Brominated Furanones Inhibit Biofilm Formation by
Salmonella enterica Serovar Typhimurium

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Salmonella enterica serovar Typhimurium is a main cause of bacterial food-borne diseases. As Salmonella can form biofilms in which it is better protected against antimicrobial agents on a wide diversity of surfaces, it is of interest to explore ways to inhibit biofilm formation. Brominated furanones, originally extracted from the marine alga Delisea pulchra, are known to interfere with biofilm formation in several pathogens. In this study, we have synthesized a small focused library of brominated furanones and tested their activity against S. enterica serovar Typhimurium biofilm formation. We show that several furanones inhibit Salmonella biofilm formation at non-growth-inhibiting concentrations. The most interesting compounds are (Z)-4-bromo-5-(bromomethyl)-3-alkyl-2(ZH)-furanones with chain lengths of two to six carbon atoms. A microarray study was performed to analyze the gene expression profiles of Salmonella in the presence of (Z)-4-bromo-5-(bromomethyl)-3-ethyl-2(ZH)-furanone. The induced genes include genes that are involved in metabolism, stress response, and drug sensitivity. Most of the repressed genes are involved in metabolism, the type III secretion system, and flagellar biosynthesis. Follow-up experiments confirmed that this furanone interferes with the synthesis of flagella by Salmonella. No evidence was found that furanones act on the currently known quorum-sensing systems in Salmonella. Interestingly, pretreatment with furanones rendered Salmonella biofilms more susceptible to antibiotic treatment. Conclusively, this work demonstrates that particular brominated furanones have potential in the prevention of biofilm formation by Salmonella serovar Typhimurium.

Salmonella enterica is a worldwide major cause of bacterial food-borne diseases. Nontyphoidal Salmonella serovars, such as Salmonella enterica serovar Typhimurium, cause a localized self-limiting gastroenteritis in humans (53). However, in immunocompromised people, Salmonella infections are often fatal if they are not treated with antibiotics. While Salmonella infections are in these cases most commonly treated using fluoroquinolones (e.g., ciprofloxacin) and extended spectrum cephalosporins (e.g., ceftaxime), there are alarming reports concerning the development of resistance against these antimicrobials (7). In addition, Salmonella is capable of forming biofilms on a variety of biotic and abiotic surfaces. These biofilms enable Salmonella to survive and spread in the environment outside the host and show an even higher tolerance to antibiotics (49). This is of concern since, according to the National Institutes of Health, in general approximately 80% of persistent bacterial infections in the United States are associated with biofilms (47). Therefore, a strong need for the development of alternative strategies to combat the spread of bacterial infections is arising (11, 59).

In recent years, halogenated furanones, a class of secondary metabolites originally extracted from the red alga Delisea pulchra, have been proven to hold great promise as antifouling products and biofilm inhibitors (15, 16, 59). It has been shown that natural brominated furanones and derivatives thereof negatively influence biofilm formation by several bacterial species, such as Pseudomonas aeruginosa (23, 24), Escherichia coli (56, 58), Bacillus subtilis (57), Staphylococcus epidermidis (28), and Streptococcus spp. (37). In addition, brominated furanones have been reported to inhibit other forms of multicellular behavior in gram-negative bacteria, such as swarming (20, 21, 54, 58) and bioluminescence (12, 13, 40), without inhibiting the growth rate of these bacteria.

These forms of multicellular behavior (biofilm formation, swarming, and bioluminescence) have been shown for many bacterial species to be regulated by so-called “quorum-sensing” (QS) systems using different classes of small signal molecules (4, 8, 10, 27, 35, 38, 48, 50). In this type of bacterial cell-cell communication, each single bacterium produces a small amount of one or more signal molecules, which are subsequently released into the environment. When the total amount of the signal molecule increases, the concentration reaches a detection limit, thereby causing the activation or repression of certain target genes. In this way, QS systems coordinate gene expression, usually in a population-density-dependent manner (18, 72, 73). In gram-negative bacteria, the best-studied QS systems use either N-acyl homoserine lactones (AHLs, produced by LuxI-type enzymes and detected by LuxR-type transcriptional activators [17, 61]) or the AI-2 class of molecules (produced by LuxS-type enzymes and detected by different types of receptors [5, 46, 63]) as signal molecules. The detection of the signal molecules results in the activation or inhibition of several target genes, thereby regulating a number...
of important biological functions, including those mentioned above.

