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Brief report

Inhibition of biofilm formation and swarming of *Escherichia coli* by (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone

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Summary

The quorum-sensing disrupter (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone (furanone) of the alga *Delisea pulchra* was found to inhibit the swarming motility of *Escherichia coli* completely at 13 μg cm^{-2} (also at 20 μg ml^{-1}) but did not inhibit its growth rate at 13–52 μg cm^{-2} or from 20 to 100 μg ml^{-1}. Swimming was not inhibited by the furanone at 20–40 μg ml^{-1}. In addition, confocal scanning laser microscopy revealed that this furanone at 60 μg ml^{-1} inhibited the biofilm formation of *E. coli*, as it decreased its thickness by 55%, reduced the number of water channels and decreased the percentage of live cells by 87%. This suggests that natural furanone may be used as a new method to control bacterial biofilms that does not involve toxicity. Furanone at 10 μg ml^{-1} also inhibited by 3300-fold the quorum sensing of *Vibrio harveyi* via autoinducer 1 (AI-1) and inhibited by 5500-fold that of *V. harveyi* via of autoinducer 2 (AI-2) as well as inhibited by 26 600-fold the quorum sensing of *E. coli* via AI-2; hence, this furanone is a non-specific intercellular signal antagonist.

Bacteria seldom live individually, and swarming (Allison and Hughes, 1991) and biofilm formation (Elvers and Lappin-Scott, 2000) are two important examples of bacterial multicellular behaviour; this behaviour enhances their chances of survival in competitive environments (Nickel *et al*., 1985; Ammendola *et al*., 1998). Multicellular behaviour also causes serious problems, such as infection and biofouling (Elvers and Lappin-Scott, 2000); hence, interest has increased in studying the mechanism of multicellular behaviour and discovering antagonists.

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Swarming exists for both Gram-positive and Gram-negative bacteria (including *Escherichia coli*; Harshey and Matsuyama, 1994) and takes place at specific surface viscosities, such as that of 0.7–0.8% agar plates for *Serratia marcescens*, and nutrient conditions (such as peptone concentrations; Allison and Hughes, 1991; Fujikawa, 1994). Once the bacteria recognize the appropriate environmental signals, they differentiate into swarming cells (which are hyperflagellated and elongated) and move quickly and co-ordinately on the surface; this leads to the different shapes of swarming colonies (Allison and Hughes, 1991).

It has been reported that quorum sensing based on cell communication is important for the normal development of swarming colonies (Eberl *et al*., 1996a) and biofilms (Davies *et al*., 1998), and swarming cells are found in biofilms (Lawrence *et al*., 1991). The quorum-sensing system is dependent on small extracellular molecules called autoinducers [AI; such as acylated homoserine lactone (AHL) for Gram-negative bacteria (Eberhard *et al*., 1981) and peptide signalling molecules for Gram-positive bacteria (Bassler, 1999)]. AI binds to a receptor protein and triggers the target genes when the culture reaches high cell density. Bioluminescence in *Vibrio fischeri* is an extensively studied example in which two genes (*luxI* and *luxR*) constitute the regulatory control system of this phenotype by controlling the concentration of the dissolvable signal *N*-3-oxohexanoyl-L-homoserine lactone (OHHL), which is known as AI-1 (Dunlap and Kuo, 1992).

In addition, a quorum-sensing system can be used to sense the population of other bacteria. For example, *Vibrio harveyi* has two autoinducer signalling systems with AI-1 (*N*-3-hydroxybutanoyl-L-homoserine lactone, encoded by *luxLM*) used for species-specific signalling and AI-2 [structure probably a furanone (Schauder *et al*., 2001), encoded by *luxS*] used for species-unspecific signalling (Bassler, 1999). A quorum-sensing system has

