Inhibition of *Bacillus anthracis* Growth and Virulence-Gene Expression by Inhibitors of Quorum-Sensing

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Density-dependent gene expression, quorum sensing (QS), involves the synthesis and detection of low-molecular-weight molecules known as autoinducers. Inhibitors of bacterial QS systems offer potential treatment of infections with highly virulent or multidrug-resistant agents. We studied the effects on *Bacillus anthracis* growth and the virulence gene (*pagA, lef,* and *cya*) expression of the QS inhibitor *(5Z)*-4-bromo-5-(bromomethylene)-3-butyl-2*(5H)*-furanone, which is naturally synthesized by the marine alga *Delisea pulchra,* as well as a related compound and synthetic derivatives. Growth of *B. anthracis* Sterne strain was substantially reduced in the presence of each furanone in a dose-dependent manner. When furanones were added to midlog-phase cultures of *B. anthracis* strains with LacZ reporters in *pagA, lef,* or *cya,* growth was inhibited, and expression of these virulence genes was inhibited to a proportionately greater extent. These data suggest that use of QS inhibitors could represent novel therapies for anthrax.

The discovery of antibiotics was the single most important advancement in the treatment of patients with bacterial infection [1]. However, antibiotics gave physicians a false sense of security, leading to both their overprescription and the development of antibiotic resistance. The emergence of multidrug-resistant bacteria and the use of pathogenic bacteria as weapons of terror have made critical the development of new effective antibacterial therapies [2].

Bacteria regulate their gene expression, at least in part, by using a population-monitoring system known as quorum sensing (QS) that involves the synthesis and detection of autoinducer signal molecules. Such QS systems permit populations of pathogenic bacteria to coordinate their actions for colonization and persistence in a host [3–9]. QS regulation of virulence gene expression is critical for clinically important bacteria, such as *Pseudomonas aeruginosa* in patients with cystic fibrosis [8]. QS systems are also involved in the regulation of genes for toxins, such as cholera toxin in *Vibrio cholerae,* and other virulence genes involved in biofilm formation and swarming [9]. Two major classes of autoinducers are known: gram-negative bacteria synthesize acylated homoserine lactones [10–12], and gram-positive bacteria synthesize short peptide chains [10, 13–15]. However, the discovery of a QS molecule, AI-2 [16], which has been identified in both gram-positive and gram-negative bacteria, suggests deep conservation and/or cross-species communication [17].

Bacillus anthracis, a gram-positive, rod-shaped bacterium that is the etiological agent of anthrax, is transmitted by its highly resistant spores. Inhalation anthrax is a severe disease, with a fatality rate >40%, despite antibiotic therapy [18]. With the emergence of *B. an-*

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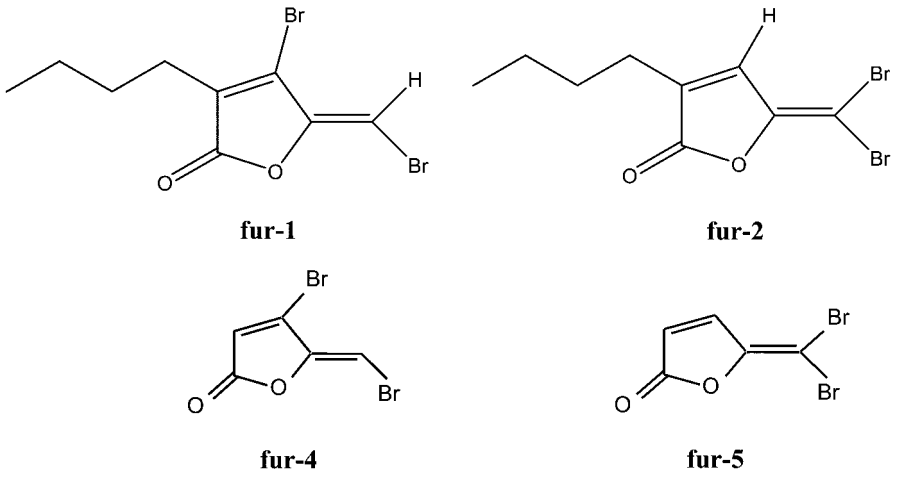

Figure 1. Structures of the 4 studied halogenated furanones (fur)

thracis spores as a weapon of terror, the development of new therapies is essential [2]. *B. anthracis* possesses genes involved in AI-2–mediated QS, and a Sterne strain derivative that is defective in AI-2 synthesis has a significant growth defect, compared with wild-type Sterne [19]. These observations suggest that the development of inhibitors to AI-2 could represent a potential new means of treating anthrax.

