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Brief Report

(*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone reduces corrosion from *Desulfotomaculum orientis*

Dacheng Ren[†] and Thomas K. Wood*
Departments of Chemical Engineering and Molecular and Cell Biology, University of Connecticut, 191 Auditorium Road, Storrs, CT 06269-3222, USA.

Summary

(*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone (furanone) from the red marine alga *Delisea pulchra* was found previously to inhibit the growth, swarming and biofilm formation of Gram-positive bacteria (Ren *et al*., 2002, *Lett Appl Microbiol* 34: 293–299). In the present study, the Gram-positive sulphate-reducing bacterium (SRB), *Desulfotomaculum orientis*, was used to study the inhibition of mild steel corrosion due to the addition of furanone. The weight loss from batch coupon experiments incubated with 40 μg ml^{-1} furanone was reduced fivefold compared with samples that lacked furanone. Analysis of the metal surface with environmental scanning electron microscopy further confirmed the protection afforded by the addition of furanone. In agreement with the corrosion inhibition, most probable number (MPN) analysis showed that 20 and 40 μg ml^{-1} furanone inhibited 58% and 96% of the *D. orientis* growth respectively. Hence, furanone has the potential to inhibit microbial-induced corrosion related to Gram-positive bacteria.

Sulphate-reducing bacteria (SRB) are a group of anaerobic bacteria that use sulphate as the terminal electron acceptor and produce hydrogen sulphide as the reduced product (Hamilton, 1985). Sulphate-reducing bacteria (both Gram-positive and Gram-negative) are a major source of microbial-induced corrosion (MIC) and are estimated to be responsible for 50% of all corrosion problems (Hamilton, 1985). Sulphate-reducing bacteria are ubiquitous and exist in marine, soil and different engineering environments (such as in chemical plants, water treatment units, heat exchangers and oil pipelines) (Hamilton, 1985), and biofilms (sessile communities with a high density of bacterial cells; Elvers and Lappin-Scott, 2000) are extensively involved in corrosion caused by SRB (Lee *et al*., 1995). As an aerobic biofilm with a thickness of 10–25 μm will generate an anaerobic environment underneath it that is favourable for SRB growth (Coulter and Russell, 1976; Costerton and Gessey, 1979), SRB-induced corrosion is commonly found with mixed bacterial species (Lee *et al*., 1995). Pitting formation is typical in SRB corrosion with the pits filled with black corrosion products (iron sulphides) (Lee *et al*., 1995).

Gram-positive bacteria are also important sources of infectious disease and are responsible for more than 60% of the nosocomial bloodstream infections in the United States, while Gram-negative bacteria are responsible for only 27% of such infections (Edmond *et al*., 1999). Hence, it would be beneficial to find novel antimicrobials for Gram-positive bacteria, and natural brominated furanones hold great potential.

Recently, several brominated furanones from the marine alga *Delisea pulchra* have been shown to inhibit the quorum sensing (control of gene expression by sensing the bacterial population; Surette and Bassler, 1998; Bassler, 1999) of Gram-negative bacteria by affecting both the species-specific and species-non-specific quorum-sensing signals autoinducer-1 and autoinducer-2, respectively (Ren *et al*., 2001), and therefore inhibit swarming and biofilm formation without affecting cell growth (Gram *et al*., 1996; Rasmussen *et al*., 2000; Ren *et al*., 2001; Hentzer *et al*., 2002). Interestingly, the furanones were found to inhibit growth of Gram-positive bacteria at a concentration non-toxic to mammalian cells (Kjelleberg, 1999). One of the best studied furanones of *D. pulchra* (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone (henceforth furanone; Fig. 1), inhibits the growth, swarming and biofilm formation of *Bacillus subtilis*

Received 29 July, 2003; revised 24 November, 2003; accepted 9 December, 2003. *For correspondence. E-mail twood@engr.uconn.edu; Tel. (+1) 860 486 2483; Fax (+1) 860 486 2959.[†]Present address: School of Chemical and Biomolecular Engineering, Cornell University, 120 Olin Hall, Ithaca, NY 14850, USA.

