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Furanone at Subinhibitory Concentrations Enhances Staphylococcal Biofilm Formation by *luxS* Repression▽

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Brominated furanones from marine algae inhibit multicellular behaviors of gram-negative bacteria such as biofilm formation and quorum sensing (QS) without affecting their growth. The interaction of furanone with QS in gram-positive bacteria is unknown. Staphylococci have two QS systems, *agr* and *luxS*, which lower biofilm formation by two different pathways, RNAIII upregulation and bacterial detachment, and polysaccharide intercellular adhesin (PIA) reduction, respectively. We synthesized natural furanone compound 2 [(5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone] from *Delisea pulchra* and three analogues to investigate their effect on biofilm formation in gram-positive bacteria. Compound 2, but not the analogues, enhanced the biofilms of *Staphylococcus epidermidis* 1457 and 047 and of *S. aureus* Newman at concentrations between 1.25 and 20 μM. We show the growth inhibition of *S. epidermidis* and *S. aureus* by free furanone and demonstrate bactericidal activity. An induction of biofilm occurred at concentrations of 10 to 20% of the MIC and correlated with an increase in PIA. The biofilm effect was *agr* independent. It was due to interference with *luxS*, as shown by reduced *luxS* expression in the presence of compound 2 and independence of the strong biofilm formation in a *luxS* mutant upon furanone addition. Poly(L-lysine)-grafted/poly(ethylene glycol)-grafted furanone was ineffective on biofilm and not bactericidal, indicating the necessity for free furanone. Free furanone was similarly toxic for murine fibroblasts as for staphylococci, excluding a therapeutic application of this compound. In summary, we observed a biofilm enhancement by furanone in staphylococci at subinhibitory concentrations, which was manifested by an increase in PIA and dependent on *luxS*.

Implant-associated infections are commonly caused by microorganisms growing in biofilms (10, 31). *Staphylococcus aureus* and *S. epidermidis* are the major causative agents of implant infections. Upon implantation, the surface of the implant is rapidly covered with host proteins and cells. If bacteria are present, they compete with host cells in a "race for the surface" (15, 41). Many approaches have been made to prevent bacterial colonization of surfaces, e.g., by coating with antifouling substances such as poly(L-lysine)-grafted-poly(ethylene glycol) copolymers (PLL-*g*-PEG) (17, 33, 42). Bacterial adherence to implant surfaces occurs in two phases (7, 32). First, staphylococci adhere via exopolysaccharides and microbial surface components recognizing adhesive matrix molecules in the wound. Then, staphylococci proliferate and accumulate in multilayers of exopolysaccharides, what is commonly described as the biofilm. The major component of staphylococcal biofilms is polysaccharide intercellular adhesin (PIA), which is encoded by the *ica* genes (13, 16). PIA production causes resistance against antibiotics (13) and makes bacteria less vulnerable by shielding them from immune defense (28). Global regulators, including the alternative sigma factor B (σ^B) (26) and the quorum-sensing (QS) system *agr*, modulate biofilm turnover by regulating a whole set of genes. It was found that *agr* upregulates RNAIII, which is associated with a reduction of biofilm (44). Clinical isolates of *S. epidermidis* often have mutations of *agr*, which is linked to more biofilm on the foreign body and less dissemination (27). Another QS system encoded by *luxS* is required for autoinducer 2 synthesis, which negatively affects biofilm by reducing *ica* gene transcription in *S. epidermidis* (46). Thus, substances acting on biofilm, e.g., via QS are drug candidates for prevention and treatment of gram-positive implant infections.

Halogenated furanones are natural compounds secreted by the alga *Delisea pulchra*. They are structurally similar to bacterial acyl homoserine lactones (AHL). AHL are released upon changes in cell population density, cross membranes and bind to transcription factors of the LuxR family, thereby inducing QS controlled genes. Gram-negative bacteria use AHL-dependent relationships for interference with eukaryotes and plants. Thus, marine algae have developed halogenated furanones as AHL antagonists, most likely in response to a negative impact of bacterial colonization (14). *Delisea pulchra* produces more than 30 furanones with a variable potential to prevent swarming of gram-negative bacteria with an AHL system, without altering their general metabolism or growth (14, 34). Furanones suppress virulence factor production and pathogenesis in *Pseudomonas* (20) and biofilm formation in *Escherichia coli* (40). In *Bacillus*

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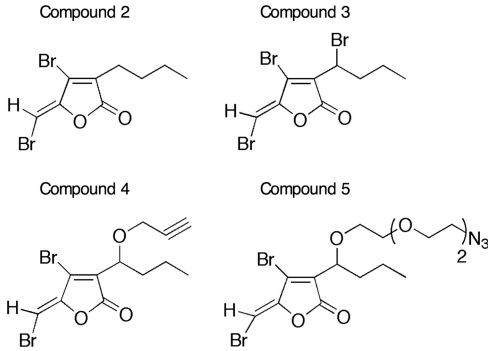

FIG. 1. Chemical structures of the furanone compounds used in the present study.

subtilis this effect was associated with a reduction in viability (39).