The red marine alga *Delisea pulchra* synthesizes halogenated furanones that are potent inhibitors of bacterial QS systems [20–27]. One of these furanones, *(5Z)*-4-bromo-5-(bromomethylene)-3-butyl-2*(5H)*-furanone (fur-1), has been reported to inhibit AI-2–mediated QS systems in *Escherichia coli* and *Vibrio harveyi* without affecting bacterial growth [22]. In addition, a recent study that used DNA microarrays indicated that 79% of *E. coli* genes repressed by fur-1 were also induced by AI-2 [26]. The attenuation of QS systems potentially offers a new means of treating bacterial infection [25–34]. In the present article, we demonstrate the effect of the naturally occurring *D. pulchra* furanones, as well as synthesized derivatives, on *B. anthracis* growth and gene expression. Our analysis revealed that growth of the Sterne strain of *B. anthracis* was substantially reduced and that its virulence genes—*pagA, lef,* and *cya*—were

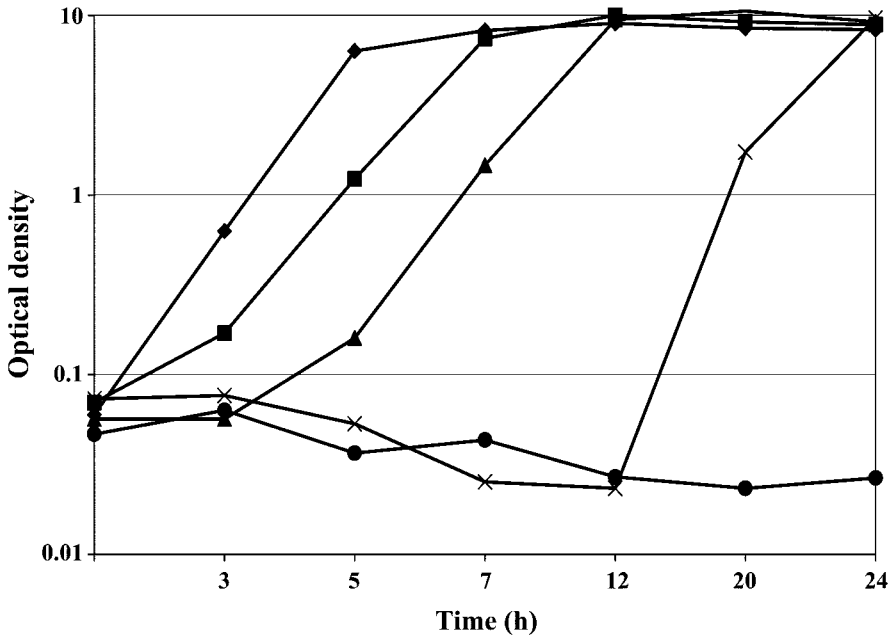

Figure 2. Effect of *(5Z)*-4-bromo-5-(bromomethylene)-3-butyl-2*(5H)*-furanone (fur-1) on *Bacillus anthracis* growth. *B. anthracis* Sterne strain $34F_2$ was incubated with 0 *(diamonds),* 5 *(squares),* 10 *(triangles),* 20 *(crosses),* or 40 *(circles)* μg/mL of fur-1 in diluent. Bacterial growth is shown as a function of optical-density measurements at 600 nm. The experiment shown is representative of 3 separate trials performed on different days.

