Oral Microbiology Immunology 2007: 22: 340–346
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ORAL MICROBIOLOGY
AND IMMUNOLOGY

Synthetic bromated furanone inhibits autoinducer-2-mediated communication and biofilm formation in oral streptococci

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Lönn-Stensrud J, Petersen FC, Benneche T, Aamdal Scheie A. Synthetic bromated furanone inhibits autoinducer-2-mediated communication and biofilm formation in oral streptococci.
Oral Microbiol Immunol 2007: 22: 340–346. © 2007 The Authors. Journal compilation © 2007 Blackwell Munksgaard.

Introduction: Autoinducer-2 (AI-2) is a widespread communication-signal molecule that allows bacteria to sense and react to environmental factors. In some streptococci AI-2 is reported to be involved in virulence expression and biofilm formation. It has earlier been shown that the alga *Delisea pulchra* produces bromated furanones, which prevent bacterial colonization of the algae.
Methods and results: We have previously published a novel and simple synthesis of (*Z*)-5-bromomethylene-2(5*H*)-furanone. In this study we showed that our synthesized furanone inhibited biofilm formation and bioluminescence induction by *Streptococcus anginosus*, *Streptococcus intermedius*, and *Streptococcus mutans*, as well as bioluminescence induction by *Vibrio harveyi* BB152.
Conclusion: We suggest that the effect is linked to interference with the AI-2 signaling pathway because adding furanone to the medium had no effect on the ability of the AI-2-defective *S. anginosus luxS* and *S. intermedius luxS* mutants to form biofilms.

Key words: autoinducer-2; biofilm formation; furanone; streptococci

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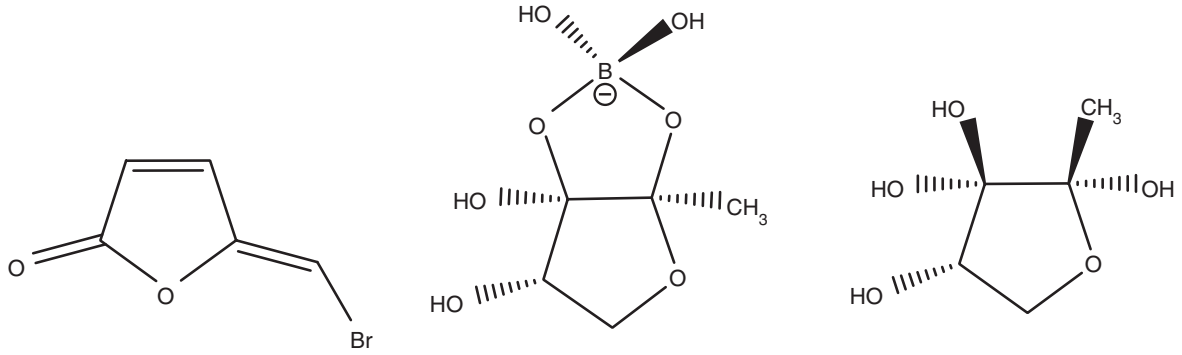
Accepted for publication December 22, 2006

Most microorganisms in nature prefer a biofilm mode of growth, and microbial biofilms are responsible for various infections in humans (10, 38). Microorganisms in biofilms show increased antimicrobial resistance, being up to 10–1000 times less susceptible to antimicrobial agents than their planktonic counterparts, thus explaining the frequent failure of antimicrobial treatments (11, 21).

The autoinducer-2 (AI-2), a furanosyl borate diester, may serve as a microbial intra-species and inter-species communication signal (8, 12, 36, 42). AI-2 is synthesized by LuxS, the product of the *luxS* gene, which is conserved in a majority of sequenced gram-positive and gram-negative bacterial genomes (7, 9, 13, 14, 24, 25, 27, 42, 43, 47). The AI-2 molecules in different species may not necessarily be identical, but are most likely derived from the same precursor 4,5-dihydroxy-2,3-pentanedione (DPD) (26). DPD is able to interconvert, consequently distinct AI-2 structures of *Vibrio harveyi* and *Salmonella typhimurium* have been identified (Fig. 1). The structure of the AI-2 produced by streptococci is unknown. AI-2 communication may regulate biofilm formation in *Escherichia coli*, *Porphyromonas gingivalis*, *S. typhimurium*, *Streptococcus gordonii*, *Streptococcus mutans* and *Streptococcus anginosus* (12, 24, 27, 34, 36, 47).