Several natural furanones were found to inhibit the growth rate of selected clinical ocular strains of *S. aureus* and *S. epidermidis* (11, 24, 25) by an unknown mechanism. Hume et al. described biofilm inhibition in *S. epidermidis* by covalently bound furanone in vitro and a reduced bacterial load on furanone-coated catheters in sheep in vivo (23).

A better understanding of the effect of free and surface-bound furanone on gram-positive bacteria is crucial for a potential clinical application of this class of substances in implant infections. Therefore, we assessed the bactericidal and biofilm-modulating activity of free and surface-bound furanone and compared it to its eukaryotic cytotoxicity. We were able to show that furanone is similarly toxic for staphylococci and eukaryotic cells, rendering a clinical application improbable. In addition, we found a biofilm-enhancing effect of furanone on several staphylococci associated with enhanced PIA production. This effect in staphylococci was related to a downregulation of the QS system *luxS*, whereas σ^B, *agr*, and RNAIII were not involved.

MATERIALS AND METHODS

Chemical substances. Tryptic soy broth (TSB) and Mueller-Hinton broth and agar were obtained from Becton Dickinson (Allschwil, Switzerland). Luria-Bertani broth, crystal violet, and poly-L-lysine (PLL) were purchased from Sigma-Aldrich (Buchs, Switzerland). A CytoTox96 kit, proteinase K, RNasin, and random primers were obtained from Promega (Dübendorf, Switzerland), and lysostaphin was purchased from Genmedics (Reutlingen, Germany). *Taq* DNA polymerase was purchased from Invitrogen (Lucerne, Switzerland). The RNeasy minikit, RNAprotect bacterial reagent, Omniscript reverse transcriptase kit, and DNase were obtained from Qiagen (Hombrechtikon, Switzerland). The rabbit immunoglobulin G (IgG) isotype antibody was purchased from Vector (Geneva, Switzerland). Horseradish peroxidase-conjugated donkey anti-rabbit IgG was from Jackson (Magden, Switzerland), and the ECL Western blotting analysis system was obtained from Amersham Biosciences/GE Healthcare (Otelfingen, Switzerland). HEPES buffer and all reagents for cell culture were obtained from Gibco/Invitrogen.

Preparation of free and surface-linked furanones. Several furanone compounds were used in the present study: 4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone (molecular mass, 310.0 g/mol), known as compound 2; 4-bromo-5-(bromomethylene)-3-(1-bromobutyl)-2(5*H*)-furanone (molecular mass, 388.9 g/mol [compound 3]); 4-bromo-5-(bromomethylene)-3-(1-(−2-propynyloxy)butyl)-2(5*H*)-furanone (molecular mass, 364.1 g/mol [compound 4]); and 4-bromo-5-(bromomethylene)-3-(1-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)butyl)-2(5*H*)-furanone (molecular mass, 483.2 g/mol [compound 5]) (Fig. 1). The synthesis of furanones, as well as the addition of functional groups on the side chain was achieved according to published methods (2, 29, 35, 38). Free furanones were dissolved in ultrapure ethanol and applied to the wells of 96-well plates. Excess ethanol was evaporated under sterile conditions, and growth medium was added to resolubilize the furanone for experiments with bacteria or fibroblasts.

The surface bound analogue of furanone compound 2 is compound 5 (which was also used in a free, unlinked form), which was grafted by click-chemistry to the polyethylene glycol (PEG) and assembled on a PLL backbone. Therefore, furanone was sequentially reacted with *N*-bromosuccinimide and 2-(2-(2-azidoethoxy)ethoxy)ethanol to a terminal azido functionalized furanone. The azido group was then reacted with a terminal acetylene function on a 3.4-kDa PEG polymer (Laysan Bio, Inc.), which was finally grafted to the 20-kDa PLL backbone. A grafting density of 80% PEG-furanone and 20% PEG was used here. For immobilization purposes, 100 μl of a compound composed of 0.1 mg of PLL-*g*-PEG-furanone/ml in HEPES buffer (10 mM in sterile NaCl 0.9%) was applied to the wells of a 96-well plate, followed by incubation for at least 30 min at room temperature under sterile conditions. Subsequently, the surfaces were rinsed with HEPES buffer followed by ultrapure water, blow drying under a stream of nitrogen, and storage in sterile containers until use. The surfaces were rehydrated for at least 30 min in HEPES buffer before use.

Bacterial strains and growth conditions. In our studies, we used *S. epidermidis* 1457 and its isogenic *ica*::*ermB* (32) and *luxS*::*ermB* (46) mutants, *S. epidermidis* 047 (18) and its isogenic *ica*::*ermB* mutant (kindly provided by F. Götz), *S. aureus* SA113 (ATCC 36665), *S. aureus* Newman (kindly provided by F. Götz) and its *agr*::*tet* mutant (ALC355 Δ*agr* [45]), *B. subtilis* (kindly provided by R. Frei), and *Pseudomonas aeruginosa* PA01 (ATCC 9027).