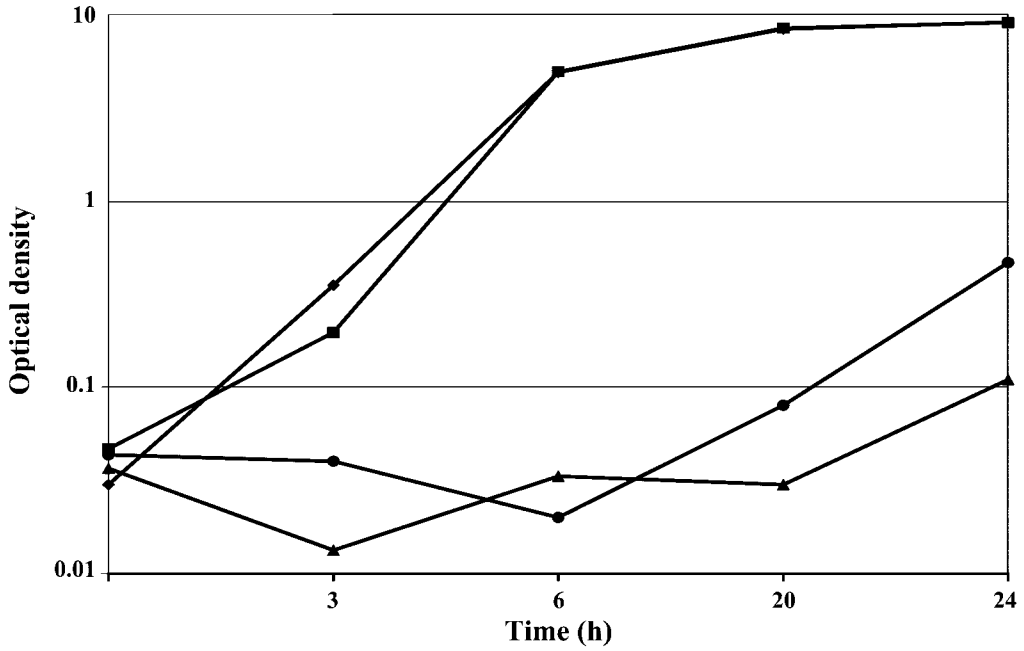

Figure 3. Effect of *(5Z)*-4-bromo-5-(bromomethylene)-3-butyl-2*(5H)*-furanone (fur-1) preincubation on growth of *Bacillus anthracis* Sterne strain $34F_2$. 0-pre, cells without preincubation with fur-1; 20-pre, cells preincubated with 20 μg/mL of fur-1 for 24 h. Survivors were then reincubated: 0-post, cells incubated without fur-1; 20-post, cells incubated with 20 μg/mL of fur-1. Cell incubation conditions were 0-pre + 0-post *(squares);* 0-pre + 20-post *(diamonds);* 20-pre + 0-post *(triangles);* and 20-pre + 20-post *(circles).* Bacterial growth is shown as a function of optical-density measurements at 600 nm. The experiment shown is representative of 3 separate trials performed on different days.

disproportionately inhibited in the presence of both the natural furanones and their derivatives. These observations suggest potential new therapeutic agents for the treatment of anthrax.

MATERIALS AND METHODS

B. anthracis *growth.* The *B. anthracis* Sterne strain $34F_2$ and strains 7702, RBAF140, RBAF143, and RBAF144 were routinely cultured in brain-heart infusion (BHI) broth at 37°C with aeration. Overnight cultures were used to inoculate 50 mL of fresh BHI medium to $OD_{600} \approx 0.03–0.05$. Before inoculation, the fresh BHI medium was supplemented with various concentrations of fur-1 dissolved in 95% ethanol, such that the final concentration of fur-1 in the medium was 5–40 μg/mL and the final ethanol concentration was 0.12%. Cells were also cocultured with 3-butyl-5-(dibromomethylene)-2-*(5H)*-furanone (fur-2), 4-bromo-5-(bromomethylene)-2*(5H)*-furanone (fur-4), and 5-(dibromomethylene)-2*(5H)*-furanone (fur-5) at similar concentrations, but methanol was used as the diluent (0.12% final concentration). Cells cultured without furanones (i.e., with only ethanol or methanol diluent) served as negative controls. Growth kinetic assays and inhibition by the furanones were conducted for 24 or 25 h. The expression of β-galactosidase (β-gal) was monitored over a 5-h period.

Furanone synthesis. Fur-1 (figure 1), originally extracted from *D. pulchra* [32, 33], was synthesized as described elsewhere [34]. This synthesis pathway also generated fur-2 as a by-product. Fur-2, a white solid, was purified by use of column chromatography (hexanes:ethyl acetate, 100:1). Its structure was confirmed with ^{1}H–nuclear magnetic resonance (NMR; 0.92 triple peaks, 1.37 multiple peaks, 1.57 multiple peaks, 2.32 triple peaks, and 7.27 signal peak) and gas chromatograph–mass spectroscopy (GC-MS; molecular weight, 310 g/mol) by comparison with known values [35]. Fur-4 and -5 were synthesized by use of a protocol described elsewhere [35]. The structures of fur-4 and -5 were confirmed with ^{1}H-NMR (6.42 single peak and 6.50 single peak for fur-4; 6.40 double peaks and 7.67 double peaks for fur-5) and GC-MS (molecular weight, 254 g/mol for both) by comparison with known values [35].