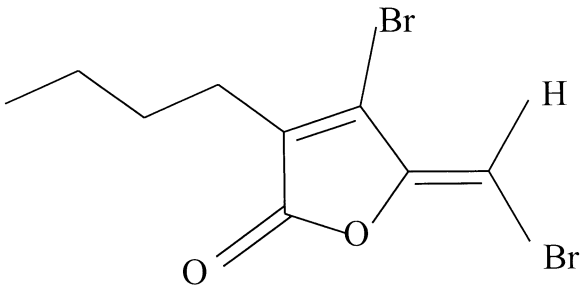

Fig. 1. Structure of (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone (furanone). Furanone was synthesized by the method of Beechan and Sims (1979) as well as by the four-step method of Manny *et al.* (1997) with three exceptions: (i) the decarboxylation was conducted by refluxing in benzene rather than toluene; (ii) a lactone formation (last step) was conducted in a water bath (100°C) rather than an oil bath (110–120°C); and (iii) 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 by ^{1}H-NMR (Bruker DRX-400 MHz; 6.24 single peak, vinylidene; 2.39 triple peaks, coupling constant J 7.2 Hz, allylic methylene; 0.93 triple peaks, coupling constant J 7.2 Hz, terminal methyl) and IR (Nicolet-Magna-IR 560; reciprocal absorbing wavelength: 2958, 1793, 1610, 1276, 1108, 1030 cm^{-1}) by comparison with literature values (Beechan and Sims, 1979; Manny *et al.*, 1997). By comparing with standard furanone synthesized by the method of Beechan and Sims (1979), the furanone product was also 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). The stocks of synthesized furanone (14.9 mg ml^{-1} in 95% ethanol) were stored at 4°C.

(Ren *et al.*, 2002). To explore further applications of this furanone, the Gram-positive SRB, *Desulfotomaculum orientis*, was used to form a biofilm on mild steel surfaces, and the ability of the furanone to inhibit *D. orientis* and reduce corrosion was investigated. This is the first report of inhibiting SRB corrosion by natural brominated furanones.

Furanone reduced corrosion with *D. orientis* in batch coupon experiments

To study the inhibition of furanone on mild steel corrosion caused by *D. orientis*, mild steel coupons were incubated with *D. orientis* and different amounts of furanone (but the same amount of diluent). The metal coupons were polished and were shiny after autoclaving. The *D. orientis* culture without furanone became slightly turbid, and the corrosion product (iron sulphides) appeared as black precipitates on the coupon surface as one or a few points (normally around the edge or under the coupons) 5 days after inoculation and expanded to cover the entire coupon as the growth of SRB continued and it developed its biofilm. The extent of the coupon surface that was covered with the black precipitate was a good indication of the extent of corrosion. Two weeks after inoculation, the black films on the coupons incubated without furanone fractured, and the whole culture started to become black. Accompanied by this change, the weight losses for the coupons incubated without furanone increased from 0.72 mg cm^{-2} per week (average in the first 2 weeks) to 1.95 mg cm^{-2} per week in the third and fourth weeks (increased 2.7-fold).

For coupons incubated with 20 μg ml^{-1} furanone, the corrosion was significantly inhibited. Although the culture turned slightly turbid, no apparent black precipitate was observed during the 4 week period of incubation. Furanone (5 μg ml^{-1}) had an intermediate effect by visual observation but did not show any inhibition by weight loss (Fig. 2). To obtain the maximum inhibition, furanone with a concentration of 40 μg ml^{-1} was also tested, and no growth of SRB was observed (by checking the culture turbidity, black precipitate and the smell of the culture).

To quantify the corrosion inhibition, three coupons for each furanone concentration were harvested every week, and the weight loss was measured. The weight loss from coupons incubated with furanone was significantly lower than for those without furanone. For example, the accumulated weight loss for the coupons incubated without furanone was 1.45 mg cm^{-2} after 2 weeks of incubation, while the coupons incubated with 40 μg ml^{-1} furanone had a weight loss of only 0.62 mg cm^{-2}. The accumulated weight loss for 4 weeks with 40 μg ml^{-1} furanone (1.32 mg cm^{-2}) was about 20% of that without furanone (5.35 mg cm^{-2}); hence, 80% of the corrosion was inhibited, and the residual corrosion noted was probably caused by the dissolved oxygen, as the upper parts of the flasks were filled with air. These results were corroborated by environmental scanning electron microscopy images (Fig. 3), which show that 40 μg ml^{-1} furanone protected the mild steel surface (polishing grooves still seen), whereas the coupons that lacked furanone show evidence of iron sulphide formation.