Bacterial strains were freshly grown in TSB for 7 h at 37°C without shaking. A 1:100 dilution was used to inoculate the overnight culture, which was further diluted into a fresh culture. After reaching the log phase, the culture was diluted to 10^5 CFU/ml in fresh medium before being used in experiments with free furanone. In experiments with covalently linked furanone, bacteria were diluted to 10^6 to 10^2 CFU/ml. Then, 100-μl portions of bacterial inocula were seeded into 96-well plates, followed by incubation for 18 h at 37°C without shaking. Bacterial numbers were estimated by optical density at 600 nm and assessed by plating serial dilutions on Mueller-Hinton agar.

Biofilm assays with crystal violet staining. Biofilm assays were performed in 96-well plates using TSB containing 0.25% glucose, with modifications as previously published (4). After incubation for 18 h at 37°C without shaking, supernatants were removed, and the plates were gently washed three times with 0.9% NaCl. For fixation of biofilm, plates were incubated for 60 min at 60°C. Each well was incubated with 100 μl of a 0.5% crystal violet solution for 20 min and washed under running tap water, and 100 μl of 33% acetic acid was added. Samples were transferred into enzyme-linked immunosorbent assay plates for reading of absorbance at 590 nm in a Molecular Devices reader (Applied Biosystems, Rotkreuz, Switzerland).

MIC determination. MIC were determined according to Clinical and Laboratory Standards Institute standards by the macrodilution method as described previously in document M7-A7 (5).

Intercellular polysaccharide adhesin slot blotting. A modification of the method was used as published by Cramton et al. (6). Briefly, *S. epidermidis* 1457 (10^5 CFU/ml) was grown in TSB with 2.5 to 20 μM compound 2. After 18 h of incubation, the supernatants were removed, adherent CFU were counted by plating, and bacteria were collected by centrifugation for 10 min at 4,000 × *g*. The bacterial pellets containing 3×10^8 to 4×10^8 CFU were resuspended in 0.5 M EDTA (pH 8.0), incubated for 5 min at 99°C, and then further incubated with 6 U of proteinase K overnight at 37°C.

Lysates corresponding to 5×10^5 CFU were transferred to a nitrocellulose membrane (Macherey-Nagel, Oensingen, Switzerland) using a Bio-Dot SF microfiltration apparatus (Bio-Rad, Reinach, Switzerland). Membranes were washed twice with Tris-buffered saline (TBS) and blocked with 10% nonfat milk for 4 h at room temperature. After being washed with TBS with 0.05% Tween (TTBS), the membranes were incubated with rabbit anti-PIA antibody (1 μg/ml; kindly provided by D. Mack, Swansea, Great Britain) or isotype-matched control antibody in 1% milk-TTBS overnight at 4°C. After another wash and incubation with the secondary horseradish-peroxidase donkey anti-rabbit IgG (0.1 μg/ml), membranes were developed with the ECL Western blotting analysis system and visualized on films (Kodak and Sigma-Aldrich).

Measurement of gene expression in biofilm. *S. epidermidis* 1457 was grown in 96-well plates with subinhibitory concentrations of free furanone compound 2 (0, 2.5, 10, and 20 μM, 48 wells per concentration). After incubation for 18 h, bacteria were resuspended, and 2 volumes of RNAprotect bacterial reagent were added. Tubes were mixed by inverting them several times and incubated 5 min at room temperature before centrifugation for 15 min at 3,800 × *g*. RNA isolation and purification was performed according to the instructions of the RNeasy minikit. Bacterial cells were disrupted with 50 U of lysostaphin, further digested

with 5 U of proteinase K and mechanically disrupted for 30 s at a speed of 6.5 m/s using a lysing matrix B tube in the FastPrep 120A instrument (Bio 101 Systems/Lucernachem, Lucerne, Switzerland). RNA was treated with DNase and quantified with a NanoDrop apparatus (ND-1000; Witec-AG, Littau, Switzerland).

For reverse transcription, 1 μg of RNA was mixed with 18 μl of Omniscript transcriptase reagent mix containing RNasin and random primers according to the instructions of the Omniscript reverse transcriptase kit. Reverse transcription was performed for 1 h at 37°C, and synthesized cDNA was chilled on ice. cDNA was kept at −20°C until use.

The following primers were used: RNAIII forward, 5′-TGAAGTTATGATG GCAGCAGAT-3′; RNAIII reverse, 5′-GTTGGGATGGCTCAACAACT-3′; gyrase B forward, 5′-TTATGGTGCTGGACAGATACA-3′; gyrase B reverse, 5′-CACCGTGAAGACCGCCAGATA-3′; σ^B forward, 5′-TTGGTATGGTTG GTCTAATAGGTGC-3′; σ^B reverse, 5′-CTGAAACTTCTAAGCGTTGTGC G-3′; *luxS* forward, 5′-TCCTATGGGTTGTCAAACTGG-3′, *luxS* reverse, 5′-C CTTCTCCGTAGATGTCATTCC-3′; 16S RNA forward, 5′-ACTTTCTGGTC TGTAACTGACGCTG-3′; and 16S RNA reverse, 5′-ACCCAACATCTCACG ACACGAG-3′. PCR was performed using *Taq* DNA polymerase with 25 amplification cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. RNA bands were visualized on a 1.2% agarose-Tris borate-EDTA gel. Quantification of signals was performed with QuantityOne (Bio-Rad) software and expressed as arbitrary units calculated from the intensity/mm^2.