The pathogen of interest in this study, Salmonella serovar Typhimurium, has been shown to contain two putative QS systems. First, Salmonella encodes a LuxR-type AHL receptor, SdiA (suppressor of cell division inhibition A), which has been shown to respond to a broad range of AHLs and AHL analogues (2, 29, 45). Since Salmonella does not posses a luxI homologue, it cannot produce its own AHLs and has therefore been hypothesized to use SdiA for the interception of AHL signals produced by other species (1, 45, 62). In response to AHLs, SdiA activates two Salmonella-specific loci, srgE (sdiA-regulated gene E) and the rck (resistance to complement killing) operon, but the exact function of SdiA in Salmonella remains unclear (1, 62). Second, S. enterica serovar Typhimurium also encodes a LuxS-type enzyme, which enables it to synthesize (S)-4,5-dihydroxy-2,3-pentanedione (DPD), which diffuses out of the cell (63). The unstable DPD spontaneously cyclizes to form the AI-2 group of signal molecules, and one of these compounds is detected by LsrB (LuxS regulated) and reinternalized by the bacteria via the Lsr ABC transporter (46, 65, 66). Inside the cell, AI-2 activates the transcription of the lsrACDRTFGE operon, of which the first four genes encode the Lsr transport apparatus. Interestingly, a Salmonella LuxS mutant can no longer form biofilms on gallstones and polystyrene (14, 51), but we have previously shown that synthetic DPD cannot complement this biofilm defect (14). Therefore, the exact functions of both SdiA and LuxS as QS systems in Salmonella remain unclear.

Since brominated furanones inhibit QS-regulated phenotypes in gram-negative bacteria, they were soon identified as QS inhibitors (13, 20, 39, 41, 56). This mode of action was confirmed for the activity of furanones on P. aeruginosa and E. coli by microarray analysis. It was shown that 80% of the P. aeruginosa genes repressed by a synthetic furanone were controlled by the AHL-mediated QS systems of this pathogen (25), while 79% of the E. coli genes that were repressed by a natural furanone were activated by AI-2 (56).

Since there have been no reports concerning the activity of halogenated furanones on Salmonella to date, we synthesized a range of brominated furanones and tested their activities on biofilm formation by Salmonella serovar Typhimurium. Additionally, we investigated the activities of combinations of furanones and antibiotics on Salmonella biofilms. Finally, we investigated the effect of the furanones on the QS systems of Salmonella and performed a microarray analysis to gain knowledge about the mode of action of these compounds.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains used in this study were E. coli DH5α (Gibco BRL), E. coli TOP10F (Invitrogen), wild-type S. enterica serovar Typhimurium strain 14028 (American Type Culture Collection), the isogenic sdiA mutant S. enterica serovar Typhimurium BA612 (2), wild-type S. enterica serovar Typhimurium SL1344 (26), and the isogenic luxS mutant S. enterica serovar Typhimurium CCMG5602 (14). The plasmids used were pPNS25 (PspG-luxCDABE; Te') (62), pFPV25.1 (PpsM-gfpMut3; Ap') (70), pCMCG5623 ([PspG-luxCDABE; Ap'] (S. De Keersmaecker, unpublished observations), pCMCG5638 (PspG-luxCDABE; Km') (14), pCMCG5836 (PspG-gfpMut3; Ap') (this study), and pCMCG5849 (PpsM-luxCDABE; Te') (this study). S. enterica serovar Typhimurium and E. coli were grown with aeration at 37°C in Luria-Bertani (LB) medium (60) or on LB plates containing 1.5% agar (Invitrogen) unless stated otherwise. Tryptic soy broth diluted 1/20 (TSB 1/20; BD Biosciences) was used for biofilm formation. Ampicillin, kanamycin, and tetracycline were used at 100, 50, and 20 μg/ml, respectively, when appropriate. Ciprofloxacin and ceftazidime were purchased from Fluka and Applied, respectively, and used at concentrations specified in the text.

Synthesis of chemical compounds. Furanones Fur-1, Fur-2, and Fur-4 to Fur-12 were synthesized as previously described (43) (Fig. 1). Furane Fur-3 was synthesized as reported by Kumar and Read (34), while the nonbrominated furanone Fur-12 was synthesized following the procedure of Gabriele et al. (19). All compounds were purified via preparative chromatography on Fluka silica gel 60 (0.040 to 0.063 mm) for flash chromatography at atmospheric or low pressure. Whenever required, end products were further purified by high-performance liquid chromatography on an Alltech Econosphere silica column (250 by 25 mm) or on a Phenomenex Gemini C18 column (250 by 10 mm) using ethyl acetate-hexane (0.05:99.95) or acetonitrile-water (67:33) as the eluent, respectively. Nuclear magnetic resonance spectra were recorded on a Bruker AMX 300 at 300 MHz (1H) and 75.5 MHz (13C). Gas chromatography-mass spectrometry analyses were performed using a gas chromatograph (Agilent Technologies 6890 N, HP-5MS column) coupled to an electron impact mass spectrometer (Agilent Technologies 5975 mass selective detector, with a 70-eV ionization voltage and 200°C ion source temperature). All furanones were stored as 50 mM stock solutions in ethanol at −20°C. 3O-C7-HSL [N-(3-oxoheptanoyl)-L-homoserine lactone] and 3O-C7-HTL [N-(3-oxoheptanoyl)-L-homoserine thialactone] were synthesized as previously reported (29). These compounds were stored as dry powders at −20°C and used as dilutions from 10 mM stock solutions in acetonitrile.