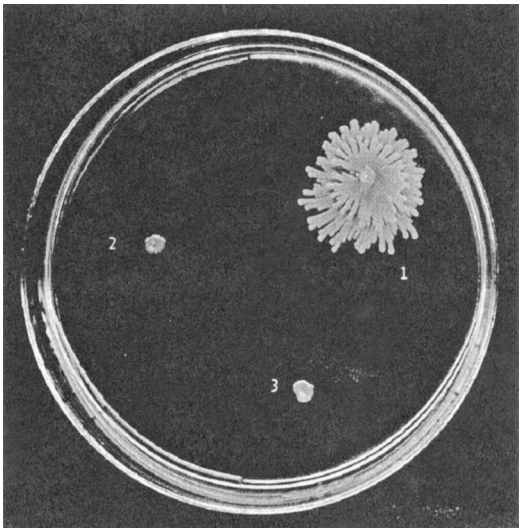

Fig. 1. Furanone inhibition of the swarming motility of *E. coli* XL1-Blue. Position 1, 0 μg cm^{-2} furanone (control); position 2, 26 μg cm^{-2} furanone; position 3, 52 μg cm^{-2} furanone. (*5Z*)-4-Bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone was synthesized as reported previously (Beechan and Sims, 1979; Manny *et al*., 1997) with the exception that the furanone was purified using column chromatography with hexane and ethyl acetate at a ratio of 100:1 (2.5 × 60 cm column; Spectrum Chromatography). The structure was verified with ^{1}H-NMR (Bruker DRX-400 MHz) and IR (Nicolet-Magna-IR 560) by comparison with literature values. By comparing with standard furanone (Beechan and Sims, 1979), the furanone product was verified routinely using thin-layer chromatography (hexane and ethyl acetate at a ratio of 20:1, $R_f = 0.8$; Silica Gel 60, F-254; Selecto Scientific). Furanone was dissolved in dichloromethane before adding to the surface of the swarming plates, and this solvent did not affect swarming. Swarming plates (20 ml in 100 × 15 mm Petri dishes) contained LB medium supplemented with 1.1% agar and were dried at 30°C for 2 h in an incubator before adding furanone dissolved in dichloromethane at various concentrations (119–2380 μg ml^{-1}) to the top of the agar plate in 40 μl (40 μl dichloromethane was added to all positions including those with no furanone). The dichloromethane evaporated rapidly, and the furanone remained as a thin layer (hydrophobic ink dissolved in dichloromethane and added to the top of agar plates did not diffuse into the agar). The plates were dried for another 3 h at 30°C, inoculated with toothpicks at the centre of the furanone-containing area from 2-day-old streaks on LB medium supplemented with 100 μg ml^{-1} ampicillin, and the swarming colonies were grown at 37°C. At least eight plates were used for each furanone concentration. Furanone was also dissolved in 0.8 and 0.9 wt% agar plates (3 ml in 35 × 10 mm Petri dishes) at 20–40 μg ml^{-1} using ethanol (final concentration in agar 0.67%) and dried for 5.5 h at 30°C.

also been reported in *E. coli* by two research groups (Surette and Bassler, 1998; Baca-Delancey *et al*., 1999); this strain has no AI-1 but does have AI-2 (synthesized by LuxS; Surette *et al*., 1999), whose structure is probably also a furanone (Schauder *et al*., 2001).

Several kinds of brominated furanones produced by the red marine alga *Delisea pulchra* inhibit AHL-mediated multicellular behaviour without affecting growth, such as the swarming motility of *Proteus mirabilis* (Gram *et al*., 1996) and *Serratia liquefaciens* (Givskov *et al*., 1996). In addition, several natural furanones (at 1–20 μg ml^{-1}) have been found to inhibit the growth rate of Gram-positive bacteria including *Staphylococcus aureus* and *Staphylococcus epidermidis* (Kjelleberg, 1999).

In this paper, *E. coli* was used to study the effect of the natural (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone (Manefield *et al*., 2000) on swarming and biofilm formation. To discern the molecular basis for the inhibition of swarming and biofilm formation, quorum-sensing mutant strains of *V. harveyi* were used to test the inhibiting effect of furanone on AI-1 and AI-2 separately. This is the first report of furanone affecting the swarming of *E. coli* and its biofilm formation. This is also the first investigation of the inhibition of furanone on the individual quorum-sensing signals AI-1 and AI-2 from *V. harveyi* as well as AI-2 from *E. coli*.

Simple tests of colony growth on LB (Maniatis *et al*., 1982) agar plates showed that furanone (13, 26, 52 μg cm^{-2}) did not slow the growth of *E. coli* XL1-Blue/pBSII KS– (henceforth XL1-Blue), as its colony size was not altered. Furanone was added to the top of the LB agar (1.5%) plates, which support growth but do not support swarming. The furanone was dissolved in dichloromethane at different concentrations and added in a constant volume of 40 μl. For cells in suspension (duplicate experiments, 100 μg ml^{-1} ampicillin present, furanone dissolved in 95% EtOH and a constant amount of ethanol added), furanone also did not affect the specific growth rate (1.17 ± 0.06 h^{-1}, no lag phase) at 20, 60 and 100 μg ml^{-1}. Similar results have been reported, in that the natural furanones extracted from *D. pulchra* have no effect on the growth of *P. mirabilis* (Gram *et al*., 1996).