Cytotoxicity of furanone in L929 cells. L929 fibroblasts were cultured in RPMI with 5% heat-inactivated fetal bovine serum, and 5×10^4 cells/ml were seeded in a 96-well plate (Falcon) containing different concentrations of free furanone. Lactate dehydrogenase (LDH) release was measured as published previously (8) in the supernatants of treated cells after 24 h and analyzed with a CytoTox96 kit according to the manufacturer's instructions (Promega).

Statistical analysis. Data were analyzed by using Prism 5.0a (GraphPad Software, Inc.), and a Mann-Whitney test was used for statistical analyses. A P value of <0.05 was considered statistically significant.

RESULTS

Effect of free furanones on gram-positive biofilms. In our study, we used one of the well-studied natural furanones, called compound 2 (12), as well as three derivatives of this substance, which were synthesized in our laboratory (Fig. 1). We first studied the effect of free compound 2 on biofilm production of several gram-positive bacteria (Fig. 2). Interestingly, free compound 2 significantly enhanced biofilm formation of the two *S. epidermidis* strains 1457 and 047, which are strong biofilm producers (19, 32), at concentrations between one-tenth and one-fifth of their MICs (Table 1). Isogenic *S. epidermidis* strains 1457 and 047, lacking the *ica* genes (Δ*ica*), were used as controls. Both strains did not form biofilm with or without furanone treatment (Fig. 2). Compound 2 also strongly enhanced biofilm of *S. aureus* Newman at subinhibitory concentrations but left the weak biofilm former *S. aureus* SA113 unaffected. Furthermore, the strong biofilm of *B. subtilis* was not influenced by furanone compound 2 (Fig. 2).

The increase of biofilm at subinhibitory furanone concentrations did not affect the viability of bacteria as shown by CFU counting (Fig. 2).

Furanone compounds 3 to 5, which represent structural analogues of compound 2, did not affect biofilm of *S. epidermidis* 1457 (Fig. 3). These results indicate that minor modification of compound 2, i.e., by an additional bromide functionality (compound 3) or by varying the side chains (compounds 4 and 5), profoundly altered the interaction with bacteria.

MIC of free furanone compound 2 for gram-positive bacteria. Furanone compound 2 was bactericidal for staphylococci at concentrations greater than 10 μM (Fig. 2). Therefore, we determined the MICs according to Clinical and Laboratory Standards Institute standard methods. *S. aureus* strains had MICs of 15 μM and were highly susceptible to soluble furanone compound 2. MICs for *S. epidermidis* strains and *B. subtilis* were higher, ranging from 30 to 65 μM and 100 μM, respectively. The minimum bactericidal concentrations (MBCs) were higher than 130 μM for all tested species except for *B. subtilis* (Table 1). Compounds 3 to 5 were only bactericidal for *S. epidermidis* 1457 at concentrations greater than 50 μM, compound 5 was the least potent (Fig. 3).

Mechanism of action of furanone on gram-positive biofilm. Staphylococcal biofilm consists mainly of PIA (13). Therefore, we quantified the effect of furanone compound 2 on biofilm by measuring PIA. In slots containing lysate from equal CFU numbers of *S. epidermidis* 1457 wild type, the amount of PIA was increased after incubation of bacteria with subinhibitory concentrations of compound 2 (Fig. 4a). Synthesis of PIA requires the enzymes encoded by the intercellular adhesion (*ica*) operon, which was found to be regulated by σ^B (26). We observed, however, unaltered σ^B expression, along with the increase in PIA (Fig. 4b).

Staphylococcal biofilm is negatively regulated by the QS system *agr* (44) with RNAIII as an important effector gene. To study whether furanone compound 2 interferes with the *agr* system, we evaluated RNAIII expression in response to compound 2. RNAIII expression of *S. epidermidis* 1457 remained unaltered at furanone concentrations ranging from 2.5 to 20 μM (Fig. 4c). In addition, we quantified biofilm formation and growth of an *agr* deletion mutant (Δ*agr*) of *S. aureus* Newman in the presence of furanone compound 2. Free compound 2 similarly enhanced biofilm formation in *S. aureus* Newman wild-type and Δ*agr* strains (Fig. 4d). The bactericidal effect of compound 2 was similar upon wild-type and mutant *S. aureus* strains (data not shown).

Alternatively, *luxS* is known to repress *ica* gene expression and thereby PIA biosynthesis (46). Therefore, we quantified *luxS* mRNA under biofilm-enhancing concentrations of furanone compound 2. *LuxS* expression was clearly reduced at 20 μM (Fig. 4e, arbitrary units of *luxS*/16S RNA: untreated, 0.783; 2.5 μM treatment, 0.762; 10 μM treatment, 0.749; and 20 μM treatment, 0.653). We next evaluated the growth and biofilm of a *luxS* deletion mutant (Δ*luxS*) of *S. epidermidis* 1457. Biofilm formation in the Δ*luxS* mutant was strong, confirming the described causal repressive relationship between the *luxS* and PIA production. Of note, the pronounced biofilm in the Δ*luxS* mutant was not influenced by furanone addition, lending further support to the idea that furanones impact biofilm formation via *luxS* (Fig. 4f). The bactericidal effect of furanone compound 2 was similar on the wild type and the Δ*luxS* mutant (data not shown).