Bromated furanones isolated from the surface of the red alga *Delisea pulchra* inhibit bacterial colonization of the algae, probably through interference with microbial communication (20). Furanones similar to those found in *D. pulchra* may interfere with bacterial behavior, induced by both AI-2 and the species-specific autoinducer-1 (AI-1) in gram-negative microorganisms, without affecting their growth (22, 23, 32). Natural furanones have also shown potential to inhibit growth and to control multicellular behavior of the gram-positive *Bacillus subtilis* (30, 33). The synthetic (*Z*)-5-bromomethylene-2(5*H*)-furanone (Fig. 1) was shown to inhibit AI-1-mediated



(*Z*)-5-bromomethylene-2(5*H*)-furanone (28)

***V. harveyi* AI-2 (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borat (*S*-THMF) (5)**

***S. typhimurium* AI-2 (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF) (18)**

Fig. 1. Chemical structure of (*Z*)-5-bromomethylene-2(5*H*)-furanone (furanone), A1-2 of *V. harveyi* and A1-2 of *S. typhimurium*.

microbial communication and to reduce the severity of *Pseudomonas aeruginosa* lung infection in mice (18, 46). We recently published a novel and simple synthesis of (*Z*)-5-bromomethylene-2(5*H*)-furanone (5). This furanone has never been tested for possible AI-2 interaction, and there are no previous studies on the connection between AI-2 and furanones in gram-positive species.

The aim of this study was first to investigate whether the synthetic (*Z*)-5-bromomethylene-2(5*H*)-furanone affected biofilm formation by *S. anginosus*, *Streptococcus intermedius*, and *S. mutans*. These species are found in biofilms on teeth. Both *S. anginosus* and *S. intermedius* are also part of the normal flora of the gastrointestinal and the genito-urinal tracts (29, 45). *S. mutans* is associated with dental caries (17), while *S. anginosus*, *S. intermedius*, and *S. mutans* may be associated with endocarditis (15, 17, 29, 45). *S. intermedius* may furthermore be associated with abscesses of the brain and liver (15, 29, 45), while *S. anginosus* may be involved in infection and cancer of the upper digestive tract (35, 37, 40). A second aim was to elucidate the mechanisms of action of the furanone through assessment of its antimicrobial activity and its ability to interfere with bacterial communication via AI-2.

Materials and methods

Furanone synthesis and preparation

(*Z*)-5-bromomethylene-2(5*H*)-furanone (Fig. 1) was synthesized according to our newly published method (5). Briefly, (*Z*)-5-bromomethylene-2(5*H*)-furanone was synthesized in four steps from the commercially available adduct between furan and maleic anhydride. A reaction between a maleic anhydride derivative and a phosphorane gave the ester (2*E*)-*tert*-butyl 2-[5-oxofuran-2(5*H*)-ylidene]acetate. The ester was then treated with trifluoroacetic acid to form the acid (2*E*)-2-[5-oxofuran-2(5*H*)-ylidene]acetic acid. Finally the (*Z*)-5-bromomethylene-2(5*H*)-furanone was prepared by bromodecarboxylation of the acid. The furanone was dissolved and diluted in absolute ethanol to a final stock concentration of 60 mM and stored at −20°C. Dilutions in double-distilled water from the stock were performed immediately before use. For the coating, the furanone was diluted to 60 μM in ethanol. Ethanol was used as a negative control in the coating assay. Up to the maximum concentration of 0.01%, as used in the antimicrobial assay, no inhibitory effect was observed.

