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Evaluation of Susceptibility of Gram-Positive and -Negative Bacteria to Human Defensins by Using Radial Diffusion Assay

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Defensins are small cationic bactericidal peptides present abundantly in the granules of polymorphonuclear neutrophils (PMNs). Human PMNs contain four defensins termed HNP-1 to HNP-4. We used a new assay system in agar plates, the radial diffusion assay, to evaluate the effects of human defensins against gram-positive and -negative bacteria. A crude mixture of HNP-1, -2, and -3 (crude HNPs) was purified from human PMN extracts by reversed-phase high-pressure liquid chromatography (RP-HPLC). The different components were later separated by RP-HPLC and gel permeation chromatography. We compared the antibacterial activities of purified HNP-1, -2, and -3 against *Escherichia coli*, *Pseudomonas aeruginosa*, methicillin-susceptible *Staphylococcus aureus*, and methicillin-resistant *S. aureus* strains using the radial diffusion assay. The antibacterial activities of HNP-1 and HNP-2 against all strains tested were similar to those of the crude HNPs, but the activity of HNP-3 was less than those of the other defensins. To quantitate the activities of HNPs against different bacteria, we defined the minimal dose of crude HNPs forming a detectable clear zone around the bacteria as the minimal inhibitory dose (MID) and determined the MIDs for 10 strains of *E. coli*, 12 strains of *P. aeruginosa*, 10 strains of methicillin-susceptible *S. aureus*, and 12 strains of methicillin-resistant *S. aureus* isolates, including clinical isolates. In general, the MIDs of the HNPs were similar against similar bacterial species. However, the MIDs for *P. aeruginosa* were higher than those for the other organisms tested. The radial diffusion assay is suitable as a screening test for measuring the susceptibilities of isolates to defensins, because it is sensitive and simple and has good reproducibility.

Polymorphonuclear neutrophils (PMNs), the most numerous phagocytes circulating in the blood, play an important role in the host defense system (26). PMNs use two different processes to kill microorganisms, namely, the oxidative and the nonoxidative mechanisms. Microorganisms are killed through exposure to reactive oxygen intermediates in the oxidative mechanism and to granule-associated microbicidal proteins in the nonoxidative mechanism (4, 24, 30). Defensins have been described as the major cationic microbicidal peptides in the granules of mammalian PMNs, in rabbit alveolar macrophages, and human and murine intestinal Paneth cells (9, 17). The microbicidal activities of defensins have been shown in the phagosomes of PMNs. On the other hand, the extraphagosomal activities of certain defensins have also been reported, namely, a cytotoxic activity against mammalian cells in culture (19), chemotactic activity against monocytes (31), opsonizing activity (6), and a few endocrine effects (29).

Human PMNs contain four defensins, designated HNP-1 to HNP-4. HNP-1, -2, and -3 peptides consist of 29 to 30 amino acids and have identical sequences except for a single amino-terminal amino acid. They are arginine-rich and contain six conserved cysteine residues that form three disulfide bonds (9, 17, 27). The HNP-1, -2, and -3 peptides constitute 5 to 7% of the protein of human PMNs and are approximately 100 times more abundant than HNP-4 (7, 9). Human defensins are active in vitro against gram-positive (2, 10) and gram-negative (2, 10, 11, 15) bacteria, fungi (16), enveloped viruses (3), and other pathogens (1, 21). Such wide activity is attributed to their ability to interact with membranes (15, 19). To our knowledge, there are only a few reports comparing and evaluating the antibacterial activities of defensins against a variety of bacterial strains of various species, including clinical isolates (2, 14). Such studies of the natural peptide antibiotics are important in order to fully understand the host defense mechanisms. In the present experiment, the radial diffusion assay (12, 18) was used to evaluate the susceptibilities of gram-positive and -negative bacteria to human defensins.