Effect of surface-bound PLL-*g*-PEG-furanone on *S. epidermidis* biofilm. To use furanone as antibacterial coating for implants, furanone may either be incorporated in a coating that releases the antibacterial compound over time or, alternatively, it has to be linked covalently to the surface. We have chosen the second option and linked furanone compound 5 covalently to the biopassive (nonfouling) surface PLL-*g*-PEG polymer with the aim of achieving a bactericidal activity due to a potential cell wall action of furanone. Surfaces were prepared by self-assembly of the PLL-*g*-PEG-furanone and subsequently exposed to *S. epidermidis* 1457. On PLL-*g*-PEG-coated surfaces, *S. epidermidis* was unable to adhere and form biofilms

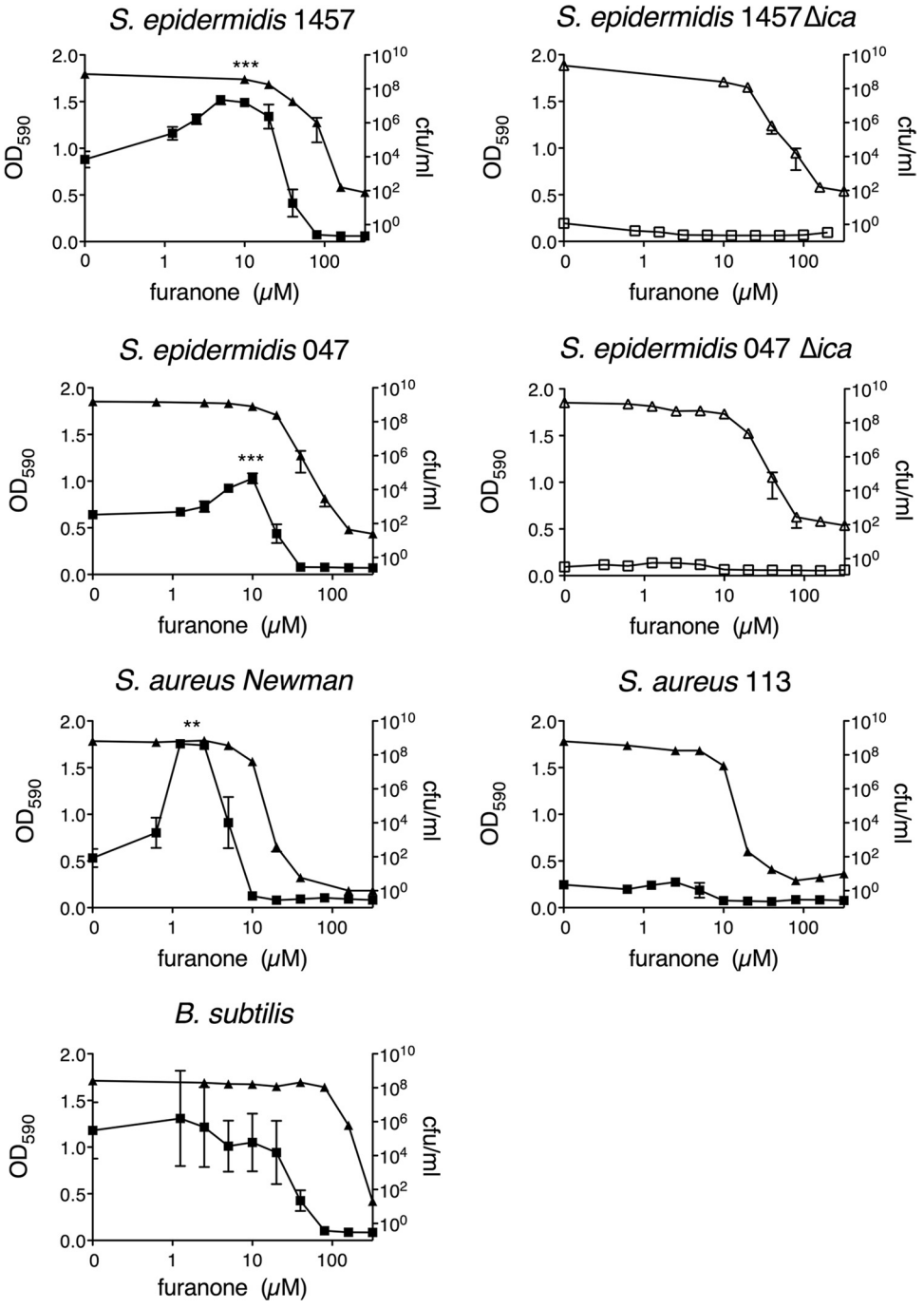

FIG. 2. Crystal violet staining of biofilms (■) and CFU counts (▲) for gram-positive bacteria grown for 18 h in 96-well plates with different concentrations of free furanone compound 2. Significant differences in the optical density with 0 μM furanone compound 2 are indicated by asterisks: **, $P < 0.01$; ***, $P < 0.001$.