Bacterial strains and media

Bacterial strains used in this study are listed in Table 1. The streptococcal cells were grown on Todd–Hewitt broth agar plates (Difco Laboratories, Detroit, MI, USA) for 48 h at 37°C in a 5% carbon dioxide aerobic atmosphere before use. For the communication-defective *S. anginosus luxS* (SA001) and *S. intermedius luxS* (SI006) mutants, the agar plates were supplemented with kanamycin (500 μg/ml). Biofilm, growth, and bioluminescence were assessed from growth in brain–heart infusion medium (BHI; Difco Laboratories) supplemented with 24 mM $NaHCO_3$, which had been shown to increase bioluminescence in *S. gordonii* (6). Kanamycin (500 μg/ml) was added to the first overnight culture of *S. anginosus luxS* and *S. intermedius luxS* mutants. Before use, *V. harveyi* BB152, which only produces AI-2, and *V. harveyi* BB170, which only senses AI-2 (2, 3), (a kind gift from Prof. B. Bassler) were grown in air on heart infusion (HI; Sigma Aldrich, St Louis, MO) agar plates supplemented with kanamycin (100 μg/ml) at 30°C for 48 h. The reporter strain *V. harveyi* BB170 and the AI-2-producing strain *V. harveyi* BB152 were grown overnight in HI medium supplemented with NaCl (20 g/l) and kanamycin (100 μg/ml), and then grown

Table 1. Bacterial strains used in this study

Strain	Relevant characteristics	Source of reference
Vibrio harveyi BB152	luxL::Tn5, AI-1 producer-, AI-2 producer+	(2, 3)
V. harveyi BB170	luxN::Tn5, AI-1 sensor-, AI-2 sensor+, reporter strain	(2, 3)
Streptococcus		
AI-2 producers		
S. anginosus NCTC10713	Type strain	NCTC
S. intermedius NCTC11324	Type strain	NCTC
S. mutans OMZ175	Serotype f	(16)
S. mutans UA159	Serotype c	(1)
AI-2 defective		
S. anginosus SA001	NCTC10713 *luxS* '::pSF151::' *luxS*	(27)
S. intermedius SI006	NCTC11324 *luxS* '::pSF151::' *luxS*	(27)

for the following night in bioluminescence assay medium containing thiamine (0.01 μg/ml) and riboflavin (1 μg/ml) (36, 39). Incubation was in a water bath with shaking at 30°C.

Biofilm assay

To assess the effect of furanone on biofilm formation, second overnight streptococcal cultures were diluted 1 : 100 in BHI containing 24 mM $NaHCO_3$. One milliliter samples were transferred to flat-bottom, 24-well polystyrene microtiter plates, as previously described (28). Furanone was added to the wells at a final concentration of 0.6 or 6.0 μM and the plates were incubated for 44 h at 37°C in a 5% CO_2 aerobic atmosphere. After 20 h of incubation, 500 μl fresh medium containing the given concentrations of furanone was added to the wells. Plates coated with 10 μg furanone were similarly incubated for 44 h. Coating was performed by adding 1 ml of 60 μM furanone to each well and allowing the solvent to evaporate for 24 h at room temperature. Biofilm quantity was assessed after removing non-adherent cells by washing the plates twice in distilled water. One milliliter of fresh medium was then added and the walls and bottoms of the wells were thoroughly scraped into the growth medium. The efficiency of removing the biofilms has been tested previously using safranin staining of any biofilm remaining on the surface after scraping. We have found the scraping method to be highly reproducible, and to effectively detach the biofilm. Biofilm formation was quantified as optical density at 600 nm (OD_{600}) after vigorous shaking to evenly disperse the cells (27).

