Effects of bacteriocins on methicillin-resistant Staphylococcus aureus biofilm

Running title: Mode of Action of Bacteriocins against MRSA Biofilm

Ken-ichi Okuda,a* Takeshi Zendo,b Shinya Sugimoto,a Tadayuki Iwase,a Akiko Tajima,a Satomi Yamada,a Kenji Sonomoto,b and Yoshimitsu Mizunoea*

aDepartment of Bacteriology, The Jikei University School of Medicine, Tokyo, Japan
bLaboratory of Microbial Technology, Division of Applied Molecular Microbiology and Biomass Chemistry, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, Fukuoka, Japan

*Address correspondence to Ken-ichi Okuda, okuda-k@jikei.ac.jp and Yoshimitsu Mizunoe, mizunoe@jikei.ac.jp.
Control of biofilms formed by microbial pathogens is an important subject for medical researchers, since the development of biofilms on foreign body surfaces often causes biofilm-associated infections in patients with indwelling medical devices. The present study examined the effects of different kinds of bacteriocins, which are ribosomally-synthesized antimicrobial peptides produced by certain bacteria, on biofilms formed by a clinical isolate of methicillin-resistant \textit{Staphylococcus aureus} (MRSA). The activities and modes of action of three bacteriocins with different structures (nisin A, lacticin Q, and nukacin ISK-1) were evaluated. Vancomycin, a glycopeptide antibiotic used in the treatment of MRSA infections, showed bactericidal activity against planktonic cells, but not against biofilm cells. Among the tested bacteriocins, nisin A showed the highest bactericidal activity against both planktonic cells and biofilm cells. Lacticin Q also showed bactericidal activity against both planktonic cells and biofilm cells, but its activity against biofilm cells was significantly lower than that of nisin A. Nukacin ISK-1 showed bacteriostatic activity against planktonic cells and did not show bactericidal activity against biofilm cells. Mode-of-action studies indicated that pore formation leading to ATP efflux is important for the bactericidal activity against biofilm cells. Our results suggest that bacteriocins that form stable pores on biofilm cells are highly potent for the treatment of MRSA biofilm infections.
A biofilm is a community of microorganisms that adheres to biotic and abiotic surfaces. In a biofilm, cells form a cluster embedded in a self-produced extracellular matrix consisting of biomolecules such as polysaccharides, proteins, nucleic acids, and lipids (1, 2). Bacterial cells in a biofilm exhibit resistance to the host’s immune system and antibiotic treatments (3-6). Therefore, once biofilms form on surfaces of medical devices such as indwelling vascular catheters, prosthetic joints, and cardiac pacemakers, they can be extremely difficult to eradicate, and invasive procedures such as removal of the infected device may be required (7). Biofilm-associated infections are often chronic or relapsing; therefore, successful prevention and treatment of these infections is a significant issue.

Bacteriocins are bacterially produced peptides or proteins that show bacteriostatic and/or bactericidal activity against other bacteria. It has been suggested that bacteriocins are valuable alternatives to antibiotics because they have many properties (e.g., significant potency, high stability, low toxicity, and availability of both broad- and narrow-spectrum bacteriocins) that make them suitable for clinical applications (8). Bacteriocins produced by Gram-positive bacteria are classified into two distinct categories: the lanthionine-containing bacteriocins (class I) and the non-lanthionine-containing bacteriocins (class II) (9). The class I bacteriocins, termed lantibiotics, are characterized by the presence of unusual amino acids such as lanthionine, 3-methyllanthionine, dehydroalanine, and dehydrobutyrine (10, 11). The lanthionine residue consists of two alanine residues cross-linked via a thioether linkage; therefore, lantibiotics are typically characterized by cyclic structures. The unusual amino acids are introduced by post-translational modification and contribute to the high
stability of the lantibiotics against many stresses (10, 11). The class II bacteriocins are small heat-stable peptides without unusual amino acids. Cotter et al. proposed a classification of class II bacteriocins into 4 groups: IIa (pediocin-like), IIb (two-peptide), IIc (cyclic), and IId (non-pediocin single linear peptides) (9). To date, many bacteriocins have been identified, and their structures, modes of action, and biosynthetic mechanisms have been studied. Nisin A, a lantibiotic produced by Lactococcus lactis (12), uses lipid II, which is a membrane-bound precursor of cell wall, as a docking molecule (13-15). Nisin A is known to possess a dual mode of action: inhibition of cell wall synthesis by masking lipid II (bacteriostatic), and membrane insertion followed by pore formation that rapidly kills cells (bactericidal). Nukacin ISK-1, a lantibiotic produced by Staphylococcus warneri ISK-1 (16), is also known to bind lipid II (17). Unlike nisin A, nukacin ISK-1 has only a bacteriostatic mode of action, suggesting that nukacin ISK-1 prevents cell wall synthesis, but does not form pores leading to cell death (18). Nisin A and nukacin ISK-1 belong to distinct subgroups of lantibiotics on the basis of their structures (Fig. 1); nisin A belongs to type AI lantibiotics (linear) and nukacin ISK-1 belongs to type AII lantibiotics (linear N-terminus and globular C-terminus) (10, 11). Lacticin Q, a class IId bacteriocin produced by Lactococcus lactis QU 5 (19), forms huge toroidal pores, causing leakage of protein molecules from target cells with lipid flip-flop (20), thus following a bactericidal mode of action. Importantly, lacticin Q does not require a specific receptor such as lipid II for its activity (21).