Plasmid construction. Standard protocols were used for buffer preparation, cloning, plasmid isolation, and E. coli competent cell preparation and transformation (60). Salmonellae were transformed as previously described (52). Cloning steps were performed using E. coli DH5α and TOP10F™.

To create pCMCG5836, the srgE promoter was cut from pJNS25 and cloned as an EcoRI fragment into the EcoRI site of pFPV25 upstream of gfpMut3. The orientation of the promotor was determined by using Ncol. Dose-response experiments with 14028/pCMCG5638 and BA612/pCMCG5836 confirmed that AHLs activate the expression of srgE in an SdiA-dependent fashion and that 3O-C7-HTL activates SdiA at lower concentrations than 3O-C7-HSL, as previously reported (29). To construct pCMCG5849, the promoter of rpsM was removed from pFPV25.1 by using EcoRI and XbaI and the fragment was blunted with Klenow polymerase. Subsequently, the fragment was cloned upstream of luxCDABE in pJNS25, after removal of the srgE promoter from this plasmid by EcoRI and blunting. A plasmid with the promoter in the right orientation was easily selected since colonies containing this plasmid were highly bioluminescent. As, which was detected with a charge-coupled device camera (Berthold Night Owl; PerkinElmer Life Science). All constructs were verified by sequencing (ABI 3100-Avant genetic analyzer) and subsequently electroporated into Salmonella strains 14028 and BA612 using a Bio-Rad gene pulser.
MIC determination. MICs were generally determined according to previously described procedures (3). Briefly, colonies of a secondary subculture of *S. enterica* serovar Typhimurium 14028 were taken from an LB agar plate and suspended into sterile distilled water until the density of a 0.5 McFarland standard was reached. This suspension was subsequently diluted 1:100 in IsoSensitest broth (Oxoid N.V.). Twofold-dilution series of the compounds were prepared in 100-μl volumes of IsoSensitest broth in microtiter plates, and 100 μl of the inoculum was added. The plates were covered with Breathable breathable sealing membranes (Greiner Bio-One N.V.) and incubated for 20 h at 37°C with aeration. The MIC is defined as the lowest concentration of the compound at which there was no detectable growth of *Salmonella*.

**Peg assay for biofilm formation.** The peg assay experiments were essentially performed as previously reported by De Keersmaecker et al. (14). Briefly, the device used for biofilm formation is a platform carrying 96 polystyrene pegs (Nunc no. 445497) that fits as a microtiter plate lid with a peg hanging into each microtiter plate well (Nunc no. 269789). For biofilm formation, twofold serial dilutions of the compounds in 100 μl liquid TSB 1/20 broth per well were performed in the microtiter plate. Subsequently, an overnight culture of *S. enterica* serovar Typhimurium 14028 was diluted 1:50 into TSB 1/20 broth and 100 μl (ca. 2 × 10^6 cells) was added to each well of the microtiter plates, resulting in a total amount of 200 μl medium per well. The pegged lid was placed on the microtiter plate, and the plate was incubated for 48 h at 16°C without shaking. The biofilms formed on the surface of the pegs, and after 24 h, the lid was transferred into a new plate with medium and the specific molecules used for testing. The optical density at 600 nm (OD_{600}) was measured for the planktonic cells in the first plate using a VERSAmax microtiter plate reader (Molecular Devices), and the growth-retarding concentration (GRC) was determined as the concentration that decreased by 10% the OD_{15} of planktonic cells in comparison to a 0% concentration. For calculation of biofilm formation, the pegs were washed once in 200 μl phosphate-buffered saline (PBS). The remaining attached bacteria were stained for 30 min with 200 μl 0.1% (wt/vol) crystal violet in an isopropanol-methanol-PBS solution (1:1:18 [vol/vol/vol]). Excess stain was rinsed off by placing the pegs in a 96-well plate filled with 200 μl distilled water per well. After the pegs were dried (30 min), the dye bound to the adherent cells was extracted with 30% glacial acetic acid. The optical density at 15 OD_{15} of each well was measured using the VERSAmax. Typically, the OD_{35} values for untreated *Salmonella* biofilms (48 h old) and controls that did not contain bacteria were ~2.0 and ~0.06, respectively. The 50% inhibitory concentration (IC_{50}) of each compound was determined from concentration gradients in three independent experiments. These values were verified in three subsequent experiments using six repeats of a single concentration per experiment.

**Determination of the number of viable biofilm cells.** To calculate viable biofilm cells numbers, the following method was used. An overnight culture was diluted 1:100 into 5 ml TSB 1/20 (ca. 5.10^6 cells), and 60 μl M-Fur-8 (from a 50 mM stock solution) or the corresponding volume of ethanol (6 μl) was added. The resulting solution was poured into a small petri dish and incubated at 16°C on a Gyro shaker (Unimix 1010; Heidolph) at 50 rpm. After 24 h, the biofilm that had formed at the bottom of the dish was gently washed with 5 ml PBS to remove unattached bacteria. Subsequently, 1.2 ml LB broth was added to the plate and all remaining cells were scraped off using a cell scraper (Greiner Bio-One N.V.). The LB broth containing the biofilm cells was pipetted out of the petri dish and vortexed, and 1/10 serial dilutions were prepared in PBS and plated onto LB agar plates. After overnight incubation at 37°C, colonies were counted and the number of viable biofilm cells was expressed as CFU per plate. For the experiments with antibiotics, biofilms were first formed in the presence of 60 μg M-Fur-8 or ethanol as described. After 24 h, the medium was replaced with 5 ml TSB 1/20 containing the appropriate amount of antibiotic or solvent and the petri dishes were incubated for an additional 24 h at 16°C with shaking. After this incubation period, the amount of viable cells present in the biofilm was determined, as described above.