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MATERIALS AND METHODS

Bacterial strains. The strains used to evaluate the antibacterial activities of human defensins included the following: *Escherichia coli* ATCC 43827 (ML-35) and nine clinical isolates, *Pseudomonas aeruginosa* ATCC 27853, PAO-1, PA103, PA103-29, and eight clinical isolates (five strains of the nonmucoid phenotype and three strains of the mucoid phenotype), methicillin-susceptible *Staphylococcus aureus* (MSSA) ATCC 25923 and nine clinical isolates, and methicillin-resistant *S. aureus* (MRSA; 12 clinical isolates). *P. aeruginosa* PAO-1, PA103, and PA103-29 were kindly provided by B. H. Iglewski, University of Rochester School of Medicine and Dentistry, Rochester, N.Y. PA103 is a hyperproducer of exotoxin A (13), and PA103-29 is its exotoxin A-deficient mutant (22). All clinical isolates were obtained from Nagasaki University Hospital, Nagasaki, Japan. Clinical isolates were named according to their origin, e.g., stool, peritoneal effusion, pus, urine, sputum, blood, pleural effusion, throat swab, and cerebrospinal fluid.

Defensins and antimicrobial agents. HNPs were purified from human PMNs, prepared from fresh buffy coats from a single donor, by using a modified system described previously by Ganz et al. (10). Briefly, we used a reversed-phase high-pressure liquid chromatography (RP-HPLC) system (Hitachi, Ltd., Tokyo, Japan) to obtain a crude mixture of the HNP-1, -2, and -3 (crude HNPs) in the first step. This was followed by RP-HPLC under another condition, replacing ion-exchange chromatography to separate HNP-3 from the mixture of HNP-1 and -2. Furthermore, we used a new prepacked gel permeation column, Super-

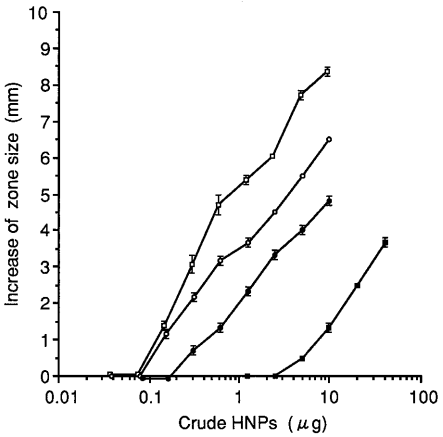

FIG. 1. Antibacterial activities of crude HNPs against *E. coli* ATCC 43827 (open squares), *P. aeruginosa* ATCC 27853 (closed squares), MSSA ATCC 25923 (open circles), and MRSA Bl-3 (closed circles). An increase in the zone size represents the zone size measured at each HNP concentration minus the size of the central well (3 mm). Data represent the means ± standard deviations of three independent experiments.

dex peptide HR 10/30 (Pharmacia Biotech, Tokyo, Japan), which has a high degree of selectivity for separating HNP-1 from HNP-2. The purities of the final products were assessed by tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (25) with a 16% acrylamide gel and acid urea-polyacrylamide gel electrophoresis (23) with 15% acrylamide, 6 M urea, and 5% acetic acid.

Penicillin G was purchased from Sigma (St. Louis, Mo.), and gentamicin was kindly supplied by Schering-Plough K. K. (Osaka, Japan). The MIC of each antimicrobial agent was determined by the National Committee for Clinical Laboratory Standards-recommended broth microdilution method (20).

Antibacterial assay. The antibacterial activities of human defensins and other antimicrobial agents were evaluated by the radial diffusion assay, a modification of the sensitive assay for defensins described by Lehrer and colleagues (12, 18). Briefly, to obtain mid-logarithmic-phase bacteria, bacteria incubated overnight were diluted 1:1,000 in Trypticase soy broth (TSB) and were incubated at 37°C until the optical density of the aliquot at 620 nm reached 0.4. The bacteria were centrifuged at 900 × *g* for 10 min at 4°C, washed once with cold 10 mM sodium phosphate buffer (SPB; pH 7.4), and resuspended in cold SPB. On the basis of previously prepared standards of the optical density at 620 nm, a volume containing 1×10^6 or 4×10^6 bacterial CFU was added to 10 ml of previously autoclaved, warm (40 to 50°C) 10 mM SPB containing 3.0 mg of TSB medium, 1% (wt/vol) low-electroendosmosis-type agarose (Sigma), and a final concentration of 0.02% Tween 20.