The emergence of methicillin-resistant Staphylococcus aureus (MRSA) is a significant public health problem worldwide (22). Recently, we reported that S. aureus MR23, a clinically isolated MRSA strain, has the potential to form a robust biofilm with a matrix primarily consisting of proteins (2). In this study, we investigated the
antibacterial effects of three bacteriocins (nisin A, lacticin Q, and nukacin ISK-1; Fig. 1) and vancomycin against planktonic and biofilm cells of *S. aureus* MR23 and several staphylococcal biofilms. In addition, mode-of-action studies of these bacteriocins and vancomycin against biofilm cells were carried out by measuring ATP efflux and membrane potential. Our data suggest that bacteriocins that form stable pores on the membrane of target cells are effective against MRSA biofilms and have the potential to prevent or cure biofilm-associated infections.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *S. aureus* and *S. epidermidis* strains were cultured in brain heart infusion (BHI; BD Bioscience, Franklin Lakes, NJ) medium at 37°C with shaking. *L. lactis* QU 5 was cultured in MRS (Oxoid, Basingstoke, United Kingdom) medium at 30°C under static conditions. *S. warneri* ISK-1 was cultured in tryptic soy broth (BD Bioscience) at 37°C with shaking.

**Bacteriocin purification.** Nisin A was purified from a commercial preparation (Sigma-Aldrich, St. Louis, MO) as described previously (23). Nukacin ISK-1 was purified from an overnight culture supernatant of *S. warneri* ISK-1 as described previously (23). Lacticin Q was purified from an overnight culture supernatant of *L. lactis* QU 5 by using the same procedure used for purification of nukacin ISK-1.

**MIC determination.** *S. aureus* and *S. epidermidis* strains were pre-cultured in BHI medium overnight, inoculated into fresh BHI medium, and incubated until an optical density of 0.05 at 595 nm was attained. Aliquots (20 μL) of serially diluted peptide
solutions (nisin A, lacticin Q, nukacin ISK-1, or vancomycin) were added to the wells of a 96-well flat-bottomed polystyrene plate (Corning, Corning, NY) containing 80 μL of the bacterial culture. After culturing for 24 h at 37°C with shaking, the absorbance at 595 nm was measured using an Infinite 200 PRO microplate reader (Tecan, Mannedorf, Switzerland). The minimum inhibitory concentrations (MICs) were defined as the lowest concentrations of the peptide that completely inhibited bacterial growth.

**Bactericidal activity against *S. aureus MR23* planktonic cells.** *S. aureus* MR23 was pre-cultured in BHI medium overnight, inoculated into fresh BHI medium, and incubated until an optical density of 0.4 at 595 nm was attained. Peptide solutions (50 μL) were added to 150 μL of the bacterial culture in a 96-well flat-bottomed polystyrene plate to a final concentration of 4× MIC and incubated at 37°C with shaking. The colony-forming units (CFUs) on BHI agar were enumerated at different time points (0, 1, 4, 8, 16, and 24 h).

**Bactericidal activity against *S. aureus MR23* biofilm cells.** *S. aureus* MR23 was pre-cultured in BHI medium overnight and diluted 1000-fold with BHI medium supplemented with 1% glucose (BHI-G). Aliquots (200 μL) of this bacterial suspension were added to the wells of a 96-well flat-bottomed polystyrene plate and incubated for 24 h at 37°C. The supernatants were removed, and the biofilms formed on the bottoms of the wells were washed twice with phosphate-buffered saline (PBS). Biofilms were incubated with 50 μL of the peptide solutions in PBS at a concentration of 4× MIC for 1 and 24 h at room temperature. After removal of supernatants, the biofilms were washed twice and then resuspended in 100 μL of PBS. The suspensions were sonicated for 30 s...
and the CFUs on BHI agar were counted.