**Epifluorescence microscopy.** Biofilms of the *Salmonella* strain 14028/pFPV25.1 (32), which constitutively expresses green fluorescent protein (GFP), were formed in the presence of compounds or ethanol on small petri dishes as described above. After 24 h, the biofilms were gently rinsed with 5 ml 0.9% NaCl and subsequently visualized using a Zeiss Axio Imager Z1 microscope with an EC Plan-Neofluar (40x/0.74, Z1) objective, and pictures were recorded using an AxioCam MRm and the AxioVision software.

**Swimming assay.** The swimming assay was adapted from Kim and Surette (32). Each swimming plate contained 30 ml of TSB 1/20 with 0.25% agar and 50 μM Fur-5 or the corresponding amount of ethanol. The plates were solidified for 2 h at room temperature and inoculated with 3 μl of an overnight culture (ca. 5.10^6 cells) by piercing the surface of the plate with the pipette tip. The plates were incubated for 5 days at 16°C, the surface of the swimming colonies was measured regularly.

**QS reporter experiments.** Competition experiments with *Salmonella* strains were essentially performed as previously described (29). Briefly, threefold serial dilutions of the furanones were prepared in triplicate in microtiter plates (transparent plates for fluorescence reporters [Greiner Bio-one] and white plates [Clinipatel Thermo Life Sciences] for luminescence reporters) in 100 μl liquid LB broth per well in the presence of 10 μM reporter system pNS25 or 40 μM reporter system pC59, in the presence of 100 μl of each reporter strain (Greiner Bio-One N.V.) with 10 μM M-Fur-8 or the corresponding amount of ethanol. The plates were solidified for 2 h at room temperature and inoculated with 3 μl of an overnight culture (ca. 5.10^6 cells) by piercing the surface of the plate with the pipette tip. The plates were incubated for 10 h at 37°C (4°C) at 16°C (5°C) at 16°C (4°C) at 16°C, respectively. For the experiments with antibiotics, biofilms were first formed in the presence of 60 μM Fur-8 or ethanol as described. After 24 h, the medium was replaced with 5 ml TSB 1/20 containing the appropriate amount of antibiotic or solvent and the petri dishes were incubated for an additional 24 h at 16°C with shaking. After this incubation period, the amount of viable cells present in the biofilm was determined, as described above.

**RESULTS**

**Synthesis and MIC determination of brominated furanones.** In spite of their reported biological effects on numerous bacterial species, brominated furanones are currently not commercially available. Therefore, studies of their biological activity...
Salmonella representative of three independent repeats, and the error bars show standard deviations of six measurements. (D to F) Growth curves of Values are representative of three independent repeats.

30% compared to that of a negative control after 24 h of incubation in TSB 1/20.

atoms. The nonbrominated furanone Fur-12 (which lacks the molecules differ in the numbers and positions of the bromine atoms (Fig. 1), as specified in Materials and Methods. These molecules require a considerable synthetic effort. Since there are currently no reports concerning the activity of brominated furanones on Salmonella, it was decided to synthesize a small focused library of 11 brominated furanones (Fur-1 to Fur-11) (Fig. 1), as specified in Materials and Methods. These molecules differ in the numbers and positions of the bromine atoms and the lengths of the alkyl chain, ranging from 0 to 12 carbon atoms. The nonbrominated furanone Fur-12 (which lacks the methylidene side chain as well) was synthesized to evaluate the necessity of the bromine atom(s) for the activity of the furanones. Of all compounds synthesized, only Fur-1 to Fur-3, Fur-6, and Fur-7 have been used previously in biological studies (20, 24, 25, 30, 41). Since brominated furanones are known to be very reactive molecules, we first determined the MIC of the furanones Fur-1 to Fur-12 in IsoSensitest broth on S. enterica serovar Typhimurium 14028. Table 1 shows that the furanones without an alkyl chain (Fur-1 to Fur-3) exhibited toxic effects on Salmonella with MICs of 500 μM, while no growth inhibition was observed for the alkylated furanones at the highest concentration tested (1 mM).