The synthesized fur-1 was dissolved in 95% ethanol to 14.9 mg/mL. Fur-2, -4, and -5 were dissolved in methanol to 10 (for fur-2) or 20 (for fur-4 and -5) mg/mL. The furanone stock solutions were stored at 4°C before experiments. Furanones were dissolved in methanol or ethanol on the basis of their optimal solubility at low diluent concentrations.

β-Gal assays. Aliquots (1 mL) collected from cultures of RBAF140, RBAF143, and RBAF144 cells were centrifuged at 8000 *g*, and pellets were snap frozen in a bath of ethanol and dry ice. Cell pellets were resuspended in 500 μL of enriched Z-buffer (Z-buffer plus freshly added 0.00027% β-mercaptoethanol) [36]. After 10 μL of toluene was added, samples were

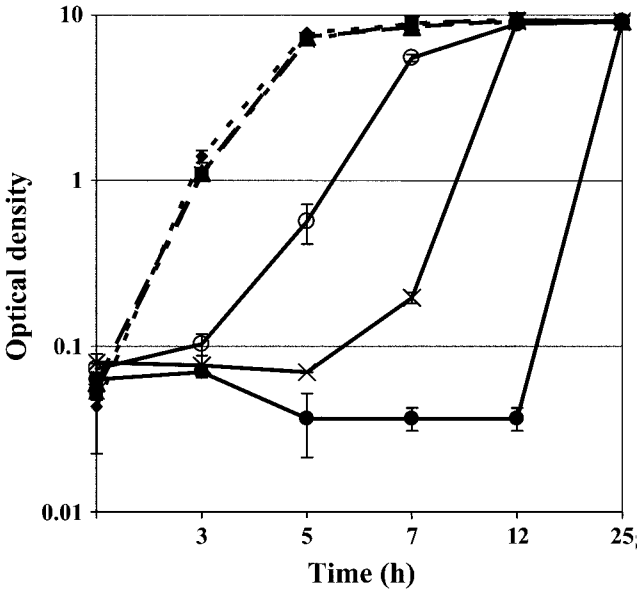

Figure 4. Effect of adding furanone derivatives to resting *Bacillus anthracis* cultures. Lines represent cells grown for 24 h in medium alone *(diamonds)* or in medium plus ethanol diluent *(squares);* methanol diluent *(triangles);* and *(5Z)*-4-bromo-5-(bromomethylene)-3-butyl-2*(5H)*-furanone(fur-1; *crosses*), 3-butyl-5-(dibromomethylene)-2-*(5H)*-furanone (fur-2; *white circles*), or 4-bromo-5-(bromomethylene)-2*(5H)*-furanone (fur-4; *black circles*). Bacterial growth is shown as a function of optical-density measurements at 600 nm. The experiment shown is representative of 3 separate trials performed on different days.

mixed for 1 min and then kept on ice for at least 1 h. Fresh enriched Z-buffer (800 μL) was aliquoted into small tubes and incubated for 15 min with 200 μL of the cell suspension; then, 200 μL of 2-nitrophenyl–β-*D*-galactopyranoside (4 mg/mL in H_2O) was added to each sample. When the sample turned yellow, the reaction was stopped with 1 mol/L Na_2CO_3, and the exposure time was recorded. Measurements at optical densities of 420 nm (β-gal absorbance) and 550 nm were taken. Miller units were calculated to compare the β-gal activity under each treatment condition, as described elsewhere [36]. All experiments were done in triplicate.

RESULTS

Inhibition of* B. anthracis *growth by fur-1. To determine the effect of fur-1 (figure 1) on *B. anthracis* growth, cultures were added to flasks that contained sterile BHI supplemented with increasing concentrations of fur-1, and the cell density was monitored for 24 h. *B. anthracis* cell growth was inhibited in a concentration-dependent manner (figure 2). Cells exposed to 40 μg/mL of fur-1 never recovered during the trial period. To determine whether the recovery from treatment at lower doses was due to acquired resistance to the inhibitor, cultures of cells that grew despite treatment with 20 μg/mL of fur-1 were reexposed to 20 μg/mL of fur-1 (figure 3). As controls, cells that had never been exposed to fur-1 were grown in medium with or without fur-1. For cells grown in the presence of 20 μg/mL of fur-1, there was an amount of growth inhibition parallel to that seen in the first experiments. These data indicate that the recovery from fur-1 treatment observed at doses $\leqslant$20 μg/mL did not lead to resistant cells.

