acts on the cytoplasmic membrane by activating a pore-forming mechanism, and as the concentration increases, the outer membrane partly disintegrates, followed by the bacterium leaking its cytoplasmic contents.

In conclusion, this study showed that cecropin B had broad activity against *H. parasuis* of several different serotypes, indicating it could be rather useful in controlling *H. parasuis* in pig herds. Both the hydrophobic and electrostatic properties of the bacteria's membrane affect the sensitivity of the strain to cecropin B. SEM and AFM results support the scenario that under low concentration, cecropin B activates a pore-forming mechanism during its interaction with bacterial cytoplasmic membranes. As the cecropin B concentration increases, the bacterial membrane is more seriously damaged.

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