After a rapid dispersion of the bacteria, the agar was poured into an agar plate to form a uniform layer approximately 2 mm deep and was punched with a 3-mm-diameter gel punch to make evenly spaced wells. Following the addition of 5 μl of control samples and antimicrobial agents or defensins to each well, the plates were incubated for 3 h at 37°C and were then overlaid with 10 ml of sterile agar consisting of a double-strength (6% [wt/vol]) solution of TSB and 1% (wt/vol) agarose. After incubation for 18 to 24 h at 37°C, the size of the clear zone surrounding each well was measured. The antibacterial activity represented the difference between the size of the clear zone surrounding wells containing the defensins or antimicrobial agents and that around wells containing the control sample (3 mm).

Evaluation of bacterial susceptibility to human defensins. To evaluate the susceptibilities of the bacterial strains to human defensins, we defined the minimal inhibitory dose (MID). The term described the minimum dose (in micrograms) of crude HNPs forming a detectable clear zone larger than the size of the central well (3 mm) under our experimental conditions. To validate the MIDs, we used a modified form of the time-kill method described by Ganz et al. (10). Briefly, approximately 10^6 of mid-logarithmic-phase bacteria were incubated in SPB supplemented with 0.3 mg of TSB per ml for 2 h at 37°C with and without crude HNPs (5 and 50 μg/ml). Viable cells (CFU per milliliter) were counted after incubation for 24 h at 37°C on Trypticase soy agar. The antibacterial activity was expressed as the percentage of growth inhibition relative to the growth on the defensin-free control (0.01% acetic acid). The limit of quantitation in this assay was a 3-log reduction (99.9%).

RESULTS

Antibacterial effects of individual purified HNPs. The antibacterial activities of the crude HNPs (Fig. 1) and individual purified HNP-1, HNP-2, and HNP-3 (Fig. 2) were evaluated by the radial diffusion assay. The activities of these peptides against *E. coli* ATCC 43827 (ML-35), *P. aeruginosa* ATCC 27853, MSSA ATCC 25923, and the clinical isolate MRSA Bl-3 were examined (Fig. 1 and 2). The results of the assay demonstrated that the size of the clear zone increased with higher doses of HNPs, although it varied from one strain to another. The increase in the size of the clear zone was directly proportional to the logarithm of the HNP dose.

The antibacterial activities of HNP-1 and HNP-2 against all tested strains were similar to those of crude HNPs, and chemically synthesized HNP-1 and HNP-2 were as active as those prepared from human PMNs (data not shown). However, the activity of HNP-3 against the same strains was less than those of crude HNPs, HNP-1 and -2 (Fig. 2). A higher dose of each HNP was required to form a clear zone around *P. aeruginosa* ATCC 27853 (Fig. 1 and 2).

Evaluation of bacterial susceptibility to human defensins. We evaluated the susceptibilities of several bacterial strains to human defensins by measuring the MID for each organism. The MIDs of the crude HNPs for the tested strains are presented in Fig. 3. While the MIDs were similar for *E. coli*, MSSA, and MRSA, it was clearly higher for *P. aeruginosa*. That is, the MID for *E. coli* ranged between 0.078 to 0.625 μg, that for MSSA was 0.156 to 0.625 μg, and that for MRSA was 0.156 to 1.25 μg; on the other hand, the MID for *P. aeruginosa* was 5 to 20 μg.

Interestingly, the MID for *E. coli* ATCC 43827 (ML-35), known as a defensin-susceptible strain (10, 15), was 0.156 μg, a value higher than that found for other strains of *E. coli* and