Furanone inhibited the growth of *D. orientis* in shake flasks

The furanone used in the present study inhibits the growth of Gram-positive bacteria, such as *B. subtilis* (Ren *et al.*, 2002), so the inhibition of *D. orientis* growth was investigated. Similar to other SRB, *D. orientis* produces H_2S as its metabolism product (Lee *et al.*, 1995). In the absence of furanone, the growth of *D. orientis* caused slight turbidity of the culture, and a strong smell of H_2S was detected when the flasks were opened. However, when furanone was added, the SRB growth was inhibited, as evidenced by the decrease in both turbidity and the H_2S smell. To quantify the inhibition of furanone on the growth of *D. orientis*, the most probable number (MPN) method (Anonymous, 1992) was used. After contact for 1 h, 20 and 40 μg ml^{-1} furanone inhibited the growth of 58% and 96% of the SRB cells respectively. Hence, the MPN data

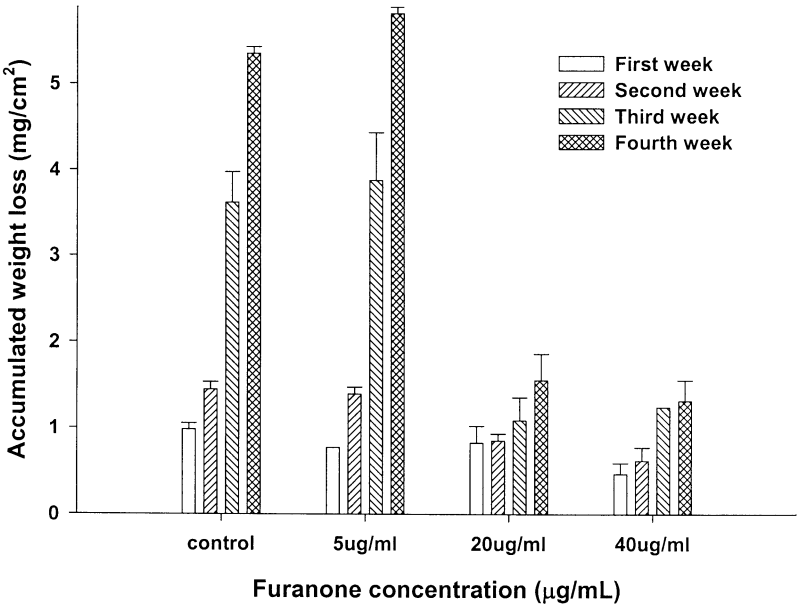

Fig. 2. Weight loss of mild steel coupons incubated with *D. orientis* and different concentrations of furanone. Error bars indicate one standard deviation. The Gram-positive SRB strain *D. orientis* was obtained from the American Type Culture Collection (ATCC 23598). *D. orientis* was grown anaerobically in 15 ml screwcap tubes containing 13 ml of modified Baars' medium (ATCC medium 1249) supplemented with 260 μl of 4% Na_2SO_3 and 260 μl of Oxyrase (Oxyrase) to maintain anaerobic conditions; each 15 ml tube was inoculated with 1 ml of –80°C stock solution and incubated at 30°C for 4–6 days. Mild steel 1010 (UNS G10100) plates (Yarde Metal) were cut into 1 inch square coupons and polished with P240 polishing paper (3M Imperial Wet-or-Dry). Each polished mild steel coupon was put in a 250 ml flask supplemented with 25 ml of modified Baars' medium. The flasks containing coupons and medium were then autoclaved for 20 min at 120°C. Each flask was inoculated with 750 μl of a 6-day-old culture of *D. orientis* and incubated at 30°C without shaking for 4 weeks. Fresh modified Baars' medium (25 ml) was added to each flask every week. To measure the weight loss, each coupon was cleaned by wiping with a rubber stopper and 0.01% chromic acid followed by washing with warm water (three times).

indicate that furanone inhibits the growth of *D. orientis* in a concentration-dependent manner.