**Brominated furanones inhibit Salmonella biofilm formation.** Brominated furanones have been shown to interfere with biofilm formation in several bacterial species (59). The activity of the furanones on biofilm formation by S. enterica serovar Typhimurium 14028 was screened using a 96-well microtiter plate assay with polystyrene pegs and crystal violet staining as described in Materials and Methods. The planktonic growth in TSB 1/20 medium was monitored after 24 h. Table 1 lists the concentrations of the furanones that were needed to inhibit the biofilm formation by 50% (IC$_{50}$). Additionally, the GRCs are given. From these experiments, it can be concluded that several furanones inhibit Salmonella biofilm formation at concentrations that do not influence the growth of planktonic cells. The furanones without an alkyl chain (Fur-1 to Fur-3) are the most active molecules regarding biofilm formation inhibition (IC$_{50}$ of 10 to 15 μM) but also delay planktonic growth at low concentrations (GRCs of 30 to 40 μM). For the alkylated furanones Fur-5, Fur-6, and Fur-8, Fig. 2A to C show a dose-response effect (IC$_{50}$ of 50, 100, and 60 μM, respectively) on

![FIG. 2. Inhibition of Salmonella biofilm formation by brominated furanones. (A to C) Biofilms were formed on polystyrene pegs in the presence of different concentrations of Fur-5 (A), Fur-6 (B), and Fur-8 (C) for 48 h at 10°C. The biofilms were stained with crystal violet, and the amount of stain was measured (bars) and compared to that of a control that was treated with the corresponding amount of ethanol (100%). The black line indicates 50% biofilm inhibition. After 24 h, the influence of the compounds on the growth of planktonic cells was determined by measuring the OD$_{600}$ (squares) in the microtiter plate and comparing it to that of an ethanol control (100%). The data are the results of one experiment, representative of three independent repeats, and the error bars show standard deviations of six measurements. (D to F) Growth curves of Salmonella in TSB 1/20 in the presence of brominated furanones. S. enterica serovar Typhimurium was grown in 200-μl volumes of TSB 1/20 in the presence of furanone (gray diamonds) or solvent (black squares) at 16°C for 12 h in a microtiter plate. The OD$_{600}$ was measured regularly. The furanones were 50 μM Fur-5 (D), 100 μM Fur-6 (E), and 60 μM Fur-8 (F). The data are the results of one experiment, representative of three independent repeats, and the error bars show standard deviations of four measurements. Means of biofilm formation that were found to be significantly different from the control by the Tukey test are indicated (*, P < 0.01; **, P < 0.001).
the amount of biofilm formed without influencing the growth of planktonic cells (Fig. 2D to F). Similarly, Fur-5, Fur-6, and Fur-8 also inhibited biofilm formation by *S. enterica* serovar Typhimurium SL1344 (data not shown). Fur-5 and Fur-8 were used for further study.

Next, we investigated whether the furanones indeed cause a decrease in the number of viable cells present in the biofilm. We grew a *Salmonella* biofilm in the presence or absence of 60 μM Fur-8 as described in Materials and Methods. After 24 h, the biofilm cells were collected and the cell number was determined by plating and colony counting. Table 2 shows that treatment with 60 μM Fur-8 reduced the number of viable biofilm cells 30-fold. Epifluorescence microscopy confirmed that brominated furanones reduce biofilm formation by *S. enterica* serovar Typhimurium, as shown for Fur-5 in Fig. 3.

**Effect of combination of furanones with antibiotics on biofilm formation.** Since Hentzer et al. (25) reported that addition of 10 μM Fur-3 increased the susceptibility of *P. aeruginosa* biofilms to treatment with the antibiotic tobramycin, we were interested to know whether the brominated furanones used in this work had similar effects on *Salmonella* biofilms. Therefore, the effect of 100 μg/ml of the antibiotics tetracycline, ciprofloxacin, or cefotaxime on the number of viable cells in *Salmonella* biofilms that were pretreated with 60 μM Fur-8 or ethanol was determined by colony counting as described in Materials and Methods. Table 2 confirms that 50- to 2,100-fold fewer viable biofilm cells were present in the plates that were pretreated with Fur-8. Ciprofloxacin was the most potent antibiotic tested.

**Identification of genes influenced by Fur-5.** We determined the *Salmonella* genes that are differentially expressed after contact with Fur-5 by DNA microarray analysis, as described in Materials and Methods. Expression profiling was performed for planktonic cultures of *S. enterica* serovar Typhimurium 14028 in the presence or absence of 50 μM Fur-5. Genes were assigned a d-value based on the level of differential expression between both conditions by means of SAM (69). Of all 4,718 genes of *S. enterica* serovar Typhimurium on the array, 150 genes (2.8%) were significantly affected by Fur-5. Fifty genes (1.1%) were repressed, while 80 genes (1.7%) were activated. The genes were classified according to their function and are presented in Tables S1 and S2 at http://www.bw.kuleuven.be/dtp/cmpg/jansens.htm. Interestingly, most of the genes that were differentially expressed are involved in metabolic processes (16 repressed and 26 activated genes). Figure S1 at the URL mentioned above represents the distribution of these metabolic genes among all classes of *S. enterica* serovar Typhimurium metabolism, as obtained by the Omics Viewer (http://biocyc.org/ov-expr.shtml), and shows that genes involved in many different metabolic processes are targeted by the furanone. Other genes that were activated are involved in heat/cold shock adaptation (e.g., *ibpA*, *ibpB*, and *grpE*), detoxification and drug/analogue sensitivity (e.g., *ahpC*, *marA*, *emrR*, and *acrA*), and broad regulatory functions (e.g., *yfiA*, *rsd*, and *fur*). Nonmetabolic genes that were repressed by Fur-5 are genes for a type III secretion system which is of importance for pathogenicity (e.g., *secaD* and *saEGH*), genes for lipopolysaccharide biosynthesis (e.g., *rfbL* and *rfbKNP*), and several motility genes. Interestingly, most of these motility genes are involved in different aspects of flagellar biosynthesis (*fljK*, *fljM*, *fljO*, and *flgCD*). In addition, the global flagellar regulator *flhD* was also found to be repressed by Fur-5 (d-value, 11.70), although not selected with the applied cutoff. This repression will decrease flagellar biosynthesis and hence might be one of the causes of the observed decreased biofilm formation.