The swarming colony of XL1-Blue was highly ordered (fractal) and periodic (Fig. 1), which suggests that there is some mechanism by which the colony structure is optimized (possibly through the autoinducer signal). It has been shown that periodic swarming colonies are more likely to appear in relatively less rich medium (Fujikawa, 1994). The swarming motility was affected markedly by the wetness of the LB agar plates (0.2–1.5% agar tested). It was found that XL1-Blue can swarm on the LB plates supplemented with 0.8–1.1% agar, whereas the 1.1% agar was optimal (Fig. 1) for swarming plates in 100 × 15 mm Petri dishes and 0.8–0.9% in 35 × 10 mm Petri dishes. If the agar concentration was < 0.8%, the cells exhibited swimming (Eberl *et al*., 1996b) through the water channels in the plates (cells seen clearly 1.5 mm below the surface of the agar). Similarly, high agar concentrations (> 1.1%) did not support swarming motility, only growth. Light microscopy (1000× total magnification, Galeen III; Cambridge Instrument) showed that the cells from the edge of swarming colonies (on 1.1% agar plates) were in bundles (as seen in swarming colonies of *P. mirabilis*; Gram *et al*., 1996) and elongated (5.1 ± 0.7 μm in length), which is similar to the

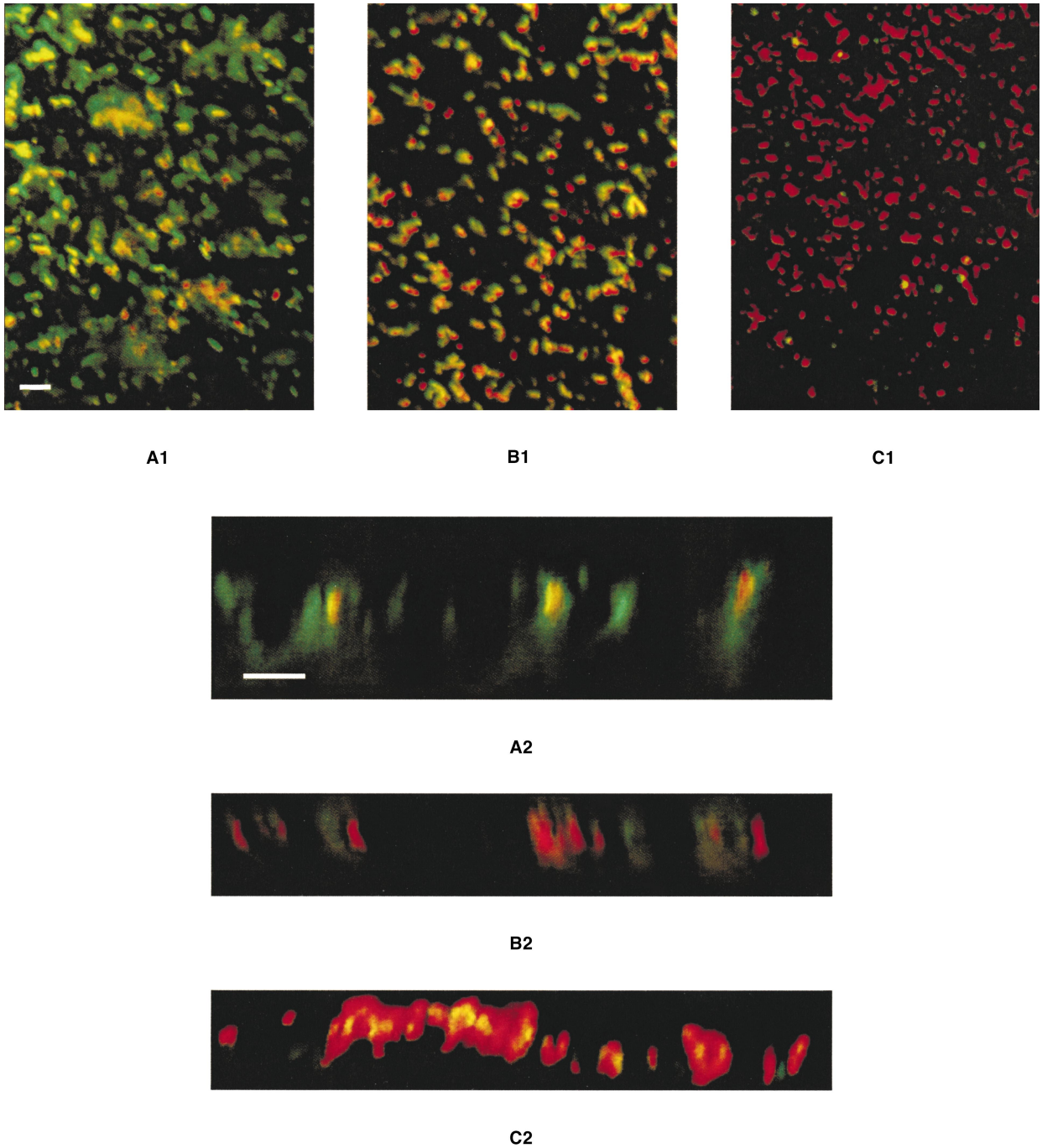

Fig. 2. Furanone inhibition of *E. coli* XL1-Blue biofilm formation (representative confocal images). Bar = 10 μm. A1 (Z section) and A2 (vertical section), no furanone; B1 (Z section) and B2 (vertical section), 30 μg ml^{-1} furanone; C1 (Z section) and C2 (vertical section), 60 μg ml^{-1} furanone. The impact of furanone on biofilm formation was discerned with SAE1018 steel coupons (25.5 mm diameter and 1.2 mm thick, polished with P240 polishing paper, 3M Imperial Wet-or-Dry). A single coupon was placed in a 100 × 15 mm Petri dish with 20 ml of LB (supplemented with 100 μg ml^{-1} ampicillin). The furanone was dissolved in 95% ethanol (14.9 mg ml^{-1}) and added to the culture at the start of the experiments with a final concentration of 30 or 60 μg ml^{-1}. The same amount of 95% ethanol (0.6%) was added to the control sample without furanone. Coupons were incubated at 37°C without shaking for 2 days, and the biofilm was visualized with the Live/Dead Baclit bacteria viability assay kit (L-7007; Molecular Probes) and confocal scanning laser microscopy (MRC 600; Bio-Rad) as described previously (Jayaraman *et al.*, 1997). Ninety biofilm positions on 16 coupons were visualized.

