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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-(bromomethylene)-3-alkyl-2(*5H*)-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-(bromomethylene)-3-ethyl-2(*5H*)-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., cefotaxime), 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

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of important biological functions, including those mentioned above.

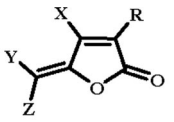
The pathogen of interest in this study, *Salmonella* serovar Typhimurium, has been shown to contain two putative QS systems. First, *Salmonella* encodes a LuxR-type AHL receptor, SdiA (*s*uppressor of cell *d*ivision *i*nhibition A), which has been shown to respond to a broad range of AHLs and AHL analogues (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* (s*diA-r*egulated *g*ene *E*) and the *rck* (*r*esistance to *c*omplement *k*illing) 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 (*L*ux*S* *r*egulated) and reinternalized by the bacteria via the Lsr ABC transporter (46, 65, 66). Inside the cell, AI-2 activates the transcription of the *lsrACDBFGE* 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 CMPG5602 (14). The plasmids used were pJNS25 (P*srgE-luxCDABE*; Tc^r) (62), pFPV25.1 (P*rpsM-gfp*mut3; Ap^r) (70), pCMPG5623 ((P*lsrA-gfp*mut3; Ap^r) (S. De Keersmaecker, unpublished observations), pCMPG5638 (P*lsrA-luxCDABE*; Km^r) (14), pCMPG5836 (P*srgE-gfp*mut3; Ap^r) (this study), and pCMPG5849 (P*rpsM-luxCDABE*; Tc^r) (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 cefotaxime were purchased from Fluka and Applichem, respectively, and used at concentrations specified in the text.



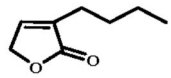
Compound	R	X	Y	Z
Fur-1	H	H	H	Br
Fur-2	H	H	Br	Br
Fur-3	H	Br	H	Br
Fur-4	C_2H_5	H	Br	Br
Fur-5	C_2H_5	Br	H	Br
Fur-6	C_4H_9	Br	H	Br
Fur-7	C_4H_9	Br	Br	Br
Fur-8	C_6H_{13}	Br	H	Br
Fur-9	C_8H_{17}	Br	H	Br
Fur-10	$C_{10}H_{21}$	Br	H	Br
Fur-11	$C_{12}H_{25}$	Br	H	Br
Fur-12				

FIG. 1. Chemical structures of the synthesized compounds.

Synthesis of chemical compounds. Furanones Fur-1, Fur-2, and Fur-4 to Fur-12 were synthesized as previously described (43) (Fig. 1). Furanone 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 C_{18} 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 (^{1}H) and 75.5 MHz (^{13}C). 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 5973 network 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-C_7-HSL [*N*-(3-oxoheptanoyl)-DL-homoserine lactone] and 3O-C_7-HTL [*N*-(3-oxoheptanoyl)-DL-homocysteine thiolactone] 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 pCMPG5836, the *srgE* promoter was cut from pJNS25 and cloned as an EcoRI fragment into the EcoRI site of pFPV25 upstream of *gfp*mut3. The orientation of the promoter was determined by using NcoI. Dose-response experiments with 14028/pCMPG5836 and BA612/pCMPG5836 confirmed that AHLs activate the expression of *srgE* in an SdiA-dependent fashion and that 3O-C_7-HTL activates SdiA at lower concentrations than 3O-C_7-HSL, as previously reported (29). To construct pCMPG5849, 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, 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 Breathseal 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 prepared 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 decreases the OD_{600} of the planktonic cells by more than 30%. For quantification 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]). 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 air dried (30 min), the dye bound to the adherent cells was extracted with 30% glacial acetic acid. The OD_{570} of 135 μl of each well was measured using the VERSAmax. Typically, the OD_{570} 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 cell numbers, the following method was used. An overnight culture was diluted 1:100 into 5 ml TSB 1/20 (ca. 5.10^7 cells), and 60 μM 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 Gyrotory shaker (Unimax 1010; Heidolph) at 50 rpm. After 24 h, the biofilm that had been formed on the bottom of the petri 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 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.