Dose dependencies of bacteriocins and vancomycin against *S. aureus* MR23 biofilm.

Biofilms were formed on a 96-well flat-bottomed polystyrene plate as described above and incubated with 50 μL of the peptide solutions in PBS at a concentration of 4× MIC for 1 h at room temperature. After removal of supernatants, the biofilms were washed twice with PBS. Living and dead cells in the biofilms were stained using a FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. After two washes with PBS, the fluorescence derived from propidium iodide (PI) was measured with an Infinite 200 PRO microplate reader at an excitation and emission wavelength of 485 and 635 nm, respectively.

Three-dimensional live/dead imaging of *S. aureus* and *S. epidermidis* biofilm. *S. aureus* and *S. epidermidis* strains were pre-cultured in BHI medium overnight and diluted 1000-fold with BHI-G medium. An aliquot of 2 mL of this bacterial suspension was added to a 35 mm glass-bottomed dish (Iwaki, Tokyo, Japan) and incubated for 24 h at 37°C. The supernatant was removed and the biofilm formed at the bottom of the dish was washed twice with PBS. The biofilm was incubated with 100 μL of peptide solution in PBS at a concentration of 4× MIC for 1 h at room temperature. After removal of the supernatant, the biofilm was washed with PBS and stained using a FilmTracer™ LIVE/DEAD® Biofilm Viability Kit according to the manufacturer’s protocol. After washing with PBS, the biofilm was observed by fluorescence microscopy (BZ9000; Keyence, Tokyo, Japan). A three-dimensional image was constructed by stacking multiple images with different Z-foci.
ATP efflux from *S. aureus* MR23 biofilm. Biofilms were formed on a 96-well flat-bottomed polystyrene plate as described above and incubated with 50 μL of the peptide solutions in PBS at a concentration of 4× MIC for 1 h at room temperature. The supernatants were filtered using a 0.22-μm centrifugal filter (Millipore, Billerica, MA) and diluted 10-fold with water. ATP concentrations of the filtrates were measured using BacTiter-Glo Reagent (Promega, Madison, WI) according to the manufacturer’s protocol. The bioluminescence response in relative light units was detected with an Infinite 200 PRO microplate reader. The ATP concentration of each sample was determined using standard ATP solutions.

Measurement of membrane potential of *S. aureus* MR23 biofilm. Biofilms were formed on a 96-well flat-bottomed polystyrene plate as described above. The membrane potentials of the biofilm cells were measured using a membrane-potential-sensitive probe, bis-(1,3-dibutylbarbituric acid)trimethine oxonol (DiBAC₄(3); Invitrogen). The biofilms were pre-incubated with 100 μL of 5 μM DiBAC₄(3) in PBS for 30 min. After removal of the supernatant, the biofilms were incubated with 50 μL of solution containing 5 μM DiBAC₄(3) and peptides at a concentration of 4× MIC in PBS for 1 h at room temperature. After two washes with PBS, the fluorescence derived from DiBAC₄(3) was measured with an Infinite 200 PRO microplate reader at an excitation and emission wavelength of 485 and 535 nm, respectively.

Binding of fluorescently labeled vancomycin to *S. aureus* MR23 biofilm. Biofilms were formed on a 96-well flat-bottomed polystyrene plate as described above. After two
washes with PBS, the biofilms were incubated with 50 μL of 40 μM BODIPY FL vancomycin (BODIPY-vancomycin; Invitrogen) dissolved in PBS for 0, 5, 15, 30, and 60 min at room temperature. After two washes with PBS, fluorescence derived from BODIPY-vancomycin was measured with an Infinite 200 PRO microplate reader at an excitation and emission wavelength of 485 and 535 nm, respectively. After 60-min incubation, the biofilms were washed with PBS to remove unbound BODIPY-vancomycin and resuspended, and the localization of BODIPY-vancomycin was analyzed by fluorescence microscopy using a GFP filter (Eclipse E600; Nikon, Tokyo, Japan).