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to examine the architecture of *S. intermedius* NCTC11324 and *S. mutans* OMZ175 biofilms formed on furanone-coated surfaces. Biofilms were formed as described above on furanone-coated polystyrene disks (Nunc, Rochester, NY, USA) immersed into separate culture wells. The discs were removed after 44 h, rinsed with distilled water, and fixed with 2.5% glutaraldehyde in 0.1 M Sørensen phosphate buffer. Samples were successively dried with 30, 50, 70 and 90% absolute alcohol for 10 min, sputtered with palladium/gold and then examined by SEM (model XL 30 ESEM; Philips, Eindoven, the Netherlands).

Antimicrobial effect of furanone

To exclude the possibility that the furanone effect was the result of a bactericidal effect, the streptococci were grown in BHI medium with various concentrations of furanone (0.6, 6.0 and 60.0 μM) for 24 h at 37°C in a 5% CO_2 aerobic atmosphere. Growth was determined every second hour by measuring OD. Cells in BHI medium without furanone were similarly assayed as negative controls. When forming biofilm in furanone-coated wells, a bactericidal effect was excluded by measuring total growth of the streptococcal cells. Total growth was assayed by measuring biofilm and planktonic cells together. To assess an antimicrobial effect on *V. harveyi*, bacterial suspensions from the bioluminescence assay with or without furanone were diluted and inoculated onto HI agar plates. The agar plates were incubated for 24 h at 30°C in air, and numbers of colony-forming units were compared.

Bioluminescence assay

AI-2 communication was assessed as the ability to induce bioluminescence in *V. harveyi* BB170. Cell-free supernatants were prepared from *V. harveyi* BB152 and streptococci by centrifuging the second overnight cultures at 8000 *g* for 10 min and then passing the supernatant through 0.2-μm filters (Schleicher Schuell, Dassel, Germany). The bioluminescence assay was performed essentially as described earlier (36, 39), with slight modifications (27). Briefly, the second overnight culture of the reporter strain *V. harveyi* BB170 was diluted 1 : 5000 in fresh bioluminescence assay medium. Filtered supernatants from *V. harveyi* BB152 and the streptococci to be tested were added to a final concentration of 10%. The ability of furanone to interfere with AI-2 from the streptococci was assessed by adding furanone to the culture supernatants at final concentrations of 0.6 μM or 6.0 μM. Bioluminescence induction was measured in a Wallac Workstation, 1420 multilabel counter in luminescence mode (Wallac Oy, Turku, Finland).

Statistical analysis

One-way analysis of variance on ranks followed by Student–Newman–Keuls method was used for multiple comparisons on biofilm formation, growth and bioluminescence induction. The level of significance was set at $P < 0.05$.

Results

Furanone inhibits streptococcal biofilm formation at a sub-antimicrobial concentration

Both 0.6 and 6.0 μM furanone added to the growth medium reduced biofilm formation by *S. anginosus* NCTC10713, *S. intermedius* NCTC11324, *S. mutans* OMZ175, and *S. mutans* UA159 (Table 2). Coating wells with furanone reduced biofilm formation by *S. anginosus*, *S. intermedius*, and *S. mutans* OMZ175 (Table 3) to a higher degree than when furanone was added to the medium. The reduction in biofilm formation on coated surfaces by *S. intermedius* NCTC11324 and *S. mutans* OMZ175 was also confirmed by SEM (Fig. 2). OD_{600} measurements of the streptococcal wild-types revealed no effect of furanone on growth at concentrations ranging from 0.6 to 6.0 μM, whereas 60 μM markedly reduced the growth (Fig. 3). Similar results were seen in the *luxS* mutants (not shown). The furanone had no effect on established biofilm at the sub-bactericidal concentration of 6 μm (not shown). Total growth in furanone-coated wells was also unaffected (not shown). Colony-forming units of

Table 2. Effect of furanone added to the growth medium on biofilm formation by *Streptococcus anginosus*, *Streptococcus intermedius*, and *Streptococcus mutans*