Furanone reduced SRB-related corrosion in continuous reactors

The batch coupon experiments indicated that furanone significantly reduced the corrosion of mild steel caused by *D. orientis*. To corroborate these results, continuous reactor studies were performed to measure the *in situ* corrosion rate by measuring electrical impedance data as described previously (Jayaraman *et al*., 1997a). EIS is a non-destructive corrosion monitoring technique that can be used to study corrosion behaviour of the metal under investigation without disturbing the properties of the biofilms and metal surface (Mansfeld, 1995; Jayaraman *et al*., 1999).

Desulfotomaculum orientis was inoculated at 3% (v/v) from a 4-day-old culture in modified Baars' medium, and 0 or 20 μg ml^{-1} furanone was added to both the reactor and the feed bottle before the inoculation of *D. orientis*. The same amount of ethanol was added to the medium without furanone. Air was supplied to the headspace of the reactors at 200 ml min^{-1}. The reactors were non-continuous (batch) for 2 days, and were then made continuous for 6 days [12 ml h^{-1}, dilution rate (D) = 0.08 h^{-1}].

It was found that 20 μg ml^{-1} furanone inhibited the growth and corrosion of a pure culture of *D. orientis* for 8 days as the metal plate incubated without furanone turned black 48 h after inoculation, but no black precipitate of iron sulphide was seen on the metal incubated with 20 μg ml^{-1} furanone until 8 days after inoculation. However, the difference in weight loss between the protected and unprotected metal plates was modest (0.93 mg cm^{-2} with 20 μg ml^{-1} furanone versus 1.1 mg cm^{-2} without furanone). The corrosion inhibition by furanone was also supported by the impedance measurements as the impedance curve for both reactors had a plateau at the beginning of the experiment; however, the impedance curve for the reactor without furanone lost the plateau on the third day after inoculation (typical curves associated with corrosion caused by SRB; Jayaraman *et al*., 1999), while the impedance curve for the reactor with 20 μg ml^{-1} furanone had a low plateau 8 days after inoculation.

As SRB in natural environments commonly exist with other species (Lee *et al*., 1995), inhibition of corrosion by furanone was also studied in the presence of a protective aerobic biofilm. In these tests, a biofilm containing aerobic bacteria was allowed to form on mild steel that, in the absence of SRB, would inhibit corrosion by removing dissolved oxygen; such biofilms have been described elsewhere (Jayaraman *et al*., 1997a,b) and are the basis of the phenomenon known as 'corrosion control using regenerative biofilms'. However, the consumption of oxygen in these biofilms by the aerobic bacteria creates an anaerobic zone that provides favourable conditions for SRB.