**Fur-5 affects flagellar biosynthesis.** To validate the microarray results on the reduction of flagellar gene expression, we studied the effect of Fur-5 on the number of flagella formed by *S. enterica* serovar Typhimurium 14028 as well as on its ability to swim. *Salmonella* was grown during 4 h in TSB 1/20 at 16°C in the presence or absence of 50 μM Fur-5, after which the flagella were stained and visualized by phase-contrast microscopy. Figure 4 shows that almost no flagella were observed in the presence of Fur-5, compared to the number in controls that

![Control](image1.png) ![50 μM Fur-5](image2.png)

**FIG. 3.** Influence of 50 μM Fur-5 on biofilm formation by *S. enterica* serovar Typhimurium 14028/pFPV25.1. Biofilms were grown on the bottom of small petri dishes for 24 h at 10°C in the presence of 50 μM Fur-5 or the corresponding amount of ethanol (control) and subsequently visualized using epifluorescence microscopy. A ×40 objective was used. The pictures are representative of the biofilms observed in three independent experiments.

**TABLE 2.** Number of viable cells present in the biofilm after 24 h of growth in the presence of 60 μM Fur-8 or ethanol added at 0 h and subsequent treatment with several antibiotics for 24 h

<table>
<thead>
<tr>
<th>Treatment</th>
<th>At 24 h&lt;sup&gt;a&lt;/sup&gt;</th>
<th>With additional 24 h of treatment</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>100 μg/ml Cip</td>
</tr>
<tr>
<td>Fur-8</td>
<td>(1.6 ± 0.5) × 10&lt;sup&gt;7&lt;/sup&gt; A</td>
<td>(1.1 ± 0.5) × 10&lt;sup&gt;7&lt;/sup&gt; A</td>
</tr>
<tr>
<td>Ethanol</td>
<td>(5.0 ± 1.0) × 10&lt;sup&gt;6&lt;/sup&gt; B</td>
<td>(3.4 ± 1.4) × 10&lt;sup&gt;6&lt;/sup&gt; BG</td>
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</table>

<sup>a</sup> The data are means ± standard deviations from three to five independent repeats. Cip, ciprofloxacin; Cef, cefotaxime; Tet, tetracycline. Means that were found to be significantly different (P < 0.05) by the Tukey test are indicated by different capital letters.

<sup>b</sup> The data represent the number of cells present in the biofilm after the first 24 h.

<sup>c</sup> The control was the corresponding amount of water.
were treated with the corresponding amount of the solvent (ethanol). For each 10 cells counted, 1.0 \pm 0.7 cell contained flagella when grown in the presence of Fur-5, while 9 \pm 1 cells contained flagella when grown in the absence of Fur-5 (P < 0.001). Swimming assays were performed using TSB 1/20 plates with 0.25% agarose at 16°C, conditions under which *Salmonella* swims very slowly. The plates were monitored for 5 days, and the surface of the swimming colonies was regularly measured. Figure 5 shows that swimming by *salmonellae* grown in the presence of 50 \mu M Fur-5 was clearly retarded compared to that of the appropriate control.

**Brominated furanones do not inhibit QS-controlled gene expression in Salmonella.** Halogenated furanones have been shown to inhibit AHL- and/or AI-2-mediated QS in several gram-negative species, such as *Chromobacterium violaceum* (44), *Erwinia carotovora* (42), *E. coli* (56, 58), *P. aeruginosa* (24, 25), *Serratia liquefaciens* (20, 54), *Vibrio fischeri* (20, 39, 41), and *Vibrio harveyi* (13, 58). It has therefore been generally accepted that halogenated furanones function in gram-negative bacteria by interference with QS. However, none of the known target genes of the two currently described QS systems in *Salmonella*, the AI-2 system and the AHL receptor SdiA, were activated or repressed in our microarray study. In addition, neither sdiA nor the genes that are necessary for the synthesis of AI-2, luxS and pfs, were differentially regulated in the presence of Fur-5 under the conditions tested.