Strain	Furanone added: control (0 μM)	0.6 μM	6.0 μM
S. anginosus			
Wild-type	0.19 (0.17–0.21)	0.16 (0.15–0.16)*	0.14 (0.14–0.14)*
luxS mutant	0.09 (0.09–0.10)	0.09 (0.09–0.10)	0.09 (0.09–0.10)
S. intermdius			
Wild-type	1.20 (1.17–1.20)	0.88 (0.82–0.91)*	0.79 (0.75–0.81)*
luxS mutant	0.92 (0.91–0.96)	0.90 (0.89–1.00)	0.91 (0.89–0.92)
S. mutans			
OMZ175	0.78 (0.73–0.88)	0.68 (0.66–0.68)*	0.58 (0.57–0.59)*
UA159	1.01 (1.91–1.10)	0.89 (0.49–0.98)*	0.39 (0.34–0.49)*

Median values correspond to the optical density of the re-suspended biofilm cells, with the 25th and 75th percentiles in parentheses. The data represent four different experiments performed in triplicate. *Significantly different from control without furanone ($P < 0.05$) as calculated by the Kruskal–Wallis one-way analysis of variance on ranks, followed by the Student–Newman–Keuls method.

Table 3. Effect of furanone coating on biofilm formation by *Streptococcus anginosus*, *Streptococcus intermedius*, and *Streptococcus mutans*

Strains	Control	With furanone coating[1]
S. anginosus		
Wild-type	0.05 (0.04–0.09)	0.03 (0.02–0.04)*
luxS mutant	0.02 (0.02–0.04)	0.02 (0.01–0.02)
S. intermedius		
Wild-type	1.06 (1.00–1.15)	0.25 (0.19–0.31)*
luxS mutant	0.77 (0.67–0.91)	0.39 (0.34–0.46)*
S. mutans OMZ175		
Wild-type	0.48 (0.43–0.51)	0.18 (0.14–0.21)*

Median values correspond to the optical density of the resuspended biofilm cells, with the 25th and 75th percentiles in parentheses. The data represent four different experiments performed in triplicate. *Significantly different from control without furanone ($P < 0.05$) as calculated by the Kruskal–Wallis one-way analysis of variance on ranks, followed by the Student–Newman–Keuls method.
[1]10 μg of furanone/well; 1 ml of 60 μM furanone was added to each well.

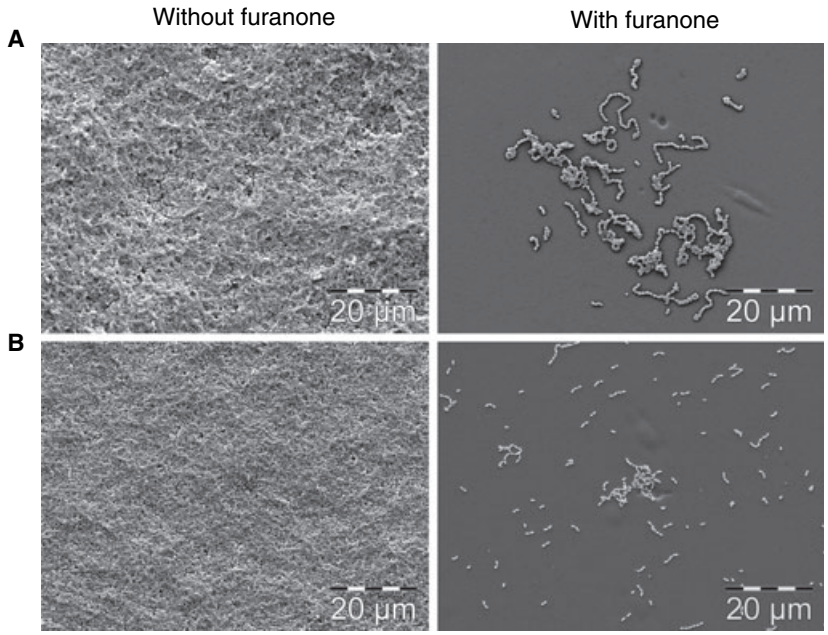


Fig. 2. Scanning electron micrographs of *Streptococcus intermedius* NCTC11324 (A) and *Streptococcus mutans* OMZ175 (B) biofilm grown in BHI containing $NaHCO_3$ on polystyrene disks with 10 μg furanone/well or without furanone.