Effect of furanone derivatives on* B. anthracis *growth. To examine the effect of other related furanones, *B. anthracis* cells grown overnight were cultured in the presence of 10 μg/mL of fur-1 (control), -2, or -4. Cells treated with fur-1 had inhibition of cell growth for ~5 h, whereas the growth inhibition due to fur-2 and -4 exposure occurred over the course of 3 and 12 h, respectively (figure 4). As expected, cells grown in either the ethanol or methanol diluent were not inhibited.

To determine whether fur-4 treatment could inhibit the growth of a log-phase *B. anthracis* culture, overnight cultures were diluted and grown for 3 h in sterile BHI, then exposed

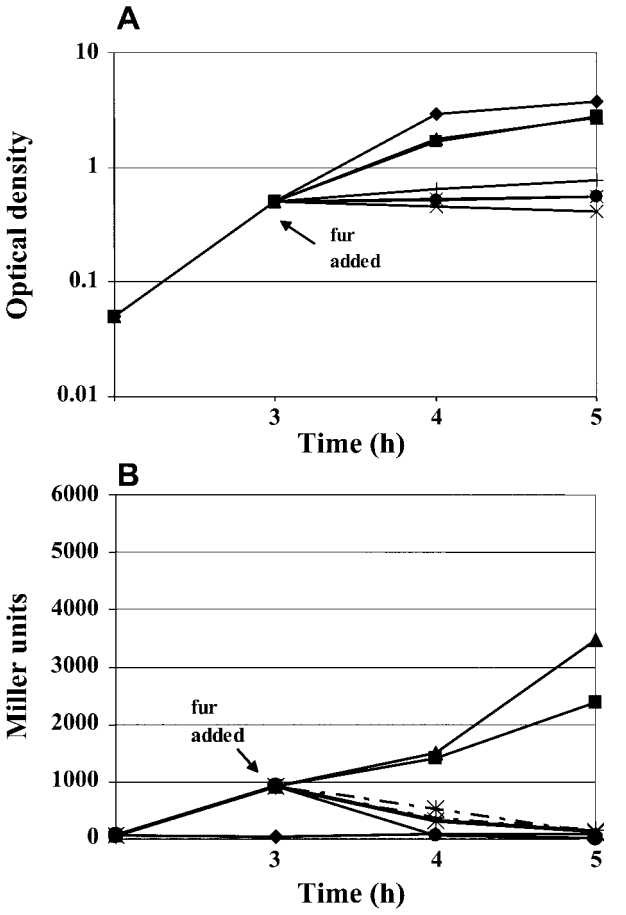

Figure 5. Effect of 4-bromo-5-(bromomethylene)-2*(5H)*-furanone (fur-4) on *Bacillus anthracis* growth during the midlog phase. Lines represent the addition of 0 *(diamonds),* 10 *(squares),* 20 *(triangles),* and 40 *(crosses)* μg/mL of fur-4. Bacterial growth is shown as a function of optical-density measurements at 600 nm. The experiment shown is representative of 2 separate trials performed on different days.