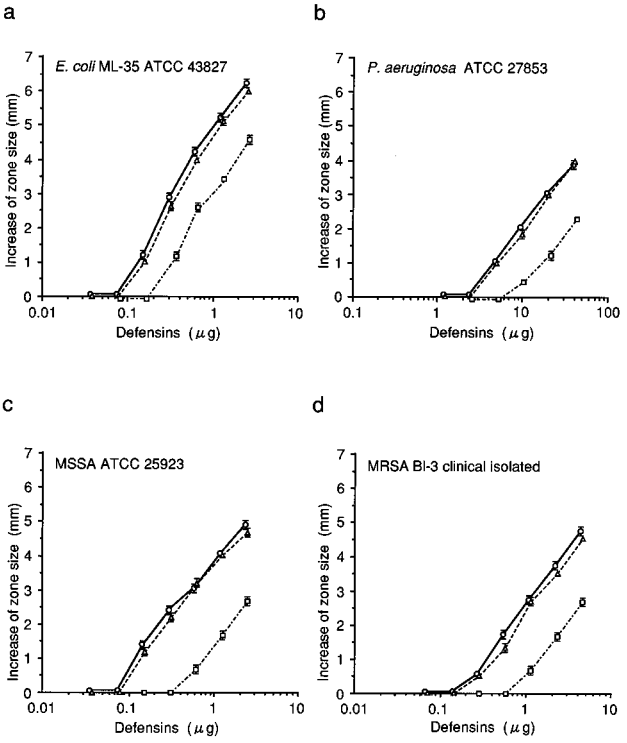

FIG. 2. Antibacterial activities of individual purified HNP-1 (circles), HNP-2 (triangles), and HNP-3 (squares) against *E. coli* ATCC 43827 (a), *P. aeruginosa* ATCC 27853 (b), MSSA ATCC 25923 (c), and MRSA Bl-3 (d). An increase in the zone size represents the zone size measured at each HNP concentration minus the size of the central well (3 mm). Data represent the means ± standard deviations of three independent experiments.

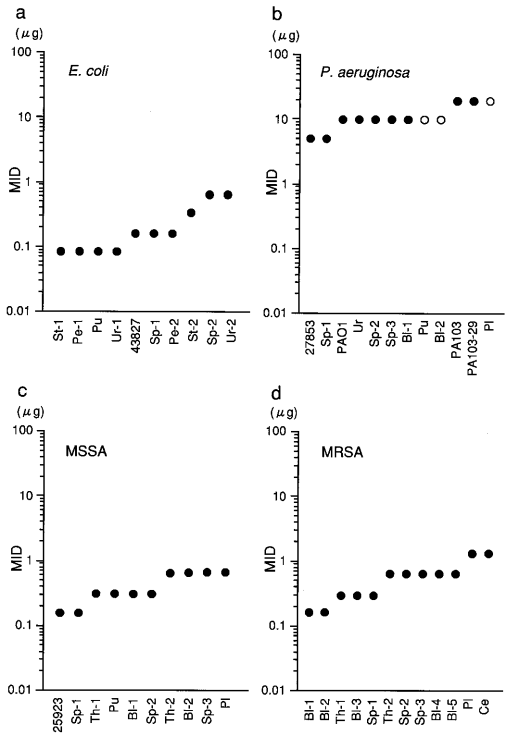

FIG. 3. MIDs of crude HNPs for *E. coli* (a), *P. aeruginosa* (closed circles, nonmucoid strains; open circles, mucoid strains) (b), MSSA (c), and MRSA (d). The numbers 43827, 27853, and 25923 indicate standard American Type Culture Collection strains. Clinical isolates were named according to their origin: St, stool; Pe, peritoneal effusion; Pu, pus; Ur, urine; Sp, sputum; Bl, blood; Pl, pleural effusion; Th, throat swab; and Ce, cerebrospinal fluid.

similar to that found for certain strains of MSSA and MRSA. The MIDs for clinical isolates obtained from various samples (sputum, blood, pus, stool, urine, throat swab, cerebrospinal fluid, and pleural and peritoneal effusions) were approximately the same among the same species. Furthermore, the MIDs for the mucoid and the nonmucoid strains of *P. aeruginosa* were similar, as were those for PA103 and PA103-29.

To validate the MID data, we used another antibacterial assay system, the time-kill assay. The results of the time-kill study are influenced by the initial bacterial input and the constitution of the buffer (10). Thus, we attempted to select an experimental condition that maximized the effects of defensins. The results obtained with this assay system demonstrated that the susceptibilities of *E. coli* ATCC 43827 (ML-35), MSSA ATCC 25923, and clinical isolate MRSA Bl-3 to crude HNPs were similar, while *P. aeruginosa* ATCC 27853 was less susceptible at both 5 and 50 μg/ml (Table 1).

Evaluation of bacterial susceptibility to other antimicrobial agents by the radial diffusion assay. In the next series of experiments we compared the antibacterial activities of the defensins with those of penicillin G and gentamicin (Fig. 4) using the radial diffusion assay. Like defensins, the size of the clear zone increased with the dose of the antimicrobial agents, although it varied among the tested strains, and the increase in size was directly proportional to the logarithm of the dose. However, the response rate, represented by the size of the zone, varied among agents and was higher than that observed with crude HNPs (Fig. 4). The MIDs and MICs of penicillin G, gentamicin, and crude HNPs for *E. coli* ATCC 43827 (ML-35), *P. aeruginosa* ATCC 27853, MSSA ATCC 25923, and MRSA Bl-3 are presented in Table 2. The results of the evaluation of bacterial susceptibility to penicillin G and gentamicin by MID determination were proportional to those measured by MIC determination, although minor inconsistencies were evident, particularly with gentamicin.