RESULTS

Susceptibility of S. aureus MR23 to bacteriocins and vancomycin under planktonic growth conditions. At first, MICs of nisin A, lacticin Q, nukacin ISK-1, and vancomycin against S. aureus MR23 were determined by the micro-liquid dilution method (Table 1). After addition of 4× MIC peptides to S. aureus MR23 cells adjusted to 10^8 CFU/mL in the culture medium, CFUs were counted following different incubation periods (Fig. 2). After the addition of nisin A and lacticin Q, which have a bactericidal mode of action, the cells were killed quickly. Incubation times for complete cidal action (i.e., no colony formation on BHI-agar) were 1 and 4 h for nisin A and lacticin Q, respectively. Nukacin ISK-1 showed bacteriostatic activity against S. aureus MR23, consistent with its previously reported mode of action (18). Vancomycin showed bactericidal activity, but the number of viable cells after 4-h incubation was 10^7 CFU/mL (Fig. 2), indicating that its killing rate is slower than that of nisin A and lacticin Q.
Antibacterial activities of bacteriocins and vancomycin against *S. aureus* MR23 biofilm cells. Biofilms of *S. aureus* MR23 formed on a 96-well plate were incubated with the peptides at a concentration of 4× MIC for 1 and 24 h; subsequently, the CFUs of the biofilms suspended in PBS were enumerated. Nisin A and lacticin Q showed bactericidal activities against biofilm cells, but complete killing (as observed for planktonic cells) was not achieved (Fig. 3). After 24-h incubation, the bactericidal activity of nisin A was about 10 times higher than that of lacticin Q. Nukacin ISK-1, which showed bacteriostatic activity against planktonic cells, did not show bactericidal activity against biofilm cells. Vancomycin showed bactericidal activity against planktonic cells but it did not show bactericidal activity against biofilm cells under the experimental conditions.

Diffusion of bacteriocins into *S. aureus* MR23 biofilm. After treatment with bacteriocins or vancomycin, the biofilms were stained with SYTO9 and PI (components of the FilmTracer™ LIVE/DEAD® Biofilm Viability Kit), which stain the living and dead cells, respectively. Subsequently, three-dimensional images of the stained biofilms were observed using fluorescence microscopy (Fig. 4). After incubation of the biofilm with PBS for 60 min, living cells constituted the majority of the biofilm (Fig. 4A). After addition of nisin A, the proportion of dead cells was significantly higher and it increased with the incubation time (Fig. 4B and 4C). Lacticin Q also increased the proportion of dead cells, but not as much as nisin A (Fig. 4D). Addition of vancomycin or nukacin ISK-1 had no effect on the viability of the biofilm cells (Fig. 4E and 4F). These results are consistent with the CFU counts of the biofilms after treatment with the peptides. It
should be noted that dead cells in the biofilms treated with nisin A and lacticin Q seemed to be uniformly distributed throughout the biofilm (Fig. 4B′, 4C′, and 4D′). If the biofilm matrix blocked the penetration of the bacteriocins, dead cells would be found primarily at the top of the biofilm. Therefore, it is suggested that both nisin A and lacticin Q penetrated the biofilm matrix and reached the deepest part of the biofilm.

Modes of action of bacteriocins against *S. aureus* MR23 biofilm. To determine the effective concentrations of the peptides against biofilm cells, serially diluted peptides were added to the biofilms and the fluorescence derived from dead cells stained by PI was measured with a fluorometer (Fig. 5). The fluorescence intensity of cells treated with nisin A was higher than that of untreated cells and it increased with nisin A concentration. The increase in fluorescence was observed even when 0.25× MIC (0.625 μM) nisin A was added. Addition of lacticin Q also increased the fluorescence intensity of the biofilm in a dose-dependent manner, but the intensity was significantly lower than that of the biofilm treated with nisin A. Nukacin ISK-1 and vancomycin did not increase the fluorescence even at 8× MIC (96 and 80 μM, respectively). None of the peptides reduced the amount of biofilm, even at 8× MIC (data not shown), indicating that they do not have destructive activity against the established *S. aureus* MR23 biofilm. Next, we analyzed the efflux of ATP molecules from the biofilm cells by luciferase assay (Fig. 6). The amount of ATP leaked from biofilm cells increased in a dose-dependent manner following treatment with nisin A or lacticin Q. Nisin A caused ATP efflux at 0.25× MIC (0.625 μM), whereas the minimum concentration of lacticin Q required for ATP efflux was 1× MIC (5.0 μM). Nukacin ISK-1 and vancomycin had no effect on ATP efflux even at 8× MIC (96 and 80 μM, respectively). In addition, to
analyze the effects of the peptides on membrane integrity, we measured the membrane potentials of the biofilm cells by using DiBAC₄(3), a membrane-potential-sensitive probe (24) (Fig. 7). As might be expected from the results of ATP efflux measurements, nisin A and lacticin Q disrupted the membrane potentials of biofilm cells, and vancomycin did not. Interestingly, nukacin ISK-1 disrupted the membrane potential of the biofilm cells but it did not cause ATP efflux.