(Fig. 5). The grafted furanone abolished the biopassive characteristics of PLL-*g*-PEG and allowed adhesion, as well as biofilm formation of *S. epidermidis* to a similar level, as found on control surfaces with the nonbiopassive PLL (Fig. 5).

Effect of free furanone compound 2 on *Pseudomonas* biofilm and the MIC. Furanones have previously been described to inhibit quorum sensing in gram-negative bacteria and reduce biofilm through interference with the AHL system by accelerating degradation of LuxR and by decreasing the DNA-binding activity of LuxR (9, 21). In view of the unexpected effects of free compound 2 in gram-positive bacteria, we tested biofilm and MIC of *P. aeruginosa* PA01 after incubation with furanone compound 2. In contrast to staphylococcal biofilms, *P. aeruginosa* biofilm was unaffected and decreased only at high furanone concentrations from 322 μM to 2.58 mM without any bactericidal activity. The observed tolerance of *P. aeruginosa* to

TABLE 1. MICs and MBCs of furanone compound 2 for different gram-positive and gram-negative bacteria

Strain	Concn (μM)	
	MIC	MBC
S. epidermidis 1457	50	>130
S. epidermidis 1457 (Δ*ica*)	65	>130
S. epidermidis 047	50	>130
S. epidermidis 047 (Δ*ica*)	30	>130
S. aureus SA113	15	>130
S. aureus Newman	15	>130
B. subtilis	100	100

furanone compound 2 was further confirmed with high MICs and MBCs of more than 130 μM.

Cytotoxicity of free furanones in L929 fibroblasts. The cytotoxicity of an antimicrobial compound is important, if the compound is to be used to prevent bacterial implant infections. Therefore, we quantified cytotoxicity of the different furanone compounds in eukaryotic L929 fibroblasts by LDH release. After 24 h, 30% cytotoxicity was reached at a concentration of 28.4 ± 8.4 μM with compound 2, at 18.6 ± 12.3 μM with compound 3, and at 38.35 ± 15.0 μM with compound 4; for compound 5 this value was 12.1 ± 5.7 μM (Fig. 6). In contrast, PLL-*g*-PEG-coated furanone compound 5 was not toxic for L929 fibroblasts (data not shown). These results indicate that murine fibroblasts and gram-positive bacteria are similarly susceptible to free furanones.

DISCUSSION

The present study was designed to investigate the effect of furanone upon biofilm in gram-positive bacteria. In many biofilm-forming bacteria, the differentiation from planktonic to sessile exopolysaccharide-producing cells is associated with the activation of a complex regulatory network in response to quorum sensing signals and/or environmental stress factors such as high osmolarity, detergents, urea, ethanol, and oxidative stress (37).

In *S. epidermidis*, the production of biofilm may be due to upregulation by the global regulator σ^B (26) or by interference with the *luxS* QS systems (46). We did not observe any change of σ^B expression levels but found *luxS* downregulated by subinhibitory concentrations of furanone. The involvement of LuxS was further confirmed by using a Δ*luxS* mutant, which generally formed more biofilm than the isogenic wild type but remained unaffected by subinhibitory furanone concentrations. Recent studies on *LuxS* function suggest that, besides its transcriptional repression of *ica* genes, it has strong metabolic effects and thus the mechanism of interaction between *luxS* and PIA under furanone treatment remains to be determined in a future study (30).

Alternatively, furanone may act by repression of *agr*, which is a global regulator of QS in *S. aureus* and *S. epidermidis* and enhances biofilm detachment via protease activation (3) and via altering biofilm structure (43). However, our data do not support an action of furanone on *agr*, since subinhibitory concentrations similarly enhanced biofilm in wild-type and Δ*agr* staphylococci. Furthermore, an *agr*-independent mechanism is supported by the fact that RNAIII expression, which is upregulated by *agr* (36) and is associated with a reduction of biofilm (44), was not modified by furanone. Finally, our observation that furanone acted similarly on biofilm in *S. epidermidis* 1457 and in *S. epidermidis* 047, which is a natural Δ*agr* mutant (43), excludes a contribution of *agr* in furanone-induced biofilm formation.

The furanone effects on staphylococcal biofilm resemble results obtained with tetracycline and quinupristin-dalfopristin (37). These substances, which were previously found to increase *S. epidermidis* biofilm at subinhibitory concentrations, are protein synthesis inhibitors acting at the ribosome. The bactericidal mechanism of action of furanone upon staphylococci is unknown; our results indicate that furanone has to enter the bacterial cell, since the PLL-*g*-PEG-grafted furanone remained without effect on staphylococci. The results of an earlier study describing biofilm inhibition in *S. epidermidis* by covalently bound furanone in vitro and a reduced bacterial load on furanone-coated catheters in sheep in vivo (23) are discrepant with our results. However, it should be pointed out that the authors of that study used a different furanone