TABLE 1. Percent inhibition of growth of *E. coli*, *P. aeruginosa*, MSSA, and MRSA by crude HNPs[a]

Organism	Concentration (μg/ml)	% Growth inhibition[b]
E. coli ATCC 43827 (ML-35)	5	93.4 ± 5.5
(MID, 0.156 μg)	50	>99.9
P. aeruginosa ATCC 27853	5	26.8 ± 15.0
(MID, 5 μg)	50	93.2 ± 5.0
MSSA ATCC 25923	5	98.7 ± 0.9
(MID, 0.156 μg)	50	>99.9
MRSA Bl-3 clinical isolate	5	94.9 ± 3.7
(MID, 0.312 μg)	50	>99.9

[a] Approximately 10^6 mid-logarithmic-phase bacteria were incubated in 10 mM SPB (pH 7.4) supplemented with 0.3 mg of TSB per ml for 2 h at 37°C with and without crude HNPs (5 and 50 μg/ml), and viable cells (CFU per milliliter) were counted after incubation for 24 h at 37°C on Trypticase soy agar.

[b] Data are percent growth inhibition relative to the growth of the defensin-free control and represent the means ± standard deviations of five independent experiments.

DISCUSSION

The microbicidal activities of defensins have been reported previously (1, 2, 10, 11, 15, 17, 21, 26). However, to our knowledge, evaluation of the susceptibilities of several clinical bacterial isolates of different species to human defensins by a screening test such as the determination of MICs has not been reported previously. It is possible that the reason for the lack of such studies is the inadequacy of the broth microdilution or the agar dilution method, recommended by the National Committee for Clinical Laboratory Standards, for the determination of MICs and evaluation of the susceptibility of bacteria to crude HNPs. In a preliminary series of experiments, we attempted to determine the MICs of human defensins using the broth microdilution method. However, we could not determine these values clearly, and they were much higher than the prospective values from the previous reports (e.g., the MICs for *E. coli* ATCC 43827 [ML-35] and *P. aeruginosa* ATCC 27853

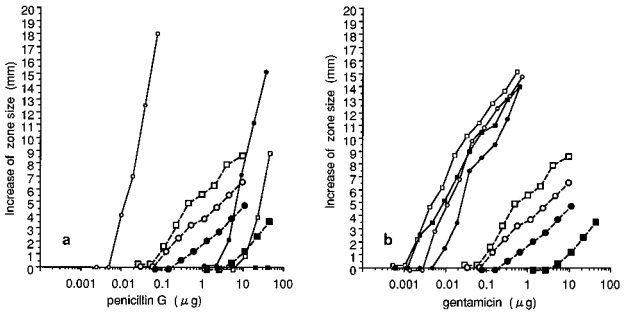

FIG. 4. Antibacterial activities of penicillin G (solid lines) (a), gentamicin (solid lines) (b), and crude HNPs (dotted lines) against *E. coli* ATCC 43827 (open squares), *P. aeruginosa* ATCC 27853 (closed squares), MSSA ATCC 25923 (open circles), and MRSA Bl-3 (closed circles). An increase in the zone size represents the zone size measured at each HNP concentration minus the size of the central well (3 mm). Against *P. aeruginosa* ATCC 27853, no detectable clear zone was shown with penicillin G at less than 40 μg. Data represent the means of three independent experiments (crude HNPs) and one representative datum (penicillin G and gentamicin).