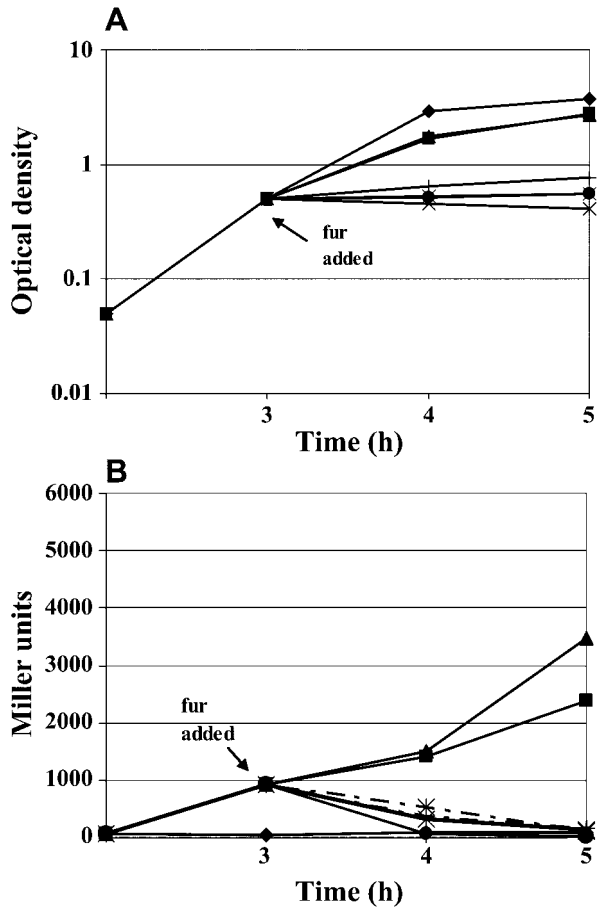

Figure 6. Effects of adding *(5Z)*-4-bromo-5-(bromomethylene)-3-butyl-2*(5H)*-furanone (fur-1) and derivatives at a concentration of 40 μg/mL on cell growth and *pagA-lacZ* expression of *Bacillus anthracis* strain RBAF140 cells in the log phase. *A,* Cells grown in R-medium [37] without bicarbonate *(diamonds)* or with 0.8% sodium bicarbonate *(squares),* diluent *(triangles),* fur-1 *(crosses),* 3-butyl-5-(dibromomethylene)-2-*(5H)*-furanone (fur-2; *asterisks*), 4-bromo-5-(bromomethylene)-2*(5H)*-furanone (fur-4; *circles*), or 5-(dibromomethylene)-2*(5H)*-furanone (fur-5; *plus signs*). *B,* Expression of the *pagA-lacZ* reporter in cells grown in the same medium as described above. The experiment shown is representative of 3 separate trials performed on different days.

to 0, 10, 20, or 40 μg/mL of fur-4 or to control medium. *B. anthracis* cell growth was inhibited with ⩾10 μg/mL of fur-4 (figure 5). These data show that fur-4 treatment can inhibit a midlog-phase culture of *B. anthracis* cells.

Inhibition of the expression of virulence genes by furanone treatment of* B. anthracis *strains. To determine whether furanone treatment could inhibit *B. anthracis* toxin expression, we studied Sterne strain derivatives with transcriptional *lacZ* fusions (RBAF140 *[pagA-lacZ]*, RBAF143 *[lef-lacZ]*, and RBAF144 *[cya-lacZ]*) [38]. As controls, cells were exposed to diluent alone (cells incubated with ethanol or methanol) and without bicarbonate, because bicarbonate is required for the activation of toxin expression. Furanones were added 3 h after inoculation, during the midlog growth phase, when the transcriptional activation of the *B. anthracis* toxin components occurs [38]. As expected, furanone treatments inhibited log-phase growth and significantly and disproportionately reduced β-gal activity in strain RBAF140 (figure 6). Treatment with fur-2 and -5 inhibited the growth of *B. anthracis* strains RBAF143 and RBAF144 in a dose-dependent manner and significantly and disproportionately reduced β-gal activity in these strains (figures 7–9). Thus, the furanone treatments substantially reduced the expression of *pagA, lef,* and *cya.*

DISCUSSION

In the present study, we examined the effect of the natural algal QS inhibitors, fur-1 and -2, and their synthetic derivatives, fur-4 and -5, on *B. anthracis* growth and virulence gene expression. Halogenated furanones are specific inhibitors of QS in sever-