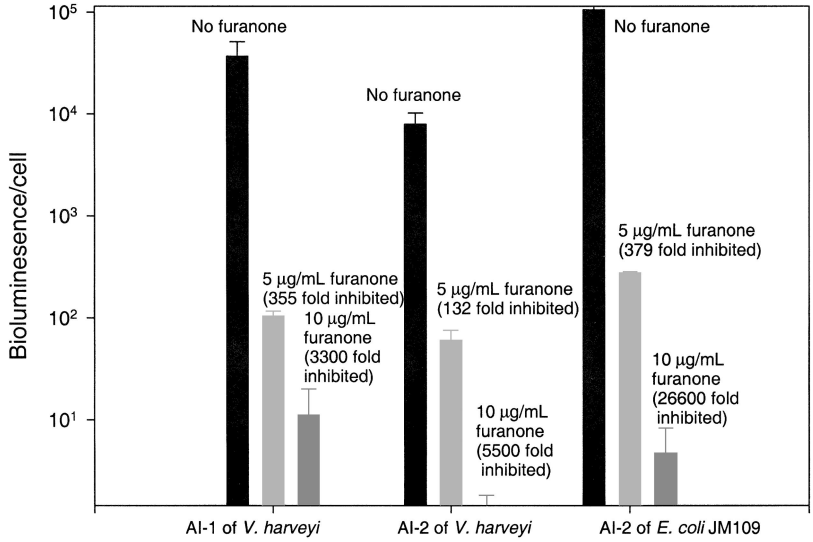

Fig. 3. Effect of furanone on the autoinduction of AI-1 (from *V. harveyi*) and AI-2 (from *V. harveyi* and *E. coli* JM109). Bacterial supernatants were assayed using the method of Surette and Bassler (1998). Briefly, *V. harveyi* BB120 (wild type) and JM109 were grown to produce AI-1 and AI-2. Reporter strains *V. harveyi* BB170 (sensor 1−, sensor 2+) and BB886 (sensor 1+, sensor 2−) were grown in AB medium overnight, diluted 1:5000 into the fresh AB medium, and then the cell-free supernatant from the *E. coli* and *V. harveyi* BB120 cultures was added at a concentration of 10% (v/v) (sterile AB or LB medium with no furanone and no supernatant was also checked as control). Furanone (5–10 μg ml^{-1}) was added to the culture 5.5 h after inoculation of the reporter cells with supernatant in order to minimize interference with the signal of the reporter cells (e.g. interference by AI-2 of the reporter is $<1\%$ of the bioluminescence signal). The bioluminescence was measured 1.5 h later with a 20/20 luminometer (Turner Design). The cell density of the *V. harveyi* reporter strains was measured 1 h after furanone addition by spreading the cells on LM plates and counting colonies after 24 h; the cell count was used to normalize the bioluminescence signal. Each experiment was conducted in duplicate, and standard deviations are shown.

previous report (Harshey and Matsuyama, 1994), whereas the cells in the swimming colonies (on 0.2–0.3% agar plates) were just vegetative cells (1.8 ± 0.4 μm in length). As the wetness of the swarming plates was critical, each plate was prepared under the same conditions including drying time and temperature. Swarming was found to be inconsistent on different plates made under the same conditions; however, as different positions on the same plate showed the same swarming motility, single plates were used containing both the control (0 μg cm^{-2} furanone) and various concentrations of furanone.

Furanone inhibited swarming when added to the surface (in 100×15 mm Petri dishes) at 13 μg cm^{-2} (3.2 and 6.5 μg cm^{-2} did not show consistent inhibition, whereas 13, 26 and 52 μg cm^{-2} consistently inhibited swarming; Fig. 1). This agrees well with the inhibition previously reported for 5 μg cm^{-2} produced by the alga (Givskov *et al*., 1996). Generally, in the absence of furanone, the swarming colony speed was about 0.8–1.1 μm s^{-1} (average speed of active and quiescent phases with the highest speed 2.9 μm s^{-1}) because the swarming started 5 h after inoculation. This corresponds well with the previous reported values for *E. coli* of 2–10 μm s^{-1} (Harshey and Matsuyama, 1994). These results were corroborated as furanone also inhibited swarming at concentrations of 20 and 40 μg ml^{-1} in both 0.8 and 0.9 wt% agar plates (in 35×10 mm Petri dishes), whereas there was no inhibition in the absence of furanone. After 16 h, 75 plates were tested and showed a fractal, swarming diameter of 22.6 ± 7.3 mm at 0 μg ml^{-1} furanone versus a non-fractal diameter of 3.5 ± 0.76 mm at 40 μg ml^{-1} furanone for 0.9 wt%, and a fractal, swarming diameter of 29.9 ± 7.1 mm at 0 μg ml^{-1} furanone versus a non-fractal diameter of 3.9 ± 0.6 mm at 40 μg ml^{-1} furanone for 0.8 wt%.

Swimming, unlike swarming, is the result of rotation of the bacterial flagella rotor and is not multicellular behaviour (Moat and Foster, 1995; Givskov *et al*., 1996). To discriminate swarming from swimming, plates of different agar concentrations were used (XL1-Blue swims on plates with agar concentrations $<0.8\%$ and swarms on 0.8–1.1% agar plates). In contrast to swarming, furanone did not affect the swimming motility of XL1-Blue, as swimming cells were seen throughout the 0.2% LB agar plates with 0, 20 and 40 μg ml^{-1} furanone in 35×10 mm Petri dishes. The swimming plates were dried at 30°C for 2 h in an incubator before inoculation with toothpicks from 1 day XL1-Blue cultures in LB medium supplemented with 100 μg ml^{-1} ampicillin. The appropriate amount of furanone was added directly to the medium, and the same amount of ethanol was added to the control plates without furanone to eliminate solvent effects (18 plates tested).