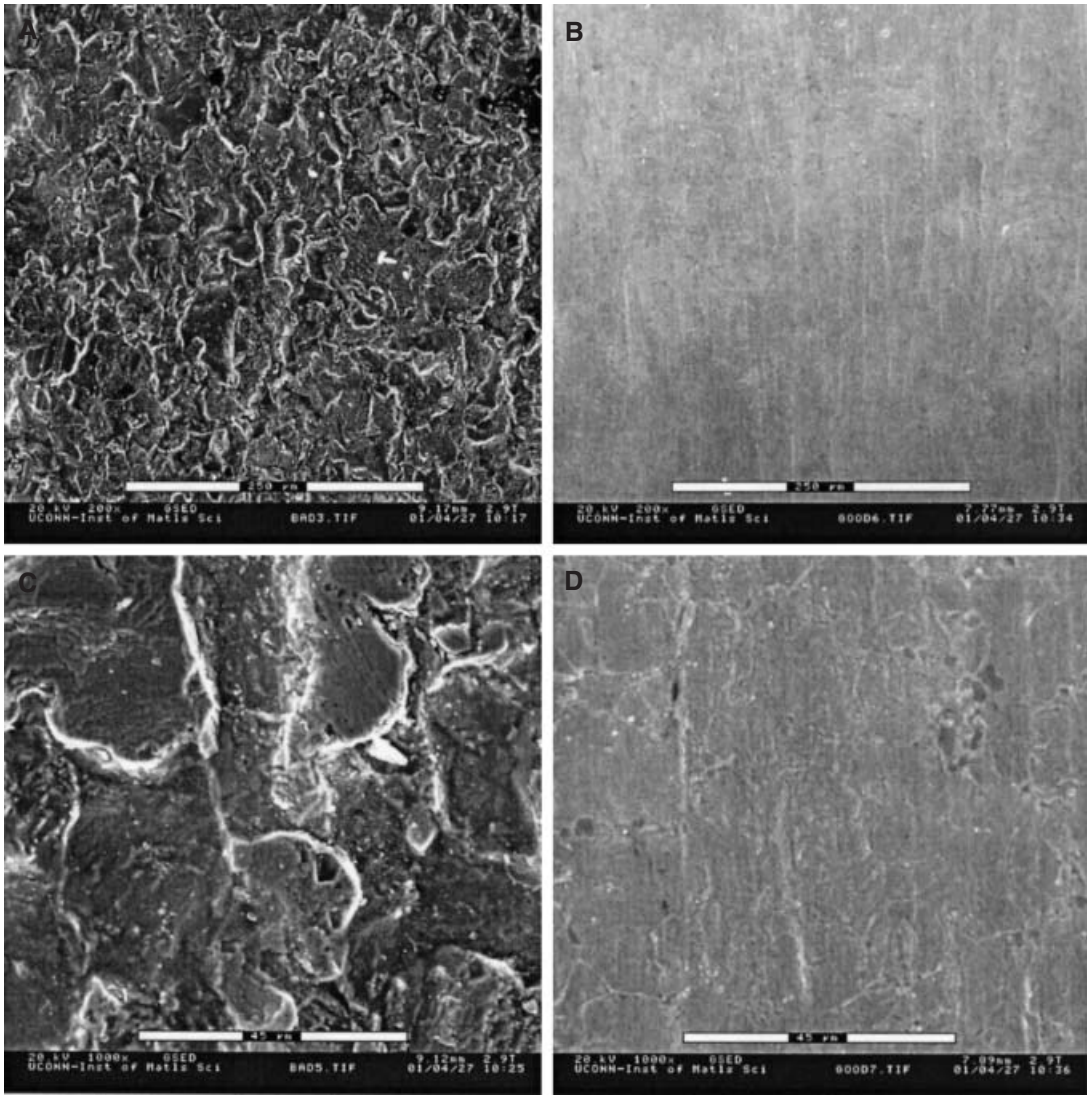

Fig. 3. Environmental scanning electron microscopy analysis of protected and unprotected mild steel coupons from batch experiments.
A. 200×, no furanone.
B. 200×, 40 μg ml^{-1} furanone.
C. 1000×, no furanone.
D. 1000×, 40 μg ml^{-1} furanone.
Scale bar 250 μm for (A) and (B), 45 μm for (C) and (D). The accumulated weight loss was measured, and the metal surface was studied by environmental scanning electron microscopy (ESEM 2020; Philips Electronic Optics).

Hence, when SRB are added, the difference in the corrosion rate between the protected metal (with furanone) and the unprotected metal (without furanone) is expected to be even more significant. Our previous results showed that furanone could inhibit the formation of biofilms, such as those of *Escherichia coli* (Ren *et al*., 2001) and *B. subtilis* (Ren *et al*., 2002), so such strains would not be appropriate for these tests. Interestingly, our previous 96-well plate biofilm assay showed that furanone promotes the biofilm formation of *Pseudomonas putida* F1 (unpublished result), which has been shown to protect steel from corrosion of dissolved oxygen (Jayaraman *et al*., 1997c). Hence, *P. putida* F1 was used to provide the protective aerobic biofilm in these tests. This experiment was performed in the same way as that for the pure *D. orientis* culture discussed above except that *P. putida* F1 was inoculated (to an optical density at 600 nm of 0.06) simultaneously with *D. orientis* (3% v/v from a 6-day-old culture), and the concentrations of furanone tested were 0, 10 and 40 μg ml^{-1}. Appropriate amounts of ethanol were

added to all the reactors and the feed bottles containing modified Baars' medium immediately after inoculating the strains. This experiment was conducted in duplicate.