Epifluorescence microscopy. Biofilms of the *Salmonella* strain 14028/pFPV25.1 (70), 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 (×40 magnification/0.75 numerical aperture) objective. Pictures and Z-stacks were recorded using an AxioCam MRm and the AxioVision software.

Transcriptome microarray analysis. An overnight culture of *Salmonella* serovar Typhimurium 14028 was diluted 1:50 in 1 liter of TSB 1/20 broth (ca. 2.10^{10} cells) and incubated with shaking (200 rpm) at 16°C. The culture was split into two at an OD_{600} of 0.1 (early exponential growth in TSB 1/20), and 50 μM Fur-5 was added to one of the cultures, while the corresponding amount of ethanol was added to the other. Samples for RNA isolation were retrieved at an OD of 0.12, subsequently immediately transferred to 0.2 volume of an ethanol-phenol solution (95:5 [vol/vol]), and finally stored at −80°C. Total RNA was isolated with a Qiagen RNeasy minikit according to the manufacturer's protocol. Contaminating genomic DNA was removed from the RNA samples with Turbo DNA-free (Ambion). Removal of DNA was checked by PCR. Prior to labeling, the concentration of total RNA was determined by measuring the A_{260} with a NanoDrop spectrophotometer (ND-1000). RNA was labeled with Cy5 and Cy3 by reverse transcription (71). Hybridizations were performed in color flip on *S. enterica* serovar Typhimurium arrays containing 70-mer oligonucleotides representing all *S. enterica* serovar Typhimurium LT2 annotated genes (Operon) spotted in duplicate on CodeLink activated slides (Amersham Biosciences), as previously reported (68). Data were Loess normalized with the LIMMA BioConductor package; no background correction was performed. Differentially expressed genes were detected by significance analysis of microarrays (SAM) (69) by means of the BioConductor siggenes package. The false discovery rate was set at 0.083. A d statistic for each gene representing a measure of differential expression (d_i) was calculated as $r_i/(s_i + s_0)$, where i is 1, 2, . . . , p genes. The parameter s_i represents the standard deviation, s_0 represents a fixed factor, and r_i represents a score which is the mean of the log ratios in the one-class case we applied.

Staining of flagella. An overnight culture of *S. enterica* serovar Typhimurium 14028 was diluted in 200 μl of TSB 1/20 (ca. 2.10^6 cells) in a microtiter plate, in the presence or absence of 50 μM Fur-5 or the corresponding amount of ethanol. The plate was covered with a breathable sealing membrane and incubated during 4 h at 16°C with shaking (200 rpm). Subsequently, the flagella were stained according to Kearns and Losick (31). The stain was prepared by mixing 10 parts mordant solution (2 g tannic acid, 10 ml 5% aqueous phenol, 10 ml saturated aqueous $AlKO_8S_2 \cdot 12\ H_2O$) with 1 part stain (12% crystal violet in ethanol). Three microliters of a sample was applied to a microscope slide and covered with a 22- by 40-mm coverslip. The slide was propped vertically, and 10 μl of dye was applied to the top edge of the coverslip to stain the sample by capillary action. Samples were observed by phase-contrast microscopy using a Zeiss Axio Imager Z1 microscope with an EC Plan-Neofluar (×100 magnification/1.3 numerical aperture) 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. 3.10^6 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 [Cliniplatel Thermo Life Sciences] for luminescence reporters) in 100 μl liquid LB broth per well in the presence of 10 nM (reporter system pJNS25) or 40 nM (reporter system pCMPG5836) 3O-C_7-HSL. Subsequently, an overnight culture of *S. enterica* serovar Typhimurium 14028/pJNS25, BA612/pJNS25, 14028/pC MPG5849, 14028/pCMPG5836, BA612/pCMPG5836, or 14028/pFPV25.1 was diluted 1:50 into LB broth and 100 μl (ca. 2. 10^6 cells) was added to each well, resulting in a total amount of 200 μl medium per well and 5 nM or 20 nM 3O-C_7-HSL. The microtiter plates were covered with breathable sealing membranes and incubated with aeration for 6 h at 37°C or 16°C (experiments at 16°C were performed in TSB 1/20 instead of LB). Following this incubation period, the luminescence or the fluorescence and the OD_{600} were measured using a charge-coupled device camera (Berthold Night Owl; PerkinElmer Life Science), a Fluoroskan Ascent fluorimeter (Thermo Life Sciences), and the VERSAmax, respectively.