Furanone significantly altered the biofilm of XL1-Blue; without furanone, the biofilm had a thickness of 22 ± 1 μm, and most of the cells were alive (76.1%, stained green). However, furanone decreased the thickness of the biofilm dramatically (14 ± 0.6 μm at 30 μg ml^{-1} furanone and 10 ± 1 μm at 60 μg ml^{-1} furanone; Fig. 2). In addition, furanone also increased the percentage of dead cells in the biofilm, as 30 and 60 μg ml^{-1} furanone decreased the percentage of living cells by 30% and 87% respectively. By looking at the vertical section of the biofilm, it was found that the structure of the biofilm with 60 μg ml^{-1} furanone was not as robust as that of the biofilm without furanone, in that it had fewer water channels and was compressed (Fig. 2A2 and C2). Furanone at 30 μg ml^{-1} had an intermediate effect on the biofilm (Fig. 2B2). Similarly, 50 μg ml^{-1} furanone caused a 42% decrease in biofilm thickness for *E. coli* JM109 (13 ± 2 μm without furanone versus 7.7 ± 0.6 μm for biofilm with 50 μg ml^{-1} furanone), although it caused only a 17% inhibition in the growth rate. The increase in dead cells in the biofilms with furanone may result from the collapse of the biofilm structure, as the furanone is clearly not toxic to these cells. This indicates that natural furanone is a new method for controlling biofilms that does not involve toxicity.

To discern whether furanone can inhibit AI-2 of *V. harveyi* directly and the possible mechanism by which furanone inhibits the multicellular behaviour of *E. coli*, the autoinducer reporter strains *V. harveyi* BB886 and BB170 were used to study the effect of furanone on the quorum-sensing autoinducers. *V. harveyi* BB886 can only sense AI-1 of *V. harveyi* (*N*-3-hydroxybutanoyl-L-homoserine lactone) but cannot sense AI-2 of *V. harveyi*. In contrast, *V. harveyi* BB170 can only sense the AI-2 but cannot sense AI-1 activity (Surette and Bassler, 1998) and was found previously to detect AI-2 activity in some *E. coli* strains, such as *E. coli* AB1157 and *E. coli* JM109 (Surette and Bassler, 1998). Hence, the inhibition of quorum sensing by furanone on AI-1 and AI-2 can be discerned separately with this system.

As the reporter strains produce and sense their own autoinducers, the time to measure the AI activity and to add furanone was chosen carefully. Based on a previous report (Surette and Bassler, 1998) and this study, the signal of the reporter strains reaches a minimum 5.5 h after inoculation (when bioluminescence per cell was stimulated 1000-fold by the AI of the supernatant relative to the signal of the reporter itself); hence, furanone was added at this time. Bioluminescence and cell density were measured 1.5 h later. It was found that 5–10 μg ml^{-1} furanone inhibited quorum sensing based on *V. harveyi* AI-2 132- to 5500-fold (Fig. 3) without affecting the growth of the reporter strain (shown by counting colonies on plates). Similarly, 5–10 μg ml^{-1} furanone inhibits quorum sensing based on *V. harveyi* AI-1 355- to 3300-fold, and quorum sensing based on *E. coli* JM109 AI-2 was inhibited 379- to 26 600-fold (Fig. 3). Under these conditions, XL1-Blue did not produce AI-1 or AI-2 activity (did not induce bioluminescence in *V. harveyi* BB886 and BB170).

The addition of 2.5 ml (10% v/v) of fresh JM109 supernatant did not reduce the inhibition of quorum sensing caused by the furanone; however, after the initial quenching of bioluminescence by the furanone for all experiments, the bioluminescence increased steadily and at the same rate as the control, which lacked furanone and AI-containing supernatant but was diluted with fresh AB medium. This indicates that the diminution of bioluminescence by the furanone masking AI-1 and AI-2 is reversible, as the signal synthesized by the growing reporter strain is able to titrate the furanone. As the addition of conditioned supernatant did not reduce the impact of the furanone, but the AI produced by the reporter strain displaced the furanone and restored bioluminescence (over a several hour period), it is not clear from these experiments about the relative affinities of furanone and AI for the AI receptors.

It was reported previously that furanone inhibits swarming by suppression of the AHL autoinduction circuit (Givskov *et al*., 1996). Using inhibition of bioluminescence from OHHL-stimulated *E. coli*/pSB403, it has also been reported that furanones inhibited bioluminescence by displacing AHLs from the AHL receptor (Manefield *et al*., 1999). As our results show that furanone inhibits the swarming and biofilm formation of *E. coli* without affecting its growth and as the inhibition of bioluminescence by furanone is reversible, it is highly possible that the furanone works in a similar manner with this strain (by displacing cell signals from receptors). Recently, it was hypothesized that furanone simultaneously inhibited both AI-1 (AHL) and AI-2 (furanone-like compound; Schauder *et al*., 2001) of *V. harveyi* by inhibiting its luminescence and virulence (phenotypes controlled by both AI-1 and AI-2; Manefield *et al*., 2000). Our results show clearly that furanone inhibits both AI-1 and AI-2 directly and independently.

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