V. harveyi grown in the presence of 0.6 or 6 μM furanone were not significantly different from the negative control, whereas furanone at a final concentration of 60 μM reduced *V. harveyi* viability by 96% (not shown).

The furanone inhibitory effect on biofilm formation is attenuated in *luxS* mutants

Biofilm formation by the communication defective *S. anginosus luxS* and *S. intermedius luxS* mutants was unaffected by furanone in the medium at concentrations of 0.6 and 6.0 μM (Table 2). When grown in furanone-coated wells, biofilm formation by the *S. anginosus luxS* mutant was not significantly affected. There was, however, a reduction in the biofilm formation by the defective *S. intermedius luxS* mutant, compared to its wild-type control, but the effect was less pronounced than in the wild-type (Table 3).

Furanone interferes with AI-2-mediated bioluminescence

AI-2 communication was assessed as the ability to induce bioluminescence in *V. harveyi* BB170 by supernatants from *V. harveyi* BB152, *S. anginosus* NCTC10713, *S. intermedius* NCTC11324, and *S. mutans* OMZ175. Furanone quenched the bioluminescence induced by all supernatants at all concentrations tested (Fig. 4).

Discussion

We showed that synthetic (*Z*)-5-bromomethylene-2(5*H*)-furanone reduced biofilm formation at sub-bactericidal concentrations. (*Z*)-5-bromomethylene-2(5*H*)-furanone was chosen because of its simple structure and because we have found an effective synthesis pathway (5). During the synthesis both the (*Z*)- and the (*E*)-isomers are formed. Interestingly, the (*Z*)-isomer inhibited AI-2 signaling in bioluminescence to a higher degree than the (*E*)-isomer (not shown), and was therefore chosen for further studies. The synthetic furanone reduced biofilm formation in *S. anginosus*, *S. intermedius*, and *S. mutans* wild-type strains, both when coated on a surface and when added to the medium. The effect was more pronounced on furanone-coated surfaces, in which the reduction was 76% and 63% in *S. intermedius* and *S. mutans* respectively. Although *S. anginosus* formed less biofilm than *S. intermedius* and *S. mutans* in our model system, the result was statistically significant. The reduced biofilm formation in the three streptococci indicates that the effect of furanone was not species specific.

We observed that streptococcal biofilm formation is reduced during growth in the presence of 0.6 and 6.0 μM furanone. A possible application for furanones is the coating of surfaces because biofilms on both biological and non-biological surfaces may be associated with infections. Therefore we examined whether our synthetic furanone would reduce biofilm formation when coated on polystyrene well surfaces and observed that biofilm formation was indeed reduced. Although 60 μM furanone showed an antimicrobial effect on planktonic streptococcal growth, no antimicrobial effect was seen when 10 μg (added as 1 ml of 60 μM furanone) was used to coat the wells.

Notably, the inhibition of biofilm formation by furanone seems not to be related to an antimicrobial effect. This suggests that the furanone will most likely not exert a selection pressure that might lead to antimicrobial resistance. Many known antimicrobial agents used both in hospitals and daily life, for example in hand soaps, toothpastes, detergents or plastics, are becoming widespread, thus enhancing the emergence of resistant microbes (41). Furanones could be used together with antibiotics against biofilm-associated infections to improve efficacy and possibly avoid the development of antibiotic resistance. Depending on the target, there may be several potential vehicles for furanone delivery.

It has been shown that furanones adsorbed physically to different biomaterials reduce *Streptococcus epidermidis* slime production (biofilm formation) (4).