To study the influence of furanones on AI-2-mediated gene expression with the reporters SL1344/pCMPG5638, CMPG5602/pCMPG5638, SL1344/pCMPG5849, SL1344/pCMPG5623, CMPG5602/pCMPG5623, and SL1344/pFPV25.1, similar experiments were conducted in the absence of 3O-C_7-HSL and the incubation period was 4 h instead of 6 h (14).

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 activ-

TABLE 1. MICs and GRCs of the compounds for *S. enterica* serovar Typhimurium 14028 and IC_{50}s on biofilm formation[a]

Compound	MIC (μM)[b]	GRC (μM)[c]	IC_{50} (μM)[d]
Fur-1	500	30	15 ± 5
Fur-2	500	40	15 ± 4
Fur-3	500	40	10 ± 3
Fur-4	−	−	−
Fur-5	−	200	50 ± 5
Fur-6	−	−	100 ± 10
Fur-7	−	−	−
Fur-8	−	150	60 ± 15
Fur-9	−	−	−
Fur-10	−	−	−
Fur-11	−	−	−
Fur-12	−	−	−

[a] The data are means ± standard deviations from three independent experiments using concentration gradients. −, no activity was observed under the conditions used at a maximal concentration of 1 mM.

[b] The MIC is the concentration needed to inhibit growth after 24 h in IsoSensitest broth.

[c] The GRC is the concentration needed to decrease the OD_{600} by more than 30% compared to that of a negative control after 24 h of incubation in TSB 1/20. Values are representative of three independent repeats.

[d] Concentration decreasing the amount of biofilm formed by 50% compared to that of an untreated control.

ity 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}s). 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}s 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}s of 50, 100, and 60 μM, respectively) on

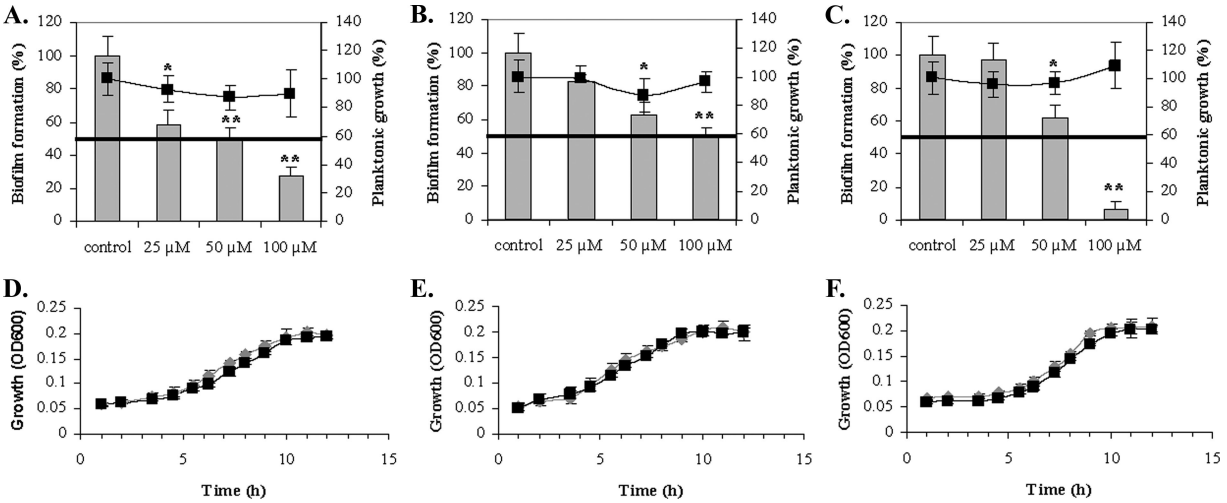


FIG. 2. Inhibition of *Salmonella* biofilm formation by brominated furanones. (A to C) Biofilms were formed on polystyrene pegs in the presence of different concentrations of Fur-5 (A), Fur-6 (B), and Fur-8 (C) for 48 h at 16°C. The biofilms were stained with crystal violet, and the amount of stain was 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 (*, $P < 0.01$; **, $P < 0.001$).