TABLE 2. MIDs and MICs of crude HNPs and other antibiotics for *E. coli*, *P. aeruginosa*, MSSA, and MRSA[a]

Antibiotic or HNP	*E. coli* ATCC 43827 (ML-35)		*P. aeruginosa* ATCC 27853		MSSA ATCC 25923		MRSA Bl-3 clinical isolate	
	MID (μg)	MIC (μg/ml)	MID (μg)	MIC (μg/ml)	MID (μg)	MIC (μg/ml)	MID (μg)	MIC (μg/ml)
Penicillin G	10	64	>40	>512	0.01	0.5	5	64
Gentamicin	0.003	1	0.003	0.5	0.005	0.25	0.02	0.25
Crude HNP	0.156		5		0.156		0.312	

[a] MIDs were determined by the radial diffusion assay; MICs were determined by the broth microdilution method.

were higher than 256 μg/ml). Moreover, the agar dilution method required large amounts of human defensins. We suspect that the standard MIC determination is not suitable for evaluation of the antimicrobial activities of defensins.

Use of the radial diffusion assay (12, 18) in the present study allowed us to assess the antibacterial activities of human defensins against several clinical isolates of *E. coli*, *P. aeruginosa*, MSSA, and MRSA. This method is very sensitive and simple and has a good reproducibility. It also allows for the evaluation of the susceptibilities of several types of bacteria by simply modifying the pH, ionic strength, or buffer composition of the bottom agar (12, 18).

Using this assay system, we first compared the antibacterial activities of crude HNPs with those of purified HNP-1, -2, and -3. Our results indicated that crude HNPs, HNP-1 and HNP-2, had similar bactericidal effects, while HNP-3, which is the least cationic, had less of a bactericidal effect against all strains tested, confirming the results of previous reports (10, 21).

We also used the MID to compare and evaluate the susceptibilities of these bacteria to human defensins. Our measurement of the MIDs for a variety of bacterial strains, including clinically isolated bacteria, revealed that the MID was almost identical for the same species, with higher MIDs seen for *P. aeruginosa* than for the other isolates tested.

Since the MID is the dose (in micrograms) of HNPs applied to the wells on the bottom agar, it is not equivalent to the MIC. Thus, MIDs are not consistent with the concentrations determined by the time-kill method in previous studies. However, in the radial diffusion assay, since the total amount, but not the concentration, of the added HNPs or other antimicrobial agents reflects their concentration in the bottom agar, we believe that the total amount, the dose, may be more suitable for expressing the amounts of the agents added.

The MID was not used to provide the absolute quantity or concentration of defensins in the human body; it was used to provide a measure of the susceptibilities of the bacteria under the assay conditions. It is possible that the actual level of defensins in the inflammatory regions is higher than those obtained under our experimental conditions. Defensins constitute 5 to 7% of the protein in human PMNs (7, 9), and the average concentration of the peptides in granules is estimated to be approximately 50 mg/ml (8).

To validate the results of MID testing, we also used the time-kill method. The results of the latter study, as summarized in Table 1, are easily influenced by the initial bacterial input and the constitution of the buffer used (10), but we selected experimental conditions that maximized the effects of defensins. The results obtained with this assay system demonstrated that *P. aeruginosa* ATCC 27853 is less susceptible to defensins than *E. coli* ATCC 43827 (ML-35), MSSA ATCC 25923, and the clinical isolate MRSA Bl-3. However, the MIDs for *P. aeruginosa* were markedly higher than those for the other species tested. This may be due to enhancement by certain factors, e.g., loss of permeation of HNPs into the agar at the high concentrations.

Using the time-kill method, Ganz et al. (10) demonstrated that human defensins are more active against *E. coli* ATCC 29648 and ML-35 than against *P. aeruginosa* PAO 579, a finding consistent with the results of our study. These results are, however, different from those obtained with rats (14) and rabbits (2, 28). In those animals, these natural antibiotics, defensins, had similar or greater effects against *P. aeruginosa* than *E. coli* and other strains.

Fields et al. (5) suggested that the activities of defensins may influence the development and severity of bacterial infections, as evidenced by the defensin hypersusceptibility of mutants of *Salmonella typhimurium*. In the present study, we compared the effects of human defensins on mucoid and nonmucoid strains of *P. aeruginosa*, against strain PA103, a hyperproducer of exotoxin A, and against strain PA103-29, the exotoxin A-deficient mutant of strain PA103. Since the MIDs for these strains were similar, it is probable that these virulence factors would not enhance bacterial survival. Furthermore, we also observed that the effects of defensins on MSSA and MRSA were similar. However, because of the variability of MIDs for the same species, especially MRSA, we speculate that defensin-hypersusceptible or -resistant variants may be isolated by screening for susceptibility to defensins.

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