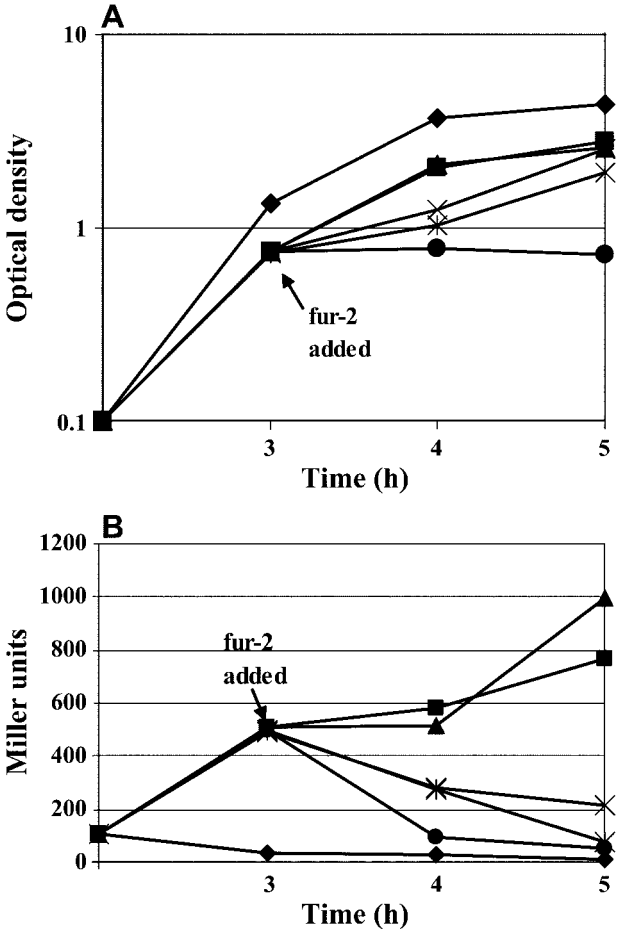

Figure 7. Effect of 3-butyl-5-(dibromomethylene)-2-*(5H)*-furanone (fur-2) on *lef-lacZ* expression in *Bacillus anthracis* strain RBAF143 in the log phase. Expression of the *lef-lacZ* reporter in cells grown in R-medium [37] without bicarbonate *(diamonds)* or with 0.8% sodium bicarbonate *(squares),* diluent alone *(triangles),* or fur-2 at concentrations of 10 *(crosses),* 20 *(asterisks),* or 40 *(circles)* μg/mL. The experiment shown is representative of 3 separate trials performed on different days.

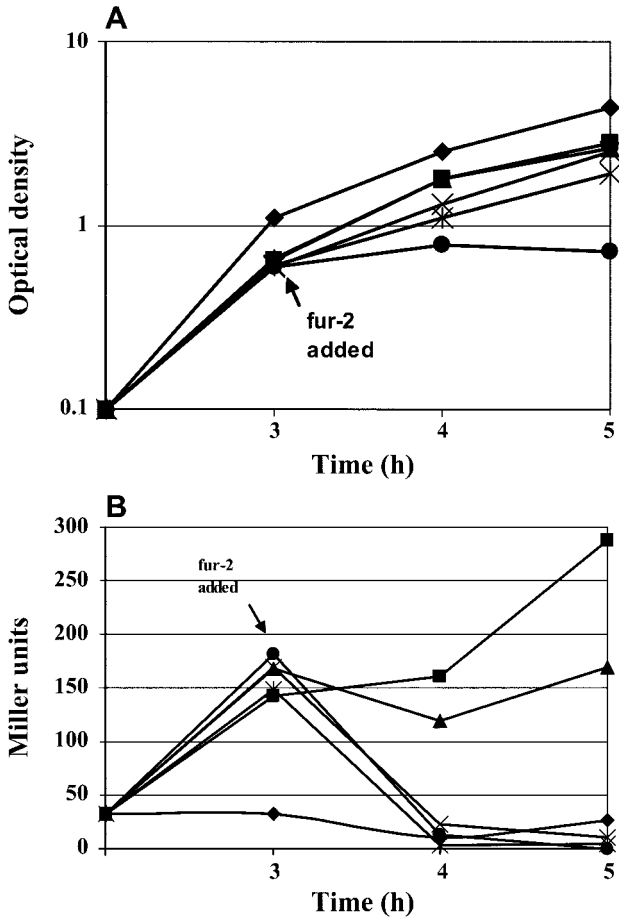

Figure 8. Effect of 3-butyl-5-(dibromomethylene)-2-*(5H)*-furanone (fur-2) on expression in *Bacillus anthracis* strain RBAF144 in the log phase. Expression of the *cya-lacZ* reporter was determined in *B. anthracis* strain RBAF144 grown in R-medium [37] without bicarbonate *(diamonds)* or with 0.8% sodium bicarbonate *(squares),* diluent alone *(triangles),* or fur-2 at concentrations of 10 *(crosses),* 20 *(asterisks),* or 40 *(circles)* μg/mL. The experiment shown is representative of 3 separate trials performed on different days.

al organisms [20, 22–27], and *B. anthracis* is subject to AI-2–dependent QS [19]. As has been seen in *Bacillus subtilis* [33], fur-1 inhibits the growth of low-density cultures of *B. anthracis* cells in a dose-dependent manner (figures 2 and 4) and that of log-phase cultures (figure 5). *B. anthracis* cells that recovered from treatment with fur-1 had no growth advantage when they were reexposed to furanone, compared with cells without such exposure (figure 3). These data suggest that *B. anthracis* cells that recover from furanone treatment have not been selected for resistance but that their recovery in the experimental conditions reflects furanone instability and/or metabolism. The resulting recovery from fur-1 treatment is more likely attributable to the latter, given that the furanones were synthesized under conditions that included exposure to sulfuric acid and heat [34]. Analysis of fur-1 by thin-layer chromatography 1 year after being synthesized revealed no additional spots, which indicates its stability under storage conditions (D.R. and T.K.W., unpublished data).