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

Treatment	No. of viable cells (CFU/plate)[a]:				
	At 24 h[b]	With additional 24 h of treatment			
		Control[c]	100 μg/ml Cip	100 μg/ml Cef	100 μg/ml Tet
Fur-8	$(1.6 \pm 0.5) \times 10^7$ A	$(1.1 \pm 0.5) \times 10^7$ A	$(3.0 \pm 0.8) \times 10^2$ C	$(3.9 \pm 2.9) \times 10^4$ D	$(1.3 \pm 0.4) \times 10^5$ F
Ethanol	$(5.0 \pm 1.0) \times 10^8$ B	$(3.4 \pm 1.4) \times 10^8$ BG	$(7.6 \pm 4.0) \times 10^4$ DF	$(1.9 \pm 0.6) \times 10^6$ E	$(2.8 \pm 0.2) \times 10^8$ G

[a] 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.
[b] The data represent the number of cells present in the biofilm after the first 24 h.
[c] The control was the corresponding amount of water.

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.

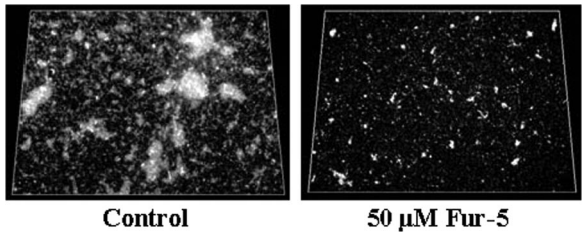


FIG. 3. Influence of 50 μM Fur-5 on biofilm formation by *S. enterica* serovar Typhimurium 14028/pFPV25.1. Biofilms were grown on the bottom of small petri dishes for 24 h at 16°C in the presence of 50 μM Fur-5 or the corresponding amount of ethanol (control) and subsequently visualized using epifluorescence microscopy. A ×40 objective was used. The pictures are representative of the biofilms observed in three independent experiments.

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, 130 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.biw.kuleuven.be/dtp/cmpg/janssens.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., *sseAD* and *ssaEGH*), genes for lipopolysaccharide biosynthesis (e.g., *rfaL* and *rfbKNP*), and several motility genes. Interestingly, most of these motility genes are involved in different aspects of flagellar biosynthesis (*fliK*, *fliM*, *fliO*, 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

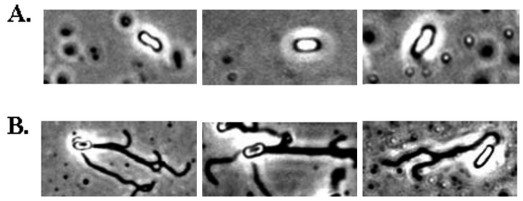


FIG. 4. Fur-5 inhibits flagellar biosynthesis by *S. enterica* serovar Typhimurium. *S. enterica* serovar Typhimurium 14028 was grown for 4 h in TSB 1/20 at 16°C in the presence of 50 μM Fur-5 (A) or the corresponding amount of ethanol (B). Subsequently, the flagella were stained as described in Materials and Methods and observed using phase-contrast microscopy. Flagellum orientation as well as the black spots in the pictures may be an artifact of staining. The pictures are representative of three independent repeats.