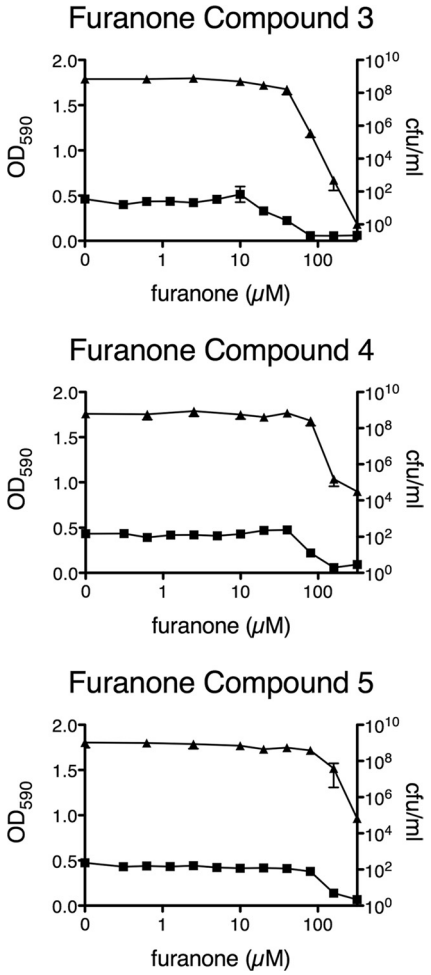

FIG. 3. Crystal violet staining of biofilms (■) and CFU count (▲) from *S. epidermidis* 1457 grown for 18 h in 96-well plates with different concentrations of free furanone compounds 3, 4, and 5. Significant differences in the optical density with 0 μM furanone are indicated by asterisks: **, $P < 0.01$; ***, $P < 0.001$.

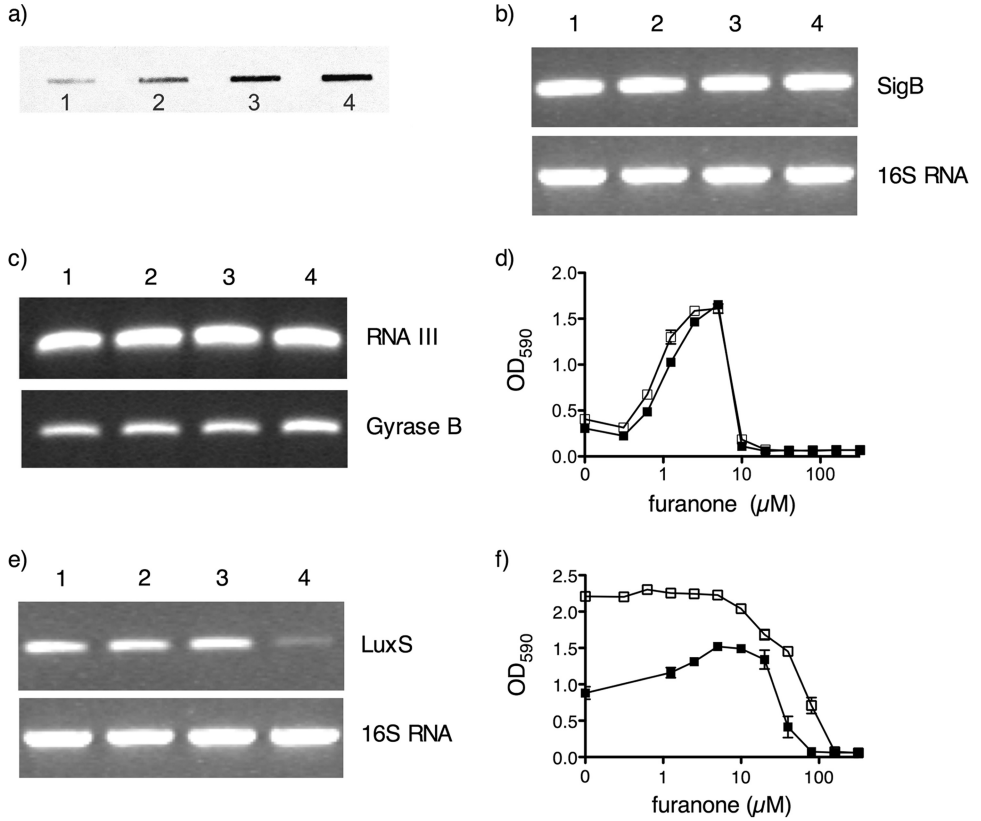

FIG. 4. (a to c and e) Anti-PIA staining of bacterial lysate using a slot blot with 5×10^5 CFU/slot (a) and expression of *sigB* and 16S RNA (b), RNAIII and *gyrB* (c), and *luxS* and 16S RNA (e) genes from *S. epidermidis* 1457 grown for 18 h in 96-well plates with different concentrations of free furanone compound 2 (lane 1, 0 μM; lane 2, 2.5 μM; lane 3, 10 μM; and lane 4, 20 μM). The 16S RNA gene and *gyrB* served as housekeeping genes. Representative results from one of three independently performed assays are shown. (d and f) Crystal violet staining of biofilms from *S. aureus* Newman wild-type (■) and Δ*agr* mutant (□) strains (d) and from *S. epidermidis* 1457 wild-type (■) and Δ*luxS* mutant (□) strains (f) grown for 18 h in 96-well plates with different concentrations of free furanone compound 2. The means ± the standard deviations of three independent experiments are shown.

compound and a different immobilization strategy. Their results may be explained by a bactericidal effect of high furanone concentrations upon *S. epidermidis*, since [^{3}H]thymidine uptake—taken as a measure of biofilm, but truly a growth indicator—was found to be reduced by furanone (23). We found a similar biofilm effect of furanone on *S. epidermidis* and *S. aureus* at least in strain Newman, which is a strong biofilm producer. In contrast, *B. subtilis* biofilm was not affected, and bactericidal activity was observed only above furanone concentrations of 50 μM, a finding that is in agreement with a reduction in viability found in a previous study (39).