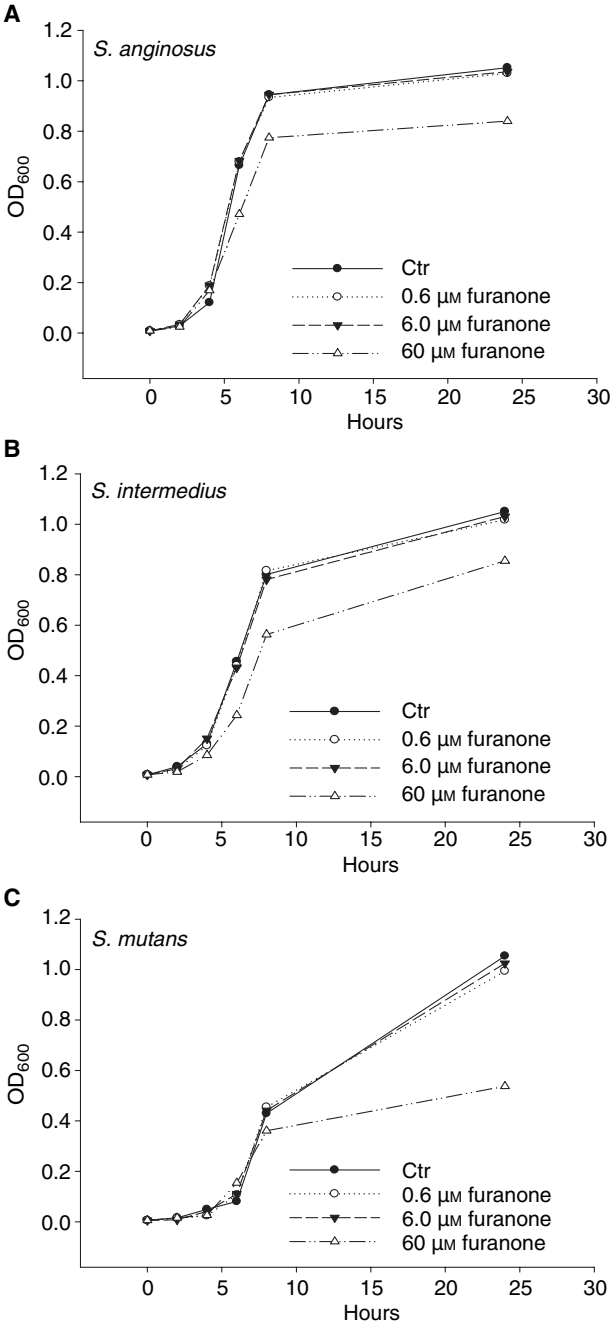


Fig. 3. Furanone effect on planktonic growth. Growth of *Streptococcus anginosus* NCTC10713 (A), *Streptococcus intermedius* NCTC11324 (B), and *Streptococcus mutans* OMZ175 (C) was measured as planktonic growth at OD_{600} for 24 h with 0.6, 6.0, and 60 μM furanone or without furanone. Results are from one experiment performed in duplicate and are representative of at least three independent experiments. Standard deviation for all time-points was <0.5%.

It has also been shown that *in vitro* biofilm formation by *S. epidermidis* was inhibited by 89% when formed on polystyrene–furanone disks and by 78% when formed on furanone-coated catheters. In the same study, an *in vivo* sheep model showed that furanones effectively controlled infection for up to 65 days (19). They suggested that furanones might be used to coat biomaterials to control *S. epidermidis* infections. Using a different furanone, we showed that furanones might control streptococcal biofilm formation. However, because surfaces in various biological environments are rapidly coated with organic pellicles, possible interference needs to be investigated.

Biofilm formation by the *S. anginosus* and the *S. intermedius* communication-defective *luxS* mutants was unaffected when furanone was added to the medium, in contrast to the wild-type strains. Furthermore, the furanone coating did not affect biofilm formation by the *S. anginosus luxS* mutant. These findings suggest that furanone interfered with AI-2 communication. In the *S. intermedius luxS* mutant however, furanone coating had an effect on biofilm formation, but this was less pronounced than the effect on the wild-type strain. This may be explained by a possible effect of furanone on other factors involved in biofilm formation by *S. intermedius*.