The regulation of virulence gene expression by QS has been characterized in several pathogenic bacteria [3–9, 15]. *B. anthracis* maximally synthesizes its virulence toxins during the transition from the late-log phase to the stationary phase of growth [38–40]. In light of evidence that the furanone treatments inhibit *B. anthracis* growth, even in high-density log-phase cultures, we focused on examining the effects of fura-

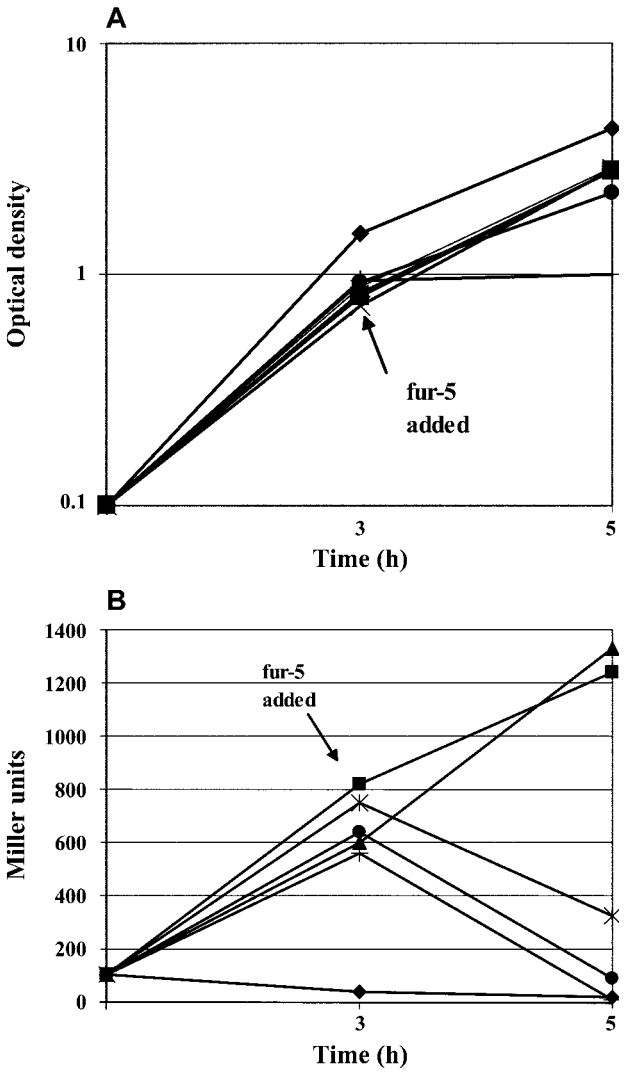

Figure 9. Effect of 5-(dibromomethylene)-2*(5H)*-furanone (fur-5) on *lef-lacZ* expression in *Bacillus anthracis* strain RBAF143 in the log phase. *A,* Growth of cells in R-medium [37] without bicarbonate *(diamonds)* or with 0.8% sodium bicarbonate *(squares),* diluent alone *(triangles),* or 3-butyl-5-(dibromomethylene)-2-*(5H)*-furanone (fur-2) at concentrations of 10 *(crosses),* 20 *(asterisks),* or 40 *(circles)* μg/mL. *B,* Expression of the *lef-lacZ* reporter in cells grown in the same medium as described above. The experiment shown is representative of 3 separate trials performed on different days.

nones on the expression of *B. anthracis* toxins. Using *B. anthracis* strains with *lacZ* fusions to the 3 toxin components (*pagA, lef,* and *cya*) [38], we have provided consistent evidence that furanone treatment of *B. anthracis* inhibits toxin transcription (figures 6–9). In addition, in cultures treated with lower concentrations of furanones, in which cells recovered from growth, toxin transcription also was suppressed (figures 6–9). These data suggest that the inhibition of toxin transcription by furanones is independent and is additive to the inhibition of *B. anthracis* growth by furanones. Thus, the treatment of patients with halogenated furanones could potentially offer a means to inhibit both *B. anthracis* growth and toxin expression. However, there are few data on the toxicity of furanones in animals, so further analysis must be done. Taken together, these data suggest that the use of halogenated furanones may be an adjuvant therapy for *B. anthracis* infection, as well as a means to study the pathogen's density-dependent gene expression.

Acknowledgments

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