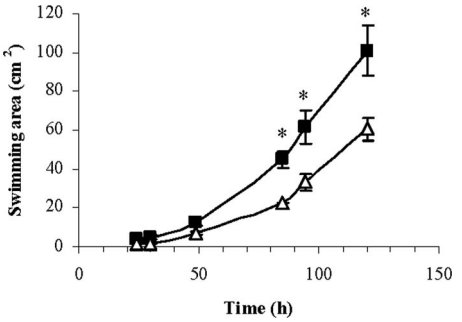


FIG. 5. Fur-5 retards swimming by *S. enterica* serovar Typhimurium. TSB 1/20 swimming plates (0.25% agar) containing 50 μM Fur-5 (open triangles) or the corresponding amount of solvent (ethanol [closed squares]) were inoculated with *S. enterica* serovar Typhimurium 14028 and incubated for 120 h at 16°C. The surface of the swimming colony was measured at six different time points. Means of the swimming area that were found to be significantly different from that of the control by the Tukey test ($P < 0.01$) are indicated by asterisks.

were treated with the corresponding amount of the solvent (ethanol). For each 10 cells counted, 1.0 ± 0.7 cell contained flagella when grown in the presence of Fur-5, while 9 ± 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 μM 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*srgE-luxCDABE*), activated by 5 nM 3O-C_7-HSL or 3O-C_7-HTL, and pCMPG5638 (P*lsrA-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*rpsM-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 methylidene side chain (Fur-5, Fur-6, and Fur-8) showed *Salmonella*

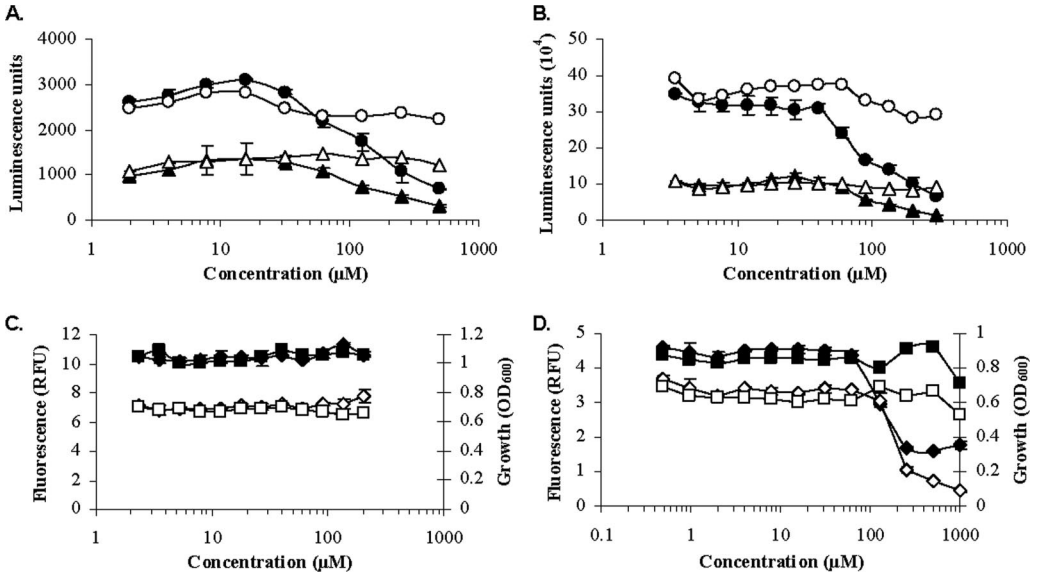


FIG. 6. Effects of furanones on reporter systems for QS activity. (A) Bioluminescence expression by AI-2 reporter pCMPG5638 in the wild-type *S. enterica* serovar Typhimurium strain SL1344 (circles) and the isogenic *luxS* mutant strain CMPG5602 (triangles) in the presence of Fur-5 (closed symbols) or 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-C_7-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-C_7-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 OD_{600} (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.

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 the first report on brominated furanones inhibiting *Salmonella* biofilm formation. Given the higher toxicity of the nonalkylated furanones (Fur-1 to Fur-3), we conclude that the furanones Fur-5, Fur-6, and Fur-8 are the most interesting compounds among those tested. Interestingly, the nonbrominated furanone Fur-12 did not show any activity under the conditions used, stressing the importance of bromination of the furanones to be active as *Salmonella* biofilm inhibitors.

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 many *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-

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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