Another indication of AI-2-specific interference is that the furanone reduced bioluminescence in *V. harveyi* BB170. Furanone reduced bioluminescence induction by *V. harveyi* BB152 in a concentration-dependent manner. We also observed a decrease in bioluminescence induced by the cell-free supernatants of the streptococci, indicating that the synthesized furanone interfered with their communication with *V. harveyi*.

Cell–cell signaling is a critical process in the formation of biofilms, including dental plaque. We have previously shown that communication via the competence-stimulating peptide is involved in biofilm formation by *S. intermedius* (28). Whether the two communication networks, AI-2 and competence-stimulating peptide, are hierarchically organized or linked in any way is presently unknown.

The mechanism of action of the furanone and the structure of streptococcal AI-2 are unknown. Induction of bioluminescence in *V. harveyi* BB170 by streptococcal supernatants indicates however, recognition of the streptococcal AI-2 by the receptor on *V. harveyi* BB170. Thus a certain molecular similarity seems likely. We suggest that, the synthetic furanone interfered with the communication by competing with AI-2, rather then by affecting the AI-2 sensing in *V. harveyi* BB170. This is also in line with results from *E. coli*, showing that furanone down-regulates about 79% of the genes that AI-2 up-regulates (31). We confirmed in this paper previous findings, that AI-2 signaling molecules are produced by *S. anginosus*, *S. intermedius*, and *S. mutans*, by the induction of bioluminescence in the reporter strain *V. harveyi* BB170 (6, 25, 27, 44, 47). To our knowledge, this is the first report in which furanone is shown to interfere with AI-2-mediated communication in streptococci.

This study shows that (*Z*)-5-bromomethylene-2(5*H*)-furanone may be a potential

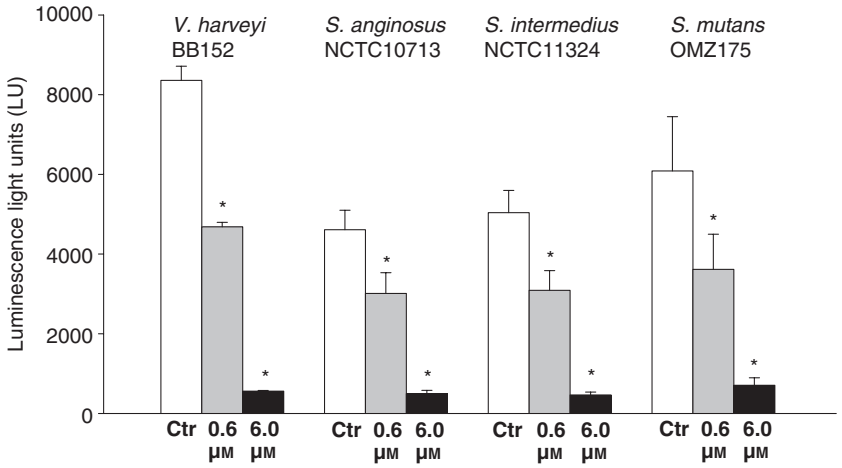


Fig. 4. Bioluminescence in the reporter strain *Vibrio harveyi* BB 170 induced by *V. harveyi* BB152, *Streptococcus anginosus* NCTC10713, *Streptococcus intermedius* NCTC11324, and *Streptococcus mutans* OMZ175 supernatants and repressed by 0.6 or 6.0 µM furanone. The results are mean values and standard errors from three independent experiments with three parallels.

inhibitor of AI-2-mediated biofilm formation by various streptococci. The furanone concentrations used for biofilm formation had no effect on total growth and therefore probably do not induce a selective pressure. This may reduce the risk of antimicrobial resistance development. Further studies should be conducted to determine whether furanone affects virulence factors other than biofilm formation.

Acknowledgment

We thank Steinar Stølen for taking the scanning electron microscopic images.

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