

Activity of Gallidermin on *Staphylococcus aureus* and *Staphylococcus epidermidis* Biofilms

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Due to their abilities to form strong biofilms, *Staphylococcus aureus* and *Staphylococcus epidermidis* are the most frequently isolated pathogens in persistent and chronic implant-associated infections. As biofilm-embedded bacteria are more resistant to antibiotics and the immune system, they are extremely difficult to treat. Therefore, biofilm-active antibiotics are a major challenge. Here we investigated the effect of the lantibiotic gallidermin on two representative biofilm-forming staphylococcal species. Gallidermin inhibits not only the growth of staphylococci in a dose-dependent manner but also efficiently prevents biofilm formation by both species. The effect on biofilm might be due to repression of biofilm-related targets, such as *ica* (intercellular adhesin) and *atl* (major autolysin). However, gallidermin's killing activity on 24-h and 5-day-old biofilms was significantly decreased. A subpopulation of 0.1 to 1.0% of cells survived, comprising "persister" cells of an unknown genetic and physiological state. Like many other antibiotics, gallidermin showed only limited activity on cells within mature biofilms.

Staphylococcus aureus and *Staphylococcus epidermidis* are widely involved in minor to severe infections. *S. aureus* has a wide range of virulence factors and can cause acute and chronic infections at many anatomical sites. In the last 2 decades, multidrug-resistant *S. aureus* and methicillin-resistant *S. aureus* (MRSA) have emerged as major causes of hospital-, community-, and livestock-acquired infections, which are increasingly difficult to manage (9, 20). In contrast to *S. aureus*, *S. epidermidis* strains produce fewer extracellular toxins than coagulase-positive strains and therefore produce fewer acute symptoms. However, despite their lower virulence, *S. epidermidis* is well adapted to forming biofilms (comprising adherence and intercellular aggregation) on surfaces of foreign bodies, such as vascular catheters, cardiac devices, ventricular catheters, and prosthetic joints (19), mainly due to its ability to form strong biofilms (18). It is therefore not surprising that *S. epidermidis* is normally associated with chronic infections. A major problem is the rising occurrence of highly virulent and multiply resistant strains. Especially when a biofilm is formed during infection, therapy is extremely difficult due to the general antibiotic resistance of a subpopulation in a biofilm community. Pathogenic staphylococci are now regarded in the scientific community as antibiotic-resistant "superbugs," because they have an amazing capacity to acquire resistance traits. Possible targets for drug development are enzymes involved in the biosynthesis of cell envelope structures, such as peptidoglycan, teichoic acids, membrane lipids, or cell wall-associated adhesins (17).

Promising drugs could be the type A lanthionine-containing peptide antibiotics (lantibiotics), which are ribosomally synthesized antibiotic peptides that contain the nonprotein amino acids lanthionine and 3-methylanthionine (51). Prominent representatives of lantibiotics are nisin (29), epidermin (1, 51), gallidermin (25), and mersacidin (3, 36). We have been working for some time to unravel the biosynthetic pathway of epidermin and gallidermin, which differ by only one amino acid (1, 14, 30, 31, 39, 41–43, 45, 49, 50). As gallidermin showed slightly higher antimicrobial activity than epidermin, we focused on gallidermin and developed a production system. The nuclear magnetic resonance structure of

gallidermin is in excellent agreement with the amphiphilic and channel-forming properties of gallidermin on membranes and its tryptic cleavage activity at the exposed site between residues 13 and 14 (12).

The mode of action of the lantibiotics has been extensively studied by the Hans-Georg Sahl group in Bonn. Originally, it was assumed that type A lantibiotics primarily kill bacteria by permeabilization of the cytoplasmic membrane in a membrane potential-dependent way. However, more recently it has been shown that nisin and epidermin also interact with the membrane-bound peptidoglycan precursors lipid I and lipid II, suggesting that they also inhibit peptidoglycan synthesis (5). The activity of lantibiotics appears to be based on different killing mechanisms that are combined in one molecule. The prototype lantibiotic nisin inhibits peptidoglycan synthesis and forms pores through a specific interaction with the cell wall precursor lipid II. Gallidermin and epidermin possess the same putative lipid II binding motif as nisin; however, both peptides are considerably shorter (22 amino acids, compared to 34 in nisin). Indeed, it has been demonstrated that the pore formation by gallidermin depends on membrane thickness and that it is the interaction with lipids I and II rather than pore formation that contributes to bacterial killing. The superior activity of gallidermin over nisin in a cell wall biosynthesis assay may explain its high killing potency (4). Recently, it was shown that nisin and gallidermin not only bind to lipid II but also to the lipid intermediates lipid III (undecaprenol-pyrophosphate-*N*-acetylglucosamine) and lipid IV (undecaprenol-pyrophosphate-*N*-acetylglucosamine-*N*-acetyl-mannosamine) of the wall teichoic

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TABLE 1 Oligonucleotide used in this study

Gene ^a	Gene name ^a	Direction	Primer sequence (5'–3') ^b
SA0905	<i>atlA</i>	Forward	GCTAATCAATCAGCGACTAC
		Reverse	<u>CTAATACGACTCACTATAGGGAGACA</u> AAATGCATGTACGAATGCG
SERP0636	<i>atlE</i>	Forward	GGGACGTCCTGAAGGTATCG
		Reverse	<u>CTAATACGACTCACTATAGGGAGAGATG</u> TTGTGCCCAAGGTGC
SA2459	<i>icaA</i>	Forward	GCCAACGCACTCAATCAAGG
		Reverse	<u>CTAATACGACTCACTATAGGGAGACTT</u> CCAAAGCCTCCCAATG
SERP2293	<i>icaA</i>	Forward	GAGGGAATCAAACAAGCATC
		Reverse	<u>CTAATACGACTCACTATAGGGAGACC</u> ATCGAACCCCTTTGTTTCC

^a Based on KEGG classification (www.genome.jp/kegg/).

^b The underlined sequence portions at the 5' ends represent the recognition sequence for the T7 RNA polymerase.

acid (WTA) biosynthesis pathway. It was shown that the specific interaction with WTA precursors promoted pore formation in artificial lipid bilayers (35).

In light of the unique mode of action of gallidermin, which targets not only peptidoglycan but also wall teichoic acid biosynthesis, we aimed to study its activity on biofilm cells. Here we show that gallidermin is able to very efficiently inhibit the formation of staphylococcal biofilms; however, its killing activity on preformed biofilms was markedly decreased, as a small percentage of a “persister” subpopulation survived.

MATERIALS AND METHODS

Determination of the MIC and MBC of gallidermin. The MICs of gallidermin against *S. aureus* and *S. epidermidis* were determined by the microdilution method, using Mueller-Hinton broth (MHB) and following the method described by the Clinical and Laboratory Standards Institute (6). Gallidermin was diluted in a 96-well microtiter plate to final concentrations ranging from 128 to 0.06 µg/ml. A 100-µl aliquot of the bacterial suspension (10⁶ CFU/ml) was inoculated and incubated at 37°C for 18 h. The MIC was determined as the lowest concentration that completely inhibited bacterial growth. The minimal bactericidal concentration (MBC) was assessed as the extract concentration that gave significant MIC values after streaking the culture on Trypticase soy agar (TSA). Experiments were carried out in triplicate.

Growth inhibition by gallidermin added to mid-logarithmic-phase cultures. Twenty-milliliter aliquots of *S. aureus* SA113 and *S. epidermidis* O47 at an optical density at 578 nm (OD₅₇₈) of 0.1 were cultured in basic medium (BM; 1% soy peptone, 0.5% yeast extract, 0.5% NaCl, 0.1% K₂HPO₄, 0.1% glucose) at 37°C with shaking at 150 rpm until mid-logarithmic phase was reached (approximately 4 h). At that time, gallidermin was added at a final concentration of 1×, 2×, 4×, and 8× MIC. The OD of the cultures was measured at the indicated time intervals. The experiment was carried out in triplicate, and the results are presented as the mean OD ± the standard deviation.

Effect of gallidermin on biofilm formation. Overnight cultures grown in Trypticase soy broth (TSB) were diluted to 10⁶ CFU/ml. A 100-µl aliquot was transferred to a 96-well microtiter plate, and 100 µl of a subinhibitory concentration of gallidermin, dissolved in TSB, was added. After incubation at 37°C for 24 h without agitation, the bacterial growth (based on the OD₆₀₀) was determined by using a microplate reader. A biofilm assay was carried out essentially as described earlier (7). Briefly, the culture supernatant was discarded, and the wells were washed twice with phosphate-buffered saline (PBS) to remove nonadherent cells. The plates were air dried, and the surface-attached cells were stained with 200 µl of 0.1% crystal violet for 30 min. Subsequently, the crystal violet was removed and the plate was washed with water. After air drying, photos were taken from the surface-attached biofilm. For quantification of the biofilm cells, 200 µl of dimethyl sulfoxide (DMSO) was added, and crystal violet-stained biofilm cells were determined at 570 nm with the microplate reader.

Viability assays of preformed biofilms cells treated with gallidermin. Microtiter plates were filled with 200 µl of 10⁶ CFU/ml in TSB and incubated at 37°C for 24 h or 5 days without shaking. For establishment of 5-day biofilms, planktonic cells were discarded daily and replaced with fresh TSB. After incubation, the medium was removed and the wells were rinsed twice with PBS. Gallidermin (200 µl of various MICs) dissolved in TSB was added. After cultivation at 37°C for 24 h, the supernatant was discarded and replaced with 200 µl of PBS supplemented with 50 µg methylthiazolotetrazolium (MTT; Sigma). This kind of indirect viability assay is based on the formation of insoluble purple formazan due to the reduction of MTT by (respiratory) reductases of living staphylococcal cells. The formazan crystals were dissolved in DMSO, and the absorbance was determined at 570 nm with a microplate reader. Viability of staphylococcal cells was also tested by the viable count method (CFU). The biofilms were prepared as described above, with the only difference that larger flat-bottom microtiter plates were used. The formed biofilms were scratched off with a sterile spatula, sonicated, and then vortexed. The suspension was diluted and plated onto a TSA plate to detect the viable count after incubation.

Transcriptional analysis of *atl* and *ica* genes. Staphylococcal cells were cultured in TSB until mid-exponential phase (after approximately 4 h). At this time, 4× MIC gallidermin dissolved in TSB was added; the control received the same amount of TSB only (see Fig. 1, below). Samples for RNA isolation were taken at 4, 6, and 8 h. Total RNA was isolated by using the acid-phenol method (16, 34) with some modifications, as described earlier (13). Briefly, 35-ml aliquots of collected samples were mixed with 15 ml of ice-cold killing buffer (20 mM Tris-HCl [pH 7.5], 5 mM MgCl₂, 20 mM NaN₃) and immediately centrifuged at 5,000 × g at 4°C for 10 min. The cells were resuspended in 1 ml of the same buffer and centrifuged at 8,000 × g at 4°C for 10 min. Five hundred microliters of lysis buffer (3 mM EDTA, 200 mM NaCl) was added to suspend the pellets. The cell suspension was transferred to a screw-cap tube containing 500 µl phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) with glass beads (diameter, 0.1 mm; Sartorius) and immediately mechanically disrupted by using a ribolyser at 6.5 m/s for 30 s. The mixture was centrifuged at 13,000 × g at 4°C for 10 min. The supernatant was extracted again with 600 µl phenol-alcohol-isoamyl alcohol. The upper phase was extracted twice with chloroform-isoamyl alcohol (25:1, vol/vol). RNA was precipitated with a 0.5× volume of absolute ethanol containing a 0.1× volume of 3 M sodium acetate at –80°C for 3 h. After centrifugation, the pellet was washed with 70% ethanol and resuspended in deionized water. RNA integrity was confirmed by agarose gel electrophoresis, and photometric measurements were made using a NanoDrop apparatus.

RNA probe preparation. Digoxigenin-labeled RNA probes of the corresponding genes were prepared by transcription with T7 RNA polymerase by using a PCR fragment as the template (16) that was generated from chromosomal DNA of *S. aureus* SA113 and *S. epidermidis* O47 and the respective oligonucleotides (Table 1). The reverse oligonucleotides contained a T7 RNA polymerase recognition site sequence at the 5' end.

Northern blot analyses. Ten micrograms of total RNA was loaded into each well. The RNA was separated under denaturing conditions in a

1% agarose gel containing 20 mM morpholinepropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA, 1.85% formaldehyde. The gel was blotted onto a nylon membrane with 20× SSPE (3 M NaCl, 0.2 M NaH_2PO_4 , 0.02 M EDTA; pH 7.4) by using a vacuum blotter for 4 h. The RNA was cross-linked to the membrane by UV for 1 min and stained with methylene blue. 16S and 23S rRNA bands on the membrane indicated the efficiency of blotting and served as a control for the quantity of RNA in each lane. RNA probes were used to detect gene-specific hybridization. Transcriptional signals were detected by using ChemiDoc (Bio-Rad).

Effect of gallidermin on autolysin activity in a zymogram. A 20-ml aliquot of a culture at an OD_{578} of 0.1 of *S. aureus* SA113 or *S. epidermidis* O47 was cultured in TSB at 37°C with shaking at 150 rpm until mid-logarithmic phase was reached (after approximately 4 h). At that time point, gallidermin was added at a final concentration of 4× MIC and cultivated for another 4 h. Then, cells were centrifuged, washed in Tris-HCl (pH 6.8), and centrifuged at 13,000 rpm for 5 min. The cell pellet was treated with sample buffer (SDS with β -mercaptoethanol), incubated at 65°C for 3 min to remove cell wall-attached proteins, and put on ice for 5 min. After centrifugation, the supernatant was electrophoresed in a 12% SDS-PAGE gel containing heat-killed *Micrococcus luteus* cells as a substrate. After electrophoresis, the PAGE gel was washed with deionized water and then incubated in regeneration buffer overnight at 37°C. The gel was stained with 0.1% methylene blue and destained with deionized water. Proteins with autolysin activity were observed as clear bands in the gel.

Statistical analysis. Statistical analysis was performed using analysis of variance (ANOVA). Comparisons between means were carried out according to Duncan's test. Differences were considered significant at a *P* level of <0.05.

RESULTS

Dose-dependent inhibition of staphylococcal growth by gallidermin. The activities of gallidermin on planktonic cells of *S. aureus* and *S. epidermidis* were investigated by determination of MIC and MBC values in MHB medium. Gallidermin inhibited the growth of both *S. aureus* and *S. epidermidis*, and the MICs were in the range between 4 and 8 $\mu\text{g}/\text{ml}$, irrespective of the resistance of strains to methicillin, such as USA300. The biofilm-positive test strains used here were *S. aureus* SA113 and *S. epidermidis* O47; they showed MIC values of 8 and 4 $\mu\text{g}/\text{ml}$, respectively, if gallidermin was present from the beginning of growth (data not shown). However, when gallidermin was added to a mid-exponential-phase culture, higher doses (on average, 2× MIC) were necessary to completely inhibit growth (Fig. 1). In contrast to the O47 strain, SA113 was not only less sensitive to gallidermin but also showed a tendency to resume growth at 1× and 2× MIC after 10 h; only at 4× and 8× MIC was growth arrested over the entire period.

Gallidermin inhibits biofilm formation. For determining the effect of gallidermin on biofilm formation, we used the quantitative microtiter plate method (7). The bacteria were cultured in microtiter plates for 24 h in the presence of subinhibitory concentrations of gallidermin (0.16× to 1× MIC), and the formed biofilm was stained with crystal violet. At 1× MIC of gallidermin, growth of *S. aureus* SA113 and *S. epidermidis* O47 was completely arrested; the O47 strain was arrested even at 0.5× MIC (Fig. 2A). However, biofilm formation, particularly of *S. aureus* SA113, was already inhibited at sublethal concentrations of gallidermin (0.16× MIC [0.5 $\mu\text{g}/\text{ml}$]; *P* < 0.05) (Fig. 2B and C). This result indicated that gallidermin already exerts an inhibiting effect on biofilm formation long before it inhibits growth and viability, probably by repression of biofilm-related genes (see below). In *S. epidermidis* O47, the effect was less pronounced than in *S. aureus*

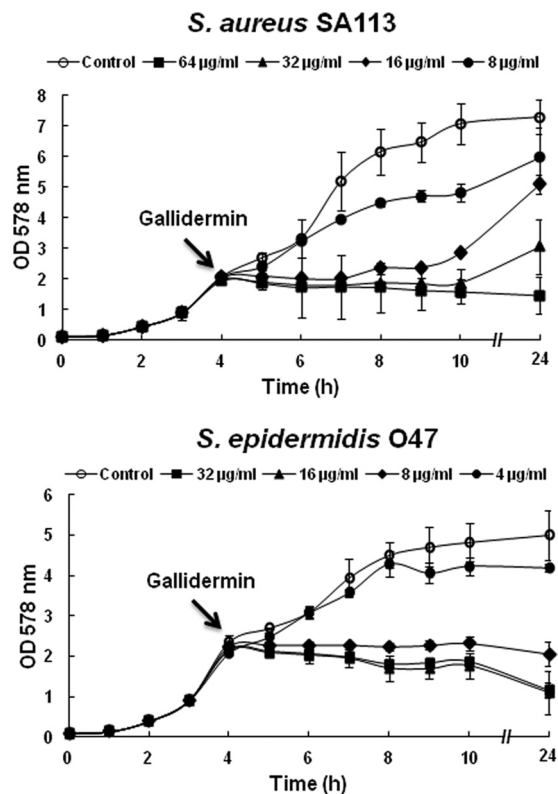


FIG 1 Growth curves of *S. aureus* SA113 and *S. epidermidis* O47 after treatment with gallidermin at 1×, 2×, 4×, and 8× MIC of gallidermin. Gallidermin was added at the mid-exponential growth phase, after 4 h of cultivation. The MIC values of gallidermin for *S. aureus* SA113 and *S. epidermidis* O47 were 8 and 4 $\mu\text{g}/\text{ml}$, respectively.

SA113; 0.5× MIC (2 $\mu\text{g}/\text{ml}$) affected both growth and biofilm formation.

Effect of gallidermin on 1- and 5-day-old preformed biofilms. Once a biofilm has been established, the cells are extremely robust against all kinds of antibiotics. We therefore studied the effect of gallidermin on cell viability in 24-h and 5-day-old biofilms. For viability testing, an indirect method (the MTT assay) and direct method (viable counting) were used. The MTT assay is based on the activities of MTT-reducing enzymes (frequently NADH-dependent dehydrogenases) as an indication of live cells. Increasing concentrations (1× to 16× MIC) of gallidermin led to a decrease in formazan production, which was more rapid in the 24-h biofilm than in the 5-day biofilms (Fig. 3A), indicating that in an old biofilm, cells are more robust and protected.

The MTT assay results correlated quite well with the viable count method results. Here too, gallidermin caused a dose-dependent (1× to 16× MIC) decrease of 3 logs in the 24-h biofilm and of 1 to 2 logs in the 5-day biofilms (Fig. 3B). *S. epidermidis* O47 survived slightly better than *S. aureus* SA113. While the bactericidal effect of gallidermin in planktonic cultures was very high (7- to 8-log reduction in CFU), it was low in biofilms, with only 1- to 3-log reductions of viable cells, depending on the age of the biofilm.

Gallidermin inhibits transcription of *atl* and *ica* genes. As shown in Fig. 2B and C, biofilm formation was inhibited already at sublethal concentrations of gallidermin, suggesting that the ex-

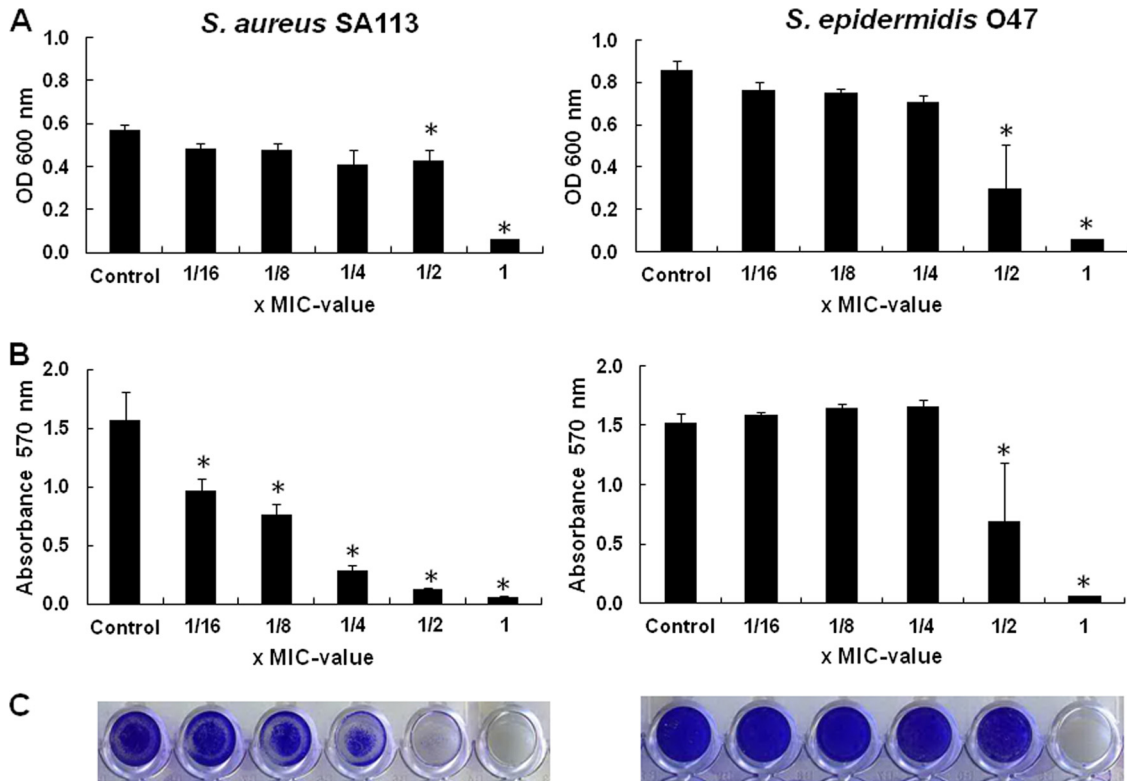


FIG 2 Biofilm formation of *S. aureus* SA113 and *S. epidermidis* O47 in the presence of subinhibitory concentrations of gallidermin. Growth and biofilm formation occurred in microtiter plates. Shown are the turbidity (the OD₆₀₀) of the cell culture (A), the absorbance (A₅₇₀) of stained cells attached to the surface after washing (B), and photos of biofilm cells attached to microtiter wells after crystal violet staining (C). Differences were considered significant at *P* < 0.05.

pression of some biofilm-related genes was affected. We therefore carried out transcription analysis (Northern blotting) for *atl* and *ica* genes. *atl* encodes the major autolysin, which is involved in attachment to a surface, one of the first steps in biofilm formation (21); *ica* (intercellular adhesin) encodes polysaccharide intercel-

lular adhesin (PIA), an exopolysaccharide composed of β-1,6-linked glucosaminoglycan (15, 22, 33). Staphylococci were grown to mid-exponential phase (4 h), gallidermin (4× MIC) was added, and RNA was isolated at 4, 6, and 8 h for Northern blot analysis. Indeed, in the presence of gallidermin, *atl* and *ica* transcripts were

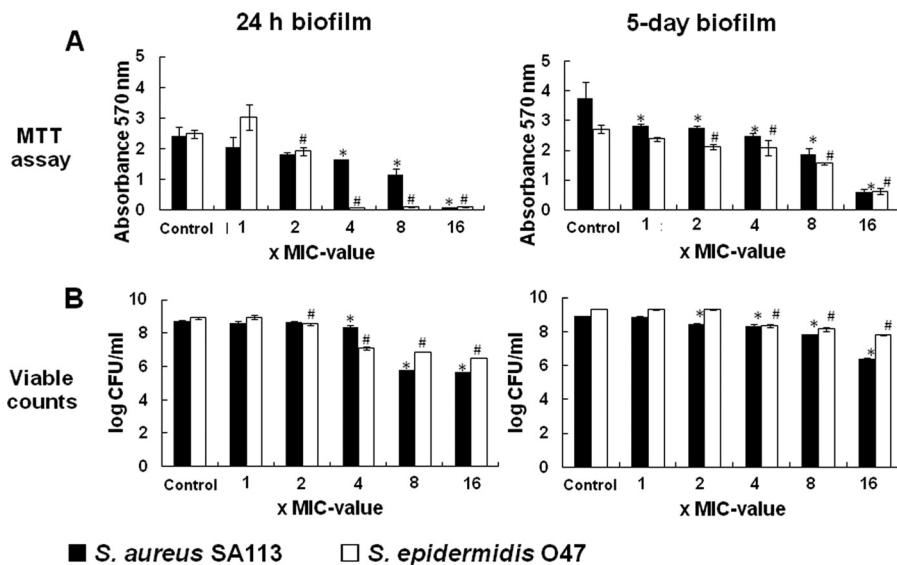


FIG 3 Gallidermin and viability of bacterial cells in 24-h and 5-day established biofilms of *S. aureus* SA113 and *S. epidermidis* O47. The viability of bacterial cells in 24-h and 5-day biofilms was detected by MTT assay (A) or viable count (B). Differences were considered significant at *P* < 0.05.

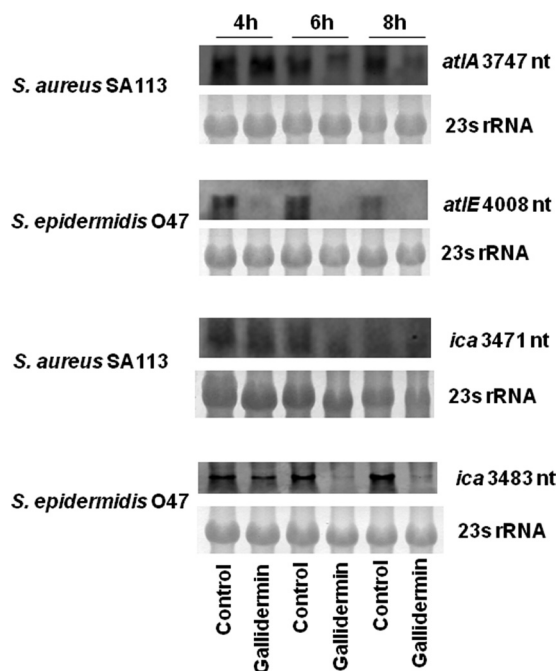


FIG 4 Effects of gallidermin on *atl* and *ica* transcription. RNA from *S. aureus* SA113 and *S. epidermidis* O47 was taken from 4-, 6-, and 8-h cultures (with or without 4× MIC gallidermin treatment) and analyzed by Northern blotting. 23S rRNA was used as a control.

markedly reduced in both *S. aureus* and *S. epidermidis* strains (Fig. 4). The inhibiting effect of gallidermin on *atl* and *ica* transcription was more pronounced in *S. epidermidis* than in *S. aureus*.

Major autolysin activity is decreased after treatment with gallidermin. To verify whether the inhibition of *atl* transcription correlated with the major autolysin activity, we analyzed the major autolysin activity pattern in a zymogram with heat-inactivated *Micrococcus luteus* cells as the substrate. Indeed, the intensities of the autolysin bands were markedly reduced in the presence of gallidermin for both strains (Fig. 5). For *S. aureus*, the characteristic lytic bands (48) with molecular masses of 138 kDa (proautolysin), 113 kDa (autolysin), and 62 kDa (amidase) almost disappeared; only the 52-kDa lytic band (most likely the glucosaminidase) was only slightly decreased compared to the untreated culture.

DISCUSSION

It has been known for some time that the lanthionine-containing peptide antibiotics epidermin and gallidermin have high bactericidal activities against many Gram-positive bacteria, including *Bacteroides fragilis*, *Micrococcus luteus*, *Propionibacterium acnes*, *S. aureus*, *S. epidermidis*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes* (25). Even the multidrug-resistant *S. aureus* (including MRSA) and oxacillin-resistant *S. aureus* (ORSA) strains, which are resistant to β-lactam antibiotics, including penicillins (methicillin, dicloxacillin, nafcillin, and oxacillin) and cephalosporins, were as susceptible as the nonresistant strains. The susceptibilities of the various staphylococcal species were quite diverse. While *S. aureus* and *S. epidermidis* showed MIC values in the range of 4 to 8 μg/ml (2 to 4 μM), some other staphylococcal species, such as nonpathogenic *S. carnosus* or *S. simulans*, are much more suscep-

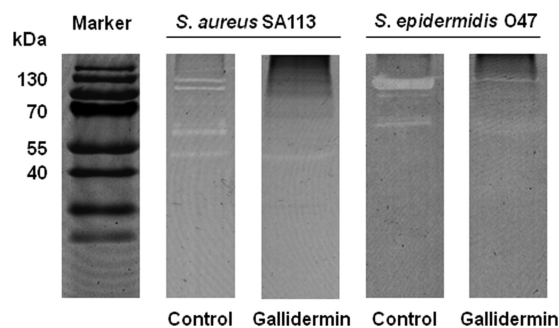


FIG 5 Autolysin zymogram profiles. *S. aureus* SA113 and *S. epidermidis* O47 were treated with or without 4× MIC of gallidermin, and the cell wall-associated proteins were separated by SDS-PAGE with heat-inactivated *Micrococcus luteus* cells as the substrate. Proteins with autolysin activity were visualized as clear (white) bands.

tible to gallidermin, with MIC values of approximately 0.3 μg/ml (0.15 μM) (4). This indicates that *S. aureus* and *S. epidermidis* have acquired some resistance traits that are lacking in the nonpathogenic species. There are several ways to improve tolerance against cationic antimicrobial peptides (CAMPs), including not only defensins but also many lantibiotics. It has been shown that modifications of the cell envelope that contribute to an increased positive charge render bacteria less susceptible to CAMPs. For example, mutants of *S. aureus* and other Gram-positive species that lack D-alanine (*dlt* mutants) in their teichoic acids (44), or *tagO* mutants that lack wall teichoic acid (2, 27, 55), or the *mprF* mutants that are unable to lysinylate lipids (10), are significantly more susceptible than the wild-type strains. As *S. aureus* and the skin-colonizing *S. epidermidis* are exposed to defensins, it is likely that they have acquired functions that increase tolerance to defensins. However, although *S. aureus* and *S. epidermidis* were less susceptible to gallidermin than the nonpathogenic species representatives, they were still sufficiently susceptible to consider gallidermin an antimicrobial therapeutic agent.

A major issue of this study was gallidermin activity against biofilms and biofilm formation. As test bacteria, we chose the well-studied biofilm-forming *S. aureus* SA113 (8) and *S. epidermidis* O47 (21). We found that gallidermin was able to completely inhibit biofilm formation at 1× MIC. For *S. aureus*, a biofilm-inhibiting effect was already observed in the sub-MIC range (Fig. 2), suggesting that biofilm-relevant functions were already affected at lower concentrations. Indeed, the transcription levels of two genes, one involved in primary adhesion (the major autolysin, *atl*) and one involved in exopolysaccharide production (the intercellular adhesin, *ica*), were significantly decreased in the presence of gallidermin (Fig. 4). The inhibition of transcription by gallidermin was also reflected by a decrease in autolytic protein bands in the zymogram (Fig. 5).

While gallidermin is a good drug for inhibiting the onset of biofilm formation, the killing activity on 24-h and 5-day-old preformed biofilms was comparatively low. Significant effects were only seen at ≥4× MIC. The number of CFU of biofilm-associated staphylococci treated with 8× MIC of gallidermin was decreased by 3 logs in the 24-h biofilms and 1 to 2 logs in the 5-day biofilms. This means that 0.1 to 1% of biofilm-associated staphylococcal cells survive gallidermin treatment. In this respect, gallidermin behaves like many other antibiotics that show good activity with

planktonic cells but low activity with already-established biofilms. There are many reports on the activities of natural compounds on staphylococcal biofilm formation. Hydroxypropyltrimethyl ammonium chloride chitosan inhibits biofilm formation as well as expression of the *icaA* gene (40). Providone iodine also inhibits biofilm formation and decreases transcription of the *ica* operon (38). The effects of natural antibacterial agents, such as berberine, carvacrol, farnesol, oregano, rhodomyrtone, and thymol, against staphylococcal biofilms have been frequently reported (24, 37, 47, 54). Oregano, carvacrol, and thymol exhibit antistaphylococcal activity in biofilms. They apparently disseminate through the biofilm matrix and lead to cell damage (37). In addition, 1% tea tree oil is able to inhibit the metabolism of biofilms formed by all test isolates of *S. aureus* (32). Those authors suggested that biofilm destruction by tea tree oil was due not only to bacteria killing but also to destruction of the extracellular matrix and clearing of the biofilms from the surface (32). However, little is known about the side effects of these natural substances.

Maintenance of a high dose of antibiofilm drugs appears crucial, at least for some antibiotics. It has been reported that subinhibitory concentrations of various antibiotics even enhance staphylococcal biofilm formation, such as erythromycin (53), furanone (28), nafcillin (11), quinupristin-dalfopristin and tetracycline (46), and vancomycin (23). Gallidermin does not fall into this class of antibiotics, as biofilm formation is inhibited by concentrations that are subinhibitory for growth.

One of the future tasks will be the characterization of the genotype and phenotype of the gallidermin survivors. Such survivors, also called persisters, are found with almost every type of antibiotic tested so far, and this is therefore a general problem (26). Little is known about how these persisters arise and about their physiological state. Do these persisters comprise dormant cells or small-colony variants (52)? Are they genetically altered, or so they pause in a certain physiological state? More investigation into these persister cells is necessary in order to be able to develop appropriate antibiotic treatments against them.

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