**Localization of fluorescently labeled vancomycin in *S. aureus* MR23 biofilm.** Vancomycin showed no bactericidal activity or membrane depolarization activity against biofilm cells. A possible explanation is that the biofilm matrix physically inhibited vancomycin penetration, so that vancomycin was not able to attack the biofilm cells. To test this hypothesis, we analyzed the localization of BODIPY-vancomycin (fluorescently labeled vancomycin) in *S. aureus* MR23 biofilm. BODIPY labeling seems to have no effect on the activity of vancomycin, since the MIC of BODIPY-vancomycin against *S. aureus* MR23 was identical to that of vancomycin (Table 1). After treatment of biofilm cells with BODIPY-vancomycin, the fluorescence derived from BODIPY-vancomycin was measured at different incubation times. The fluorescence increased with the incubation time and peaked after 30 min (Fig. 8A). After 60 min, the biofilm was suspended in PBS and observed by fluorescence microscopy. Fluorescence was observed at the surface of virtually every cell, and in some cells, the division septa were also stained (Fig. 8B). These results suggest that vancomycin can penetrate *S. aureus* MR23 biofilm and bind to lipid II, a target molecule for vancomycin located at the division septum.
Effects of bacteriocins against other staphylococcal biofilms

Activities of bacteriocins and vancomycin against biofilms of several clinical isolates of MRSA (S. aureus MR10 and MR11) and S. epidermidis (S. epidermidis SE4 and SE21) (Sato et al., unpublished data), and S. aureus SH1000 (25), a laboratory strain frequently used for biofilm assays, were investigated. The MICs of the peptides were shown in Table 1. After treatments of the biofilms with peptides at a concentration of 4× MIC for 1 followed by staining with SYTO9 and PI, live/dead images were observed. By way of exception, biofilms of SE4 and SE21 were treated with 20 μM lacticin Q since MICs of lacticin Q against these two strains were not determined at the highest concentration (Table 1). The X-Y sections of the three-dimensional images were shown in Fig. 9. Among the tested peptides, nisin A showed the highest activity against all biofilms, and dead cells constituted the majority of the biofilms after treatments with nisin A. Nukacin ISK-1 and vancomycin did not affect the viability of the biofilm cells as observed in assays using MR23 biofilm. MR10 biofilm was less sensitive to lacticin Q compared with MR11 and SH1000 biofilms, even though MICs of lacticin Q against the three strains were identical. Interestingly, lacticin Q was slightly active against SE4 biofilm at 20 μM, which is a concentration below MIC. These results suggested that biofilm formations affected susceptibility against lacticin Q and/or antimicrobial property of lacticin Q.

DISCUSSION

In this study, we investigated the effects of bacteriocins with different structures against a clinical isolate of MRSA. Among the tested bacteriocins, nisin A showed the highest bactericidal activities against both planktonic cells and biofilm cells.
In case of growing planktonic cells, nisin A and lacticin Q completely killed $10^8$ cells/mL at 4× MIC (10 and 20 μM, respectively) after 4-h incubation. However, 4× MIC nisin A and lacticin Q could not completely kill biofilm cells after 24-h incubation. These results suggest that *S. aureus* MR23 is less susceptible to bacteriocins when growing as a biofilm compared to when growing under planktonic conditions. Although mechanisms underlying bacteriocin resistance of biofilm cells are not understood, nonspecific interactions between bacteriocin molecules and the components of the biofilm matrix and/or the physiological state of the biofilm cells might affect bacteriocin activity. Notably, the bactericidal activity of nisin A against MR23 biofilm cells was about 10-fold higher than that of lacticin Q (Fig. 3). Additionally, nisin A was significantly more effective than lacticin Q against biofilms of several strains of *S. aureus* and *S. epidermidis* (Fig. 9). These results suggested that nisin A attacks biofilm cells more effectively than lacticin Q. What is the key factor influencing the superior bactericidal activity of nisin A against biofilm cells? Nisin A and lacticin Q represent different classes of bacteriocins (class Ia and class IIId, respectively) and have different structures, molecular weights, and modes of action. One possibility is that lipid II, a docking molecule for nisin A, contributes to the high activity of nisin A against biofilm cells. Nisin A is able to act against an anionic model membrane lacking lipid II (26-28). However, the presence of lipid II dramatically enhances the efficiency of nisin A, making nisin A active against susceptible cells at nanomolar concentrations (13). Mode-of-action studies with living cells and artificial membranes demonstrated that lipid II is not required for the activity of lacticin Q (21). Lacticin Q forms toroidal pores on the target membranes in the absence of a specific receptor (20), and the pore-forming activity depends on the physicochemical features of the membrane components (29).
the biofilm, the cells are surrounded by a matrix consisting of various kinds of macromolecules bonded by weak interactions such as hydrogen bonds; hydrophobic, electrostatic, and van der Waals interactions; and divalent cation bridges (1). In such environments, specific docking molecules like lipid II may contribute to specific binding to target cells.