The metal plates in the continuous reactors containing *P. putida* F1 and SRB but lacking furanone (the two control reactors) turned black 12 h after inoculation as a result of corrosion caused by *D. orientis*. The two reactors with the two bacterial strains and 40 μg ml^{-1} furanone were apparently protected well, and no black precipitate of corrosion product was seen on the metal surface (which was covered by the protective *P. putida* F1 biofilm) before the reactors were opened (after 8 days). After opening the reactors and removing the cultures, however, a black precipitate was seen on the metal surface for both these two reactors (with 40 μg ml^{-1} furanone). This suggests that SRB were still present. However, the two reactors with 10 μg ml^{-1} furanone showed a slightly different result. The mild steel plate in one of them turned black 24 h after inoculation, and the other turned black 4 days after inoculation. From the impedance data, it is estimated that the furanone (40 μg ml^{-1}) protected the metal for the first 8 days after inoculation. In the first 3 days, impedance curves for all six reactors showed a low-impedance plateau. After 3 days, however, the impedance curves of the control reactors (no furanone) had no plateau, while the impedance for protected metal (with 40 μg ml^{-1} furanone) had a low-impedance plateau for the first 8 days after inoculation, indicating the inhibition of SRB corrosion. A furanone concentration of 10 μg ml^{-1} had intermediate effects. Although the impedance data and visible observations suggest that the metal was protected by the addition of furanone, the weight loss data did not show an apparent difference in corrosion (about 0.5 mg cm^{-2} for all samples). The weight loss for the protected metal incubated with the mixed biofilm consisting of *D. orientis* and *P. putida* (0.5 mg cm^{-2} at 10 days after inoculation) was lower than that for the protected metal incubated with the pure *D. orientis* culture (0.9 mg cm^{-2} at 8 days after inoculation), which suggests that the addition of *P. putida* was beneficial for corrosion reduction.

Although both the weight loss in the batch coupon experiments and the impedance measurements in the continuous experiments showed that furanone significantly inhibits microbial-induced corrosion (MIC) caused by *D. orientis*, the weight loss data in continuous reactors did not show a significant difference. This is probably caused by the difference in culture conditions. Air was supplied to the continuous reactors at 200 ml min^{-1}, but the batch cultures did not have air other than that from the original headspace (the flasks were sealed). In addition, the flow condition was different because of the continuous addition of nutrients and removal of wastes from the continuous reactors (12 ml min^{-1}). Sulphate-reducing bacteria corrosion is a complicated process, and the corrosion rate is dependent on chemical and physical properties of the corrosion products, but is not directly related to the SRB activity (sulphate consumed in unit area and time) (Lee *et al*., 1995). It has been reported previously that there is no difference in weight loss or electrochemical data when the SRB activity is 2–32 mg sulphur m^{-2} h^{-1} (Lee *et al*., 1995). This may help to explain the absence of difference in weight loss in our continuous corrosion experiments.

It was noticed that the black film of iron sulphide did not stay on the coupon during the whole incubation period (batch cultures). The rupture of the film is an indication of the change in corrosion product (iron sulphide) from mackinawite (FeS_{1-x}) to smythite (Fe_3S_4) and pyrrhotite ($Fe_{1-x}S$), and this change accelerates the corrosion speed (Hamilton, 1985). In agreement with this, the weight loss speed increased 2.7-fold accompanying the rupture of the black film in our coupon experiments (Fig. 2). After 2 days of batch incubation, the continuous reactors were operated for 6 days (for the pure *D. orientis* culture) or 8 days (with mixed biofilms), but the rupture of the initial black film (in the reactors without furanone) started only about 3 days (for the pure *D. orientis* culture) or 5 days (with mixed biofilms) before the experiment was stopped. Hence, longer incubation may lead to higher weight loss in the reactors without furanone and, therefore, increase the difference in weight loss between the protected and unprotected samples. Unfortunately, this was not tested because of the large amount of furanone needed for long-period continuous experiments.

Although furanone has been shown to be a broad antagonist against Gram-positive bacteria (Kjelleberg, 1999) including *D. orientis*, as shown in the present study, the mechanism of inhibition has not been investigated. Using DNA microarrays, we have found that class I and III heat shock genes as well as 59 genes of unknown function are induced by 5 μg ml^{-1} furanone in Gram-positive *B. subtilis* (results not shown), and this may help to explain the toxicity of furanone on Gram-positive strains. However, as *B. subtilis* and *D. orientis* are genetically different, further work on *D. orientis* at a genetic level is necessary.

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