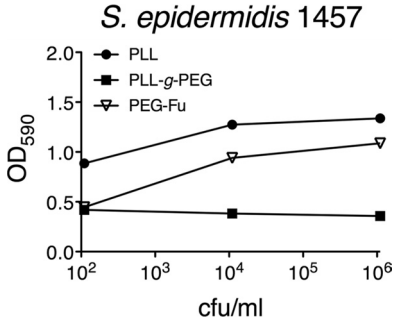

FIG. 5. Crystal violet staining of biofilms from *S. epidermidis* 1457 grown for 18 h in 96-well plates with different covalent coatings.

The potential application of furanone as a bactericidal substance against staphylococci depends on the therapeutic window between the MIC for bacteria and toxicity in eukaryotic cells. We found a potent bactericidal activity for the compounds 2 and 3 and thus confirmed previous results showing antibacterial activity (24, 25). In addition, our data demonstrate that *S. epidermidis* is less susceptible than *S. aureus*. We also found no variability of MIC among *S. epidermidis* strains and no difference of MIC between *ica*-positive and -negative strains. These findings indicate that the bactericidal action of furanone is independent of biofilm and uses an unknown mechanism, which is common to several types of gram-positive bacteria and absent in gram-negative bacteria. We could confirm the previously described biofilm-reducing effect for *Pseudomonas* in rich growth medium without bactericidal activity (22) by using high furanone concentrations. The bactericidal action of the different furanone compounds on gram-positive bacteria occurred, however, at a similar concentration as cytotoxicity for murine fibroblasts. Interestingly, compound 5, which was the least bactericidal, was the most toxic furanone compound in fibroblasts. Therefore, these particular furanones

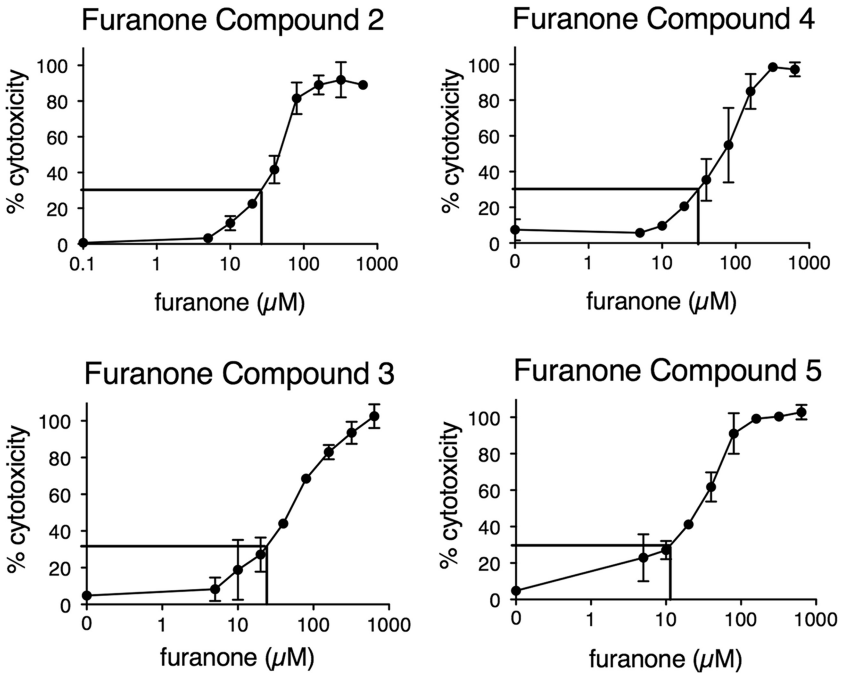

FIG. 6. Cytotoxicity of free furanone compounds in L929 fibroblasts. The LDH release after 24 h was determined, and the 30% cytotoxicity is indicated.

are not suitable for further development to drugs with systemic application, as similarly shown for other furanones (24, 25). However, a topical application, e.g., in the treatment of burns, is conceivable. Alternatively, further chemical modifications may yield compounds that have a therapeutic window. Baveja et al. found human granulocyte viability unaltered after incubation with a polystyrene-bound furanone (1). This may indicate that covalently bound furanone was inactive or the concentration of immobilized furanone was very low. In addition, human myeloid cells may be less susceptible to furanone than murine fibroblasts.

In summary, we show that free, but not surface-bound furanone is toxic for staphylococci and eukaryotic cells to a similar extent. Furthermore, subinhibitory concentrations of furanone compound 2, but none of its analogues, enhance biofilm formation of staphylococci via a *luxS*-suppressing mechanism, leading to increased PIA production.

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