To confirm these results, we studied the expression of the known target genes srgE and lsrA of SdiA and AI-2, respectively, in the presence and absence of Fur-5 or Fur-8. All experiments were performed in TSB 1/20 at 16°C as well as in LB at 37°C. The latter conditions were included as controls since the activities of the reporter systems used have been previously described for these standard conditions (Fig. 6) (14, 62) and because it has been reported that SdiA activation is specifically dependent on the presence of AHLs at this temperature (62). However, all reporter systems were also active in TSB 1/20 at 16°C and similar results were obtained under both conditions. None of the tested furanones inhibited the luminescence reporter systems pJNS25 (*P. pseudoflava*-luxCDABE), activated by 5 nM 3O-C\(_7\)-HSL or 3O-C\(_7\)-HTL, and pCMPG5638 (*P. rettgeri*-luxCDABE) at concentrations that did not inhibit their background activities in the isogenic mutants BA612 and CMPG5602, as exemplified by the AI-2 reporter pCMPG5638 in Fig. 6A. The same concentrations needed to inhibit the reporter systems also inhibited the activity of pCMPG5849 (*P. putida*-luxCDABE), which constitutively expresses luminescence, as exemplified by the SdiA reporter pJNS25 in Fig. 6B. Fur-5 and Fur-8 also did not show activity at non-growth-inhibiting concentrations on GFP-reporter systems for srgE and lsrA activity, as exemplified by the SdiA reporter pCMPG5836 in Fig. 6C and D. In addition, the effect of the furanones on biofilm formation could not be rescued by the simultaneous addition of 3O-C\(_7\)-HSL, 3O-C\(_7\)-HTL, or synthetic DPD (data not shown). Therefore, we have found no evidence that furanones act on the QS systems that are currently reported to be present in *Salmonella*.

**DISCUSSION**

Since the 1970s, microbiologists have realized that bacteria grow predominantly as biofilms in a large diversity of environments, rather than as free-living planktonic cells (22). Within these biofilms, the bacteria are better protected from external stress factors like antibacterial agents and the immune system of the host (9).

In this study, we have synthesized and screened a small focused library of brominated furanones for their activities against *Salmonella* biofilm formation. Since we envisaged the main application of compounds that inhibit biofilm formation in the environment outside the host, in order to limit the spread and the survival of this pathogen, we studied *Salmonella* biofilm formation under nutrient-poor conditions at 16°C. We focused on differences in the alkyl chain lengths of the furanones to investigate whether this feature is important for their activity in *Salmonella*. The following structure-activity relationship can be derived from the results depicted in Table 1. Furanones without an alkyl chain (Fur-1 to Fur-3) were the strongest biofilm inhibitors but were also more toxic for *Salmonella* than the alkylated furanones, which might be correlated with the higher water solubility of the nonalkylated compounds. Of the 3-alkylated furanones, only molecules with one bromine atom on the ring structure and one on the methyldiene side chain (Fur-5, Fur-6, and Fur-8) showed *Salmonella*
biofilm-inhibiting activities. No activity could be detected when a dibromomethylidene substituent was present (Fur-4 and Fur-7). Since the observed activities were limited to molecules with alkyl chains up to six carbon atoms long, Fur-9 to Fur-11 probably are too little water soluble to be biologically active in our experimental setup. To the best of our knowledge, this is probably are too little water soluble to be biologically active in our experimental setup. To the best of our knowledge, this is probably are too little water soluble to be biologically active in ourexperimental setup. To the best of our knowledge, this is probably are too little water soluble to be biologically active.

In addition, we explored the activities of the brominated furanones in combination with antibiotics. Table 2 shows that interesting effects were observed for the three antibiotics tested, since the combined treatment with Fur-8 and antibiotic resulted in a stronger decrease in the number of viable cells than would be expected. However, it should be remarked that the observed effects were different for the three antibiotics used. The effect of the addition of Fur-8 was most pronounced in combination with tetracycline and least pronounced in combination with cefotaxime. As expected, ciprofloxacin was the most potent antibiotic tested both with and without the furanone. It is, however, of interest to note that the tested antibiotics were unable to kill all biofilm cells at concentrations that were 100 to 1,000 times higher than the MICs for planktonic cells. Similar observations have been made previously by others using different experimental setups (49, 64) and are presumably caused by a combination of several different factors, one of which is the observation that a small subpopulation of the biofilm consists of dormant, nongrowing “persister” cells that are tolerant to antibiotics (9, 22, 36).

Next, we aimed at gaining insight into the mode of action of the brominated furanones on S. enterica serovar Typhimurium by studying the Salmonella genes that are differentially expressed in the presence of Fur-5 via microarray analysis. Since we hypothesize that the furanones prevent planktonic cells from forming a biofilm, this study focused on the gene expression of planktonic cells rather than biofilm cells. Similar gene expression analyses have previously been performed to study the effects of Fur-3 on P. aeruginosa (25) and the effects of Fur-6 on E. coli (56) and the gram-positive pathogen Bacillus subtilis (55). When comparing the differential gene expression profiles of the four species after treatment with furanones, it is clear that the contact with furanones is experienced as a stress factor, since some genes involved in drug sensitivity and stress response are upregulated in all species (e.g., marA in both Salmonella and E. coli). This suggests that the furanones affect the global stress response of the bacteria, but this results in growth retardation only in B. subtilis (brominated furanones are generally known as inhibitors of the growth of gram-positive bacteria) (33). Interestingly, Fur-5 affects the expression of several Salmonella genes that are involved in metabolism. Such genes can also be found among the genes that are differently regulated by furanones in the three other species. Most interestingly, Fur-5 inhibited the expression of several Salmonella genes that are involved in different stages of the flagellar bio-

**FIG. 6.** Effects of furanones on reporter systems for QS activity. (A) Bioluminescence expression by AI-2 reporter pCMPG5638 in the wild-type S. enterica serovar Typhimurium strain SL1344 (circles) and the isogenic luxS mutant strain CMPG5602 (triangles) in the presence of Fur-5 (closed symbols) or the corresponding amount of ethanol (open symbols) after 4 h of incubation at 37°C in LB medium. (B) Bioluminescence expression by the SdiA reporter in S. enterica serovar Typhimurium 14028/pJNS25 (triangles), in the presence of 5 nM 3O-C7-HSL, and the constitutive luminescence system in S. enterica serovar Typhimurium 14028/pCMPG5849 (circles), in the presence of Fur-5 (closed symbols) or the corresponding amount of ethanol (open symbols) after 6 h of incubation at 37°C in LB medium. (C and D) The GFP reporter system for SdiA activity, 14028/pCMPG5836, was grown in the presence of 20 nM 3O-C7-HSL and a concentration gradient of the furanone Fur-5 (C [diamonds]), Fur-8 (D [diamonds]), or ethanol (squares) for 6 h at 37°C in LB medium. Subsequently, the growth of the cells was determined as the OD600 (open symbols) and the fluorescence expression was measured as relative fluorescence units (RFU [closed symbols]). All data are from one experiment, representative of three independent repeats, and the error bars show standard deviations of two measurements.
synthesis (6). Similarly, it has been shown that furanones inhibit the expression of flagellar biosynthesis genes in *E. coli* (56), although no phenotypic analysis was performed. We focused on the flagellar biosynthesis to validate our microarray data. Therefore, we studied the number of flagella formed in the presence of the furanone and showed that almost no flagella were present after an incubation time of 4 h in the presence of Fur-5, while several flagella were formed per cell in the absence of the furanone (Fig. 4). These results were confirmed by swimming experiments which showed that Fur-5 retards swimming of *Salmonella* cells. Since it has been shown that the presence of functional flagella is of importance for the formation of a normal biofilm by *Salmonella* (67), it is possible that interference with the flagellar assembly causes the observed biofilm defect. This would imply that furanones have less influence on already established *Salmonella* biofilms, which has been confirmed in preliminary experiments (data not shown). However, it still remains to be determined whether the furanones have a specific target in *Salmonella*. The interference with the flagellar biosynthesis might be caused by an interaction of the furanones with such a specific target or by the more global metabolic effect that was observed. Further experiments to unravel the mode of action of the furanones are currently ongoing in our laboratories.

Since brominated furanones are generally considered to interfere with QS systems in gram-negative bacteria (15), it was surprising that none of the known target genes of the SdiA- and the AI-2-mediated QS systems of *Salmonella* were differentially regulated by Fur-5. Several experiments with gene fusion reporter systems to measure the activity of these QS systems corroborated this finding. All experiments were performed at both 16°C and 37°C, as it has been shown for SdiA that this system is selectively activated by AHLs at 37°C but not at lower temperatures (62). However, similar results were obtained under both conditions. Whereas in a number of bacterial species brominated furanones have been reported to exert their effects by interfering with QS systems, we have found no evidence of a link between the effects of the furanones on *Salmonella* biofilm formation and the QS systems that are so far identified in *Salmonella*. There are several possible explanations for our observations: (i) the furanones target another yet unknown *Salmonella* QS system, (ii) the target of the furanones is not part of a *Salmonella* QS system, or (iii) the observed inhibition of biofilm formation results from a combination of effects on several different targets.

In conclusion, we have shown that several brominated furanones have inhibitory effects on *Salmonella* biofilm formation. Additionally, pretreatment with furanones results in fewer biofilm cells surviving the treatment with several different antibiotics. In an effort to unravel the working mechanism of the furanones, we have determined the differential gene expression of *Salmonella* in the presence of a furanone. This analysis led to the finding that the furanones interfere with flagellar biosynthesis. Since our data suggest that the brominated furanones do not inhibit *Salmonella* biofilm formation by interference with the two putative QS systems of *S. enterica* serovar Typhimurium, we are currently investigating the specific targets of the furanones.

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FURANONES INHIBIT SALMONELLA BIOFILM FORMATION