Differential Gene Expression To Investigate the Effect of (5Z)-4-Bromo-5-(Bromomethylene)-3-Butyl-2(5H)-Furanone on *Bacillus subtilis*

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(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. Using the gram-positive bacterium *Bacillus subtilis* as a test organism, we observed cell killing by 20 μg of furanone per ml, while 5 μg of furanone per ml inhibited growth approximately twofold without killing the cells. To discover the mechanism of this inhibition on a genetic level and to investigate furanone as a novel antibiotic, full-genome DNA microarrays were used to analyze the gene expression profiles of *B. subtilis* grown with and without 5 μg of furanone per ml. This agent induced 92 genes more than fivefold ($P < 0.05$) and repressed 15 genes more than fivefold ($P < 0.05$). The induced genes include genes involved in stress responses (such as the class III heat shock genes *clpC*, *clpE*, and *ctsR* and the class I heat shock genes *groES*, but no class II or IV heat shock genes), fatty acid biosynthesis, lichenan degradation, transport, and metabolism, as well as 59 genes with unknown functions. The microarray results for four genes were confirmed by RNA dot blotting. Mutation of a stress response gene, *clpC*, caused *B. subtilis* to be much more sensitive to 5 μg of furanone per ml (there was no growth in 8 h, while the wild-type strain grew to the stationary phase in 8 h) and confirmed the importance of the induction of this gene as identified by the microarray analysis.

Gram-positive bacteria are important causes of infectious diseases 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 (7). 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 [3, 34]) of gram-negative bacteria by affecting both the species-specific (16) and non-species-specific (26) quorum-sensing signals autoinducer 1 (AI-1) (acylated homoserine lactones) and AI-2, respectively. As a consequence, these agents inhibit swarming and biofilm formation of gram-negative bacteria without affecting cell growth (10, 12, 24, 26). Interestingly, the furanones were also found to inhibit growth of gram-positive bacteria at concentrations not toxic to mammalian cells (14). One of the best-studied natural furanones of *D. pulchra*, (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone (referred to as furanone below) (Fig. 1), inhibits the growth, swarming, and biofilm formation of *Bacillus subtilis* (25) in a manner distinct from that of *Pseudomonas aeruginosa* since

gram-positive bacteria do not have an acylated homoserine lactone signaling system (3).

While there have been several studies of the inhibitory effect of furanone on the phenotypes of gram-negative bacteria (16, 24, 26), little is known about the mechanism by which furanone inhibits the growth of gram-positive bacteria. To initiate an investigation of this mechanism on a genetic basis, DNA microarrays were used here to obtain the gene expression profiles of a model gram-positive bacterium, *B. subtilis*, with and without furanone. DNA microarrays have been used to monitor global gene expression profiles in response to different stimuli (31), including heat shock and other stresses (11, 38, 41), quorum sensing (5, 32), anaerobic metabolism (39), sporulation (8), and biofilm formation (26b, 28, 33, 37). DNA microarrays have also been used to study the global gene expression of *B. subtilis* in response to the antibiotic bacitracin (19). The microarray data obtained in the present study suggest that some type of stress response was induced by the furanone, and a mutant with a deletion in one of the induced stress response genes, *clpC*, was found to be more sensitive to growth inhibition by furanone. The identified induced genes are also candidates for drug discovery because they may be general genes used by gram-positive bacteria to thwart antibiotics.

MATERIALS AND METHODS

Bacterial strains and culture medium. Wild-type *B. subtilis* JH642 (*pheA1 trpC2*) was obtained from the *Bacillus* Genetic Stock Center (Columbus, Ohio). *B. subtilis* 168 (*trpC2*) and its *clpC* mutant *B. subtilis* QB4756 (*trpC2 ΔclpC::spc*) were kindly provided by Tarek Msadek (Institut Pasteur, Paris, France). Luria-Bertani (LB) medium (27) containing 10 g of tryptone per liter, 5 g of yeast

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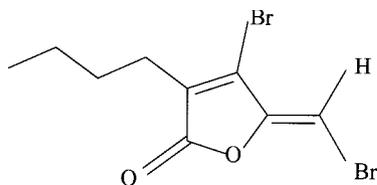
FIG. 1. Structure of furanone.

extract per liter, and 10 g of NaCl per liter was used to culture the *B. subtilis* strains.

Furanone synthesis. Furanone (Fig. 1) was synthesized by the method of Beechan and Sims (4), as well as by the four-step method of Manny et al. (18), with the following three changes: (i) the decarboxylation step was conducted by refluxing in benzene rather than in toluene, (ii) the lactone formation step (the last step) was conducted in a water bath (100°C) rather than in an oil bath (110 to 120°C), and (iii) the furanone was purified by column chromatography with hexane and ethyl acetate at a ratio of 100:1 (2.5- by 60-cm column; Spectrum Chromatography, Houston, Tex.). The structure of the purified product was verified by ^{1}H nuclear magnetic resonance (DRX-400MHz; Bruker Billerica, Mass.) (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 infrared analysis (Nicolet-Magna-IR 560; Nicolet-Magna, Madison, Wis.) (reciprocal absorbing wavelengths, 2,958, 1,793, 1,610, 1,276, 1,108, and 1,030 cm^{-1}) by comparison with previously published furanone spectra (4, 18). The identity of the furanone product synthesized by the method of Manny et al. (18) and the product synthesized by the method of Beechan and Sims (4) was also verified by thin-layer chromatography (hexane/ethyl acetate ratio, 20:1; R_f, 0.8; Silica Gel 60, F-254; Selecto Scientific, Suwanee, Ga.). Furanone stock solutions (14.9 mg/ml in 95% ethanol) were stored at 4°C.

Furanone and *B. subtilis* gene expression studied with DNA microarrays. To identify which genes are affected by addition of furanone to gram-positive bacteria, *B. subtilis* JH642 was grown in LB medium overnight at 37°C and diluted 1:100 into fresh 37°C LB medium. When the cells reached an optical density at 600 nm (OD$_{600}$) of 0.8, the culture was split equally into three portions, and 0, 5, or 20 μg of furanone per ml was added (the same amount of ethanol was added to all samples to eliminate the effect of solvent). Furanone at a concentration of 5 μg/ml decreased the rate of cell growth approximately twofold, but it did not kill the cells, while 20 μg of furanone per ml had a stronger inhibitory effect (see Results). After incubation for 15 min, the cells were harvested by centrifugation for 15 s (maximum speed) in −80°C mini bead beater tubes (catalog no. 10832; Biospec Products, Bartlesville, Okla.). The cell pellets were flash frozen in a dry ice-ethanol bath and stored at −80°C until RNA was isolated.

Total RNA isolation. To lyse the cells, 1.0 ml of RLT buffer (QIAGEN, Inc., Valencia, Calif.) and 0.2 ml of 0.1-mm zirconia/silica beads (Biospec) were added to the frozen tubes containing cell pellets. The tubes were closed tightly and beaten for 60 s at the maximum speed in a mini bead beater (catalog no. 3110BX; Biospec). Total RNA was isolated by using the protocol of an RNeasy mini kit (QIAGEN), including on-column DNase digestion with RNase-free DNase I (QIAGEN). The OD$_{260}$ was used to quantify the RNA yield, and the OD$_{280}$ was used to quantify the protein; hence, OD$_{260}$/OD$_{280}$ and 23S rRNA/16S rRNA ratios were determined to check the purity and integrity of the RNA (RNeasy mini kit handbook; QIAGEN).

DNA microarrays. The *B. subtilis* DNA microarrays were prepared as described previously (39). Each gene probe was synthesized by PCR and was the size of the full open reading frame (200 to 2,000 nucleotides). The double-stranded PCR products were denatured in 50% dimethyl sulfoxide and spotted onto aminosilane slides (Full Moon Biosystems, Sunnyvale, Calif.) as probes to hybridize with the mRNA-derived cDNA samples. It has been shown that each array can detect 4,020 of the 4,100 *B. subtilis* open reading frames (39). Each gene has two spots per slide.

Synthesis of Cy3- or Cy5-labeled cDNA. To convert the total RNA into labeled cDNA, reverse transcription was performed in a 1.5-ml microcentrifuge tube to which 10 μg of total RNA and 6 μg of random hexamer primers (Invitrogen Corp., Carlsbad, Calif.) were added, and the volume was adjusted to 24 μl with RNase-free water (Invitrogen Corp.). The mixture was incubated for 10 min at 70°C and then for 10 min at room temperature for annealing. To this mixture, 8 μl of 5× SuperScript II reaction buffer (Invitrogen Corp.), 4 μl of 0.1 M dithiothreitol (Invitrogen Corp.), 1 μl of a deoxynucleoside triphosphate mixture (2 mM dATP, 2 mM dGTP, 2 mM dTTP, and 1 mM dCTP), 1 μl of 0.5 mM Cy3-

or Cy5-labeled dCTP (Amersham Biosciences, Piscataway, N.J.), and 2 μl of SuperScript II reverse transcriptase (10 U/μl; Invitrogen Corp.) were added to make cDNA. cDNA synthesis was conducted at 42°C for 2 h and stopped by heating at 94°C for 5 min. After cDNA synthesis, the RNA template was removed with 2 μl of 2.5 M NaOH. The pH was neutralized with 10 μl of 2 M HEPES buffer, and the cDNA was purified with a Qiaquick PCR mini kit (QIAGEN, Inc.). The efficiency of labeling was checked by determining the absorbance at 260 nm for the cDNA concentration, the absorbance at 550 nm for Cy3 incorporation, and the absorbance at 650 nm for Cy5 incorporation.

Hybridization and washing. The control RNA samples (containing no furanone) and the test RNA samples (containing 5 μg of furanone per ml) were each labeled with both the Cy3 and Cy5 dyes to remove artifacts related to different labeling efficiencies. Each experiment required two slides; the Cy3-labeled no-furanone sample and the Cy5-labeled sample with furanone were hybridized on the first slide, and, similarly, the Cy5-labeled no-furanone sample and the Cy3-labeled sample with furanone were hybridized on the second slide. Since each gene had two spots on a slide, the two hybridizations generated eight data points for each gene (four points for the furanone sample and four points for the no-furanone sample).

The DNA microarrays were incubated in a prehybridization solution containing 3.5× SSC (Invitrogen), 0.1% sodium dodecyl sulfate (SDS) (Invitrogen), and 0.1% bovine serum albumin (Invitrogen) at 45°C for 20 min (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The arrays were rinsed with double-distilled water and spun dry by centrifugation. The labeled cDNA (6 μg) was concentrated to 10 μl (total volume) and mixed with 10 μl of 4× cDNA hybridization solution (Full Moon Biosystems) and 20 μl of formamide (EM Science, Gibbstown, N.J.). The hybridization mixture was heated at 95°C for 2 min and added to the DNA microarrays; each array was covered with a coverslip (Corning, Big Flats, N.Y.) and incubated overnight at 37°C for hybridization. When the hybridization was finished, the coverslips were removed in 1× SSC–0.1% SDS at room temperature, and the arrays were washed once for 5 min in 1× SSC–0.1% SDS at 40°C, twice for 10 min in 0.1× SSC–0.1% SDS at 40°C, and twice for 1 min in 0.1× SSC at 40°C. The arrays were quickly rinsed by dipping them in room temperature double-distilled water and then spun dry by centrifugation.

Image and data analysis. The hybridized slides were scanned with a Generation III array scanner (Molecular Dynamics Corp.), and 570 and 670 nm were used to quantify the cDNA probes labeled with Cy3 and Cy5 separately. The signal was quantified with Array Vision 4.0 or 6.0 software (Imaging Research, St. Catherines, Ontario, Canada). Genes were identified as differentially expressed (i.e., induced or repressed) if the expression ratio was greater than fivefold (one standard deviation) and the P value (as determined by a t test) was less than 0