It has been reported that nukacin ISK-1, a bacteriocin belonging to class I, is bacteriostatic against planktonic cells and uses lipid II as a docking molecule (17, 18). In this study, nukacin ISK-1 did not show bactericidal activity against biofilm cells, although it disrupted the membrane potential of biofilm cells (Fig. 7). According to a previous report, SA-FF22, a bacteriocin produced by *Streptococcus pyogenes* FF22, with a structure similar to that of nukacin ISK-1 (30), disrupts membrane potential by forming small short-lived pores (0.5–0.6 nm in diameter and with lifespans of a few milliseconds) (31). Therefore, nukacin ISK-1 may have penetrated the biofilm, forming small short-lived pores that allow small ions to pass. The levels of ATP efflux from biofilms were well correlated with the bactericidal activity of each bacteriocin (Fig. 3–5). For effective killing of biofilm cells, a pore size large enough for ATP to pass (>1.5 nm in diameter) seems to be required. Indeed, nisin A forms large stable pores (2–2.5 nm in diameter and with lifespans of a few seconds) on membranes containing lipid II (32), and lacticin Q forms huge toroidal pores (4.6–6.6 nm in diameter) on membranes without lipid II (20).

Vancomycin is widely used as a first-line therapy for serious MRSA infections. We have shown that vancomycin exhibits bactericidal activity against growing planktonic cells of *S. aureus* MR23, but not against biofilm cells. It has been reported that vancomycin inhibits cell wall synthesis by binding to the \( N\)-acyl-d-Ala-d-Ala
moiety of lipid II and prevents cross-linking of peptidoglycan chains, thereby leading to bacterial cell death by osmotic lysis (33). Although detailed molecular mechanisms underlying antibiotic resistance of biofilms remain unclear, one of the possibilities is that the matrix inhibits penetration of antimicrobial agents (3, 34). On the other hand, a recent study that analyzed the diffusion of BODIPY-vancomycin into an S. aureus biofilm by using time-resolved fluorescence microscopy suggested that the biofilm matrix is not an obstacle to the diffusion of molecules the size of antibiotics (35). The authors demonstrated that BODIPY-vancomycin penetrates into the deepest layer of the biofilm within minutes. In an earlier study, another group reported that penetration of BODIPY-vancomycin to the deepest layer took more than 1 h (36). The difference in penetration time in the two studies might be due to differences between the components of the biofilm matrix produced by the S. aureus strains used in the study. We estimated the penetration of BODIPY-vancomycin by measuring fluorescence intensity. We found that fluorescence reached a steady state after 30-min incubation of the biofilm with BODIPY-vancomycin (Fig. 8A). Notably, BODIPY-vancomycin localized not only at cell surfaces but also at division septa (Fig. 8B). Since the division septum is the only site of active cell wall biosynthesis of S. aureus, antibiotics that inhibit the late steps of cell wall synthesis must be able to access lipid II at the septum (37, 38). Our results suggest that vancomycin penetrated the biofilm matrix and functionally bound to its molecular target. The lower susceptibility of cells in biofilms to vancomycin may be explained by the slow growth rate of cells (3, 7, 39-41).

In conclusion, we have demonstrated that nisin A is significantly effective against MRSA and other staphylococcal biofilms. To prevent biofilm-associated infections in patients using central venous catheters, an antibiotic-lock technique has
been attempted whereby the lumen of the catheter is flushed and then filled with an antibiotic solution. It has been demonstrated that an antibiotic-lock of central venous catheters employing a vancomycin-containing solution prevents endoluminal catheter-related infection (42-44). In such clinical applications, pore-forming bacteriocins which have similar modes of actions to nisin A might be more effective than antibiotics for the prevention and treatment of biofilm-associated infections.

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FIGURE LEGENDS

FIG 1 Structures of bacteriocins
The shaded residues indicate modified amino acids. Ala-S-Ala, lanthionine; Abu-S-Ala, 3-methyllanthionine; Dha, dehydroalanine; Dhb, dehydrobutyrine; fMet, formylmethionine.

**FIG 2** Bactericidal activities of bacteriocins and vancomycin against planktonic cells

Peptides at final concentrations of 4× MIC were incubated with *S. aureus* MR23 in BHI medium and further incubated at 37°C with shaking. The CFUs were enumerated following different incubation periods (0, 1, 4, and 24 h). Data points represent the means and standard deviations of triplicate determinations.

**FIG 3** Bactericidal activities of bacteriocins and vancomycin against biofilm cells

Peptide solutions at a concentration of 4× MIC were incubated with preformed biofilms of *S. aureus* MR23 at room temperature. The CFUs of biofilm cells were measured after 1-h (white bars) and 24-h (black bars) incubation. The means and standard deviations of triplicate determinations are presented.

**FIG 4** Three-dimensional images of *S. aureus* MR23 biofilms after treatment with bacteriocins and vancomycin

Biofilms formed on 35-mm glass-bottomed dishes were incubated with peptide solutions at a concentration of 4× MIC for 1 h at room temperature. After washing, the living and dead cells in the biofilm were stained by SYTO9 (green) and PI (red), respectively. A, PBS (60 min); B, nisin A (5 min); C, nisin A (60 min); D, lacticin Q (60 min); E, nukacin ISK-1 (60 min); F, vancomycin (60 min). A′–D′, enlarged views of X-Z sections of corresponding panels. Scale bars, 5 μm.
FIG 5 Dose-dependent effects of bacteriocins and vancomycin against *S. aureus* MR23 biofilm

Peptide solutions at a concentration of 4× MIC were incubated with preformed *S. aureus* MR23 biofilm for 1 h at room temperature. Subsequently, biofilms were stained using a FilmTracer™ LIVE/DEAD® Biofilm Viability Kit. Fluorescence derived from PI was measured at an excitation and emission wavelength of 485 and 635 nm, respectively. The means and standard deviations of triplicate determinations are presented. A.U., arbitrary units.

FIG 6 ATP efflux from *S. aureus* MR23 biofilm cells

After incubation of *S. aureus* MR23 biofilm cells with bacteriocins and vancomycin at a concentration of 4× MIC for 1 h at room temperature, the amount of ATP in the supernatants was measured by luciferase assay. ATP concentrations were determined using standard ATP solutions. The means and standard deviations of triplicate determinations are presented.

FIG 7 Membrane potential of *S. aureus* MR23 biofilm after incubation with bacteriocins and vancomycin

Fluorescence derived from DiBAC₄(3), a membrane-potential-sensitive probe, was measured at an excitation and emission wavelength of 485 and 535 nm, respectively. The means and standard deviations of triplicate determinations are presented. A.U., arbitrary units.
FIG 8 Localization analysis of BODIPY-vancomycin

*S. aureus* MR23 biofilm was incubated with BODIPY-vancomycin at a concentration of 4× MIC for 0, 5, 15, 30, and 60 min at room temperature. Fluorescence derived from BODIPY-vancomycin was measured at an excitation and emission wavelength of 485 and 535 nm, respectively. The means and standard deviations of triplicate determinations are presented. A.U., arbitrary units (A). After 60-min incubation with BODIPY-vancomycin, the biofilm resuspended in PBS was observed by fluorescence microscopy (B). Arrows indicate division septa of the cells. Insert shows enlargement of boxed area. Scale bar, 1 μm.

FIG 9 Live/dead images of staphylococcal biofilms after treatment with bacteriocins and vancomycin

Biofilms formed on 35-mm glass-bottomed dishes were incubated with peptide solutions at a concentration of 4× MIC for 1 h at room temperature. By way of exception, biofilms of SE4 and SE21 were treated with 20 μM lacticin Q. After washing, the living and dead cells in the biofilm were stained by SYTO9 (green) and PI (red), respectively. X-Z sections of three-dimensional images were shown. MR10, *S. aureus* MR10; MR11, *S. aureus* MR11; SH1000, *S. aureus* SH1000; SE4, *S. epidermidis* SE4; SE21, *S. epidermidis* SE21. Scale bar, 5 μm.
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FIG 1 Structures of bacteriocins
The shaded residues indicate modified amino acids. Ala-S-Ala, lanthionine; Abu-S-Ala, 3-methyllanthionine; Dha, dehydroalanine; Dhb, dehydrobutyrine; fMet, formylmethionine.
FIG 2 Bactericidal activities of bacteriocins and vancomycin against planktonic cells
Peptides at final concentrations of 4× MIC were incubated with *S. aureus* MR23 in BHI medium and further incubated at 37°C with shaking. The CFUs were enumerated following different incubation periods. Data points represent the means and standard deviations of triplicate determinations.
FIG 3 Bactericidal activities of bacteriocins and vancomycin against biofilm cells
Peptide solutions at a concentration of 4× MIC were incubated with preformed biofilms of *S. aureus* MR23 at room temperature. The CFUs of biofilm cells were measured after 1-h (white bars) and 24-h (black bars) incubation. The means and standard deviations of triplicate determinations are presented.
FIG 4 Three-dimensional images of *S. aureus* MR23 biofilms after treatment with bacteriocins and vancomycin

Biofilms formed on 35-mm glass-bottomed dishes were incubated with peptide solutions at a concentration of 4× MIC for 1 h at room temperature. After washing, the living and dead cells in the biofilm were stained by SYTO9 (green) and PI (red), respectively. A, PBS (60 min); B, nisin A (5 min); C, nisin A (60 min); D, lacticin Q (60 min); E, nukacin ISK-1 (60 min); F, vancomycin (60 min). A′–D′, enlarged views of X-Z sections of corresponding panels. Scale bars, 5 μm.
FIG 5 Dose-dependent effects of bacteriocins and vancomycin against *S. aureus* MR23 biofilm
Peptide solutions at a concentration of 4× MIC were incubated with preformed *S. aureus* MR23 biofilm for 1 h at room temperature. Subsequently, biofilms were stained using a FilmTracer™ LIVE/DEAD® Biofilm Viability Kit. Fluorescence derived from PI was measured at an excitation and emission wavelength of 485 and 635 nm, respectively. The means and standard deviations of triplicate determinations are presented. A.U., arbitrary units.
FIG 6 ATP efflux from *S. aureus* MR23 biofilm cells
After incubation of *S. aureus* MR23 biofilm cells with bacteriocins and vancomycin at a concentration of 4× MIC for 1 h at room temperature, the amount of ATP in the supernatants was measured by luciferase assay. ATP concentrations were determined using standard ATP solutions. The means and standard deviations of triplicate determinations are presented.
FIG 7 Membrane potential of *S. aureus* MR23 biofilm after incubation with bacteriocins and vancomycin

Fluorescence derived from DiBAC$_4$(3), a membrane-potential-sensitive probe, was measured at an excitation and emission wavelength of 485 and 535 nm, respectively. The means and standard deviations of triplicate determinations are presented. A.U., arbitrary units.
FIG 8 Localization analysis of BODIPY-vancomycin.

*S. aureus* MR23 biofilm was incubated with BODIPY-vancomycin at a concentration of 4× MIC for 0, 5, 15, 30, and 60 min at room temperature. Fluorescence derived from BODIPY-vancomycin was measured at an excitation and emission wavelength of 485 and 535 nm, respectively. The means and standard deviations of triplicate determinations are presented. A.U., arbitrary units (A). After 60-min incubation with BODIPY-vancomycin, the biofilm resuspended in PBS was observed by fluorescence microscopy (B). Arrows indicate division septa of the cells. Insert shows enlargement of boxed area. Scale bar, 1 μm.
FIG 9 Live/dead images of Staphylococcal biofilms after treatment with bacteriocins and vancomycin

Biofilms formed on 35-mm glass-bottomed dishes were incubated with peptide solutions at a concentration of 4× MIC for 1 h at room temperature. By way of exception, biofilms of SE4 and SE21 were treated with 20 μM lacticin Q. After washing, the living and dead cells in the biofilm were stained by SYTO9 (green) and PI (red), respectively. X-Z sections of three-dimensional images were shown. MR10, *S. aureus* MR10; MR11, *S. aureus* MR11; SH1000, *S. aureus* SH1000; SE4, *S. epidermidis* SE4; SE21, *S. epidermidis* SE21. Scale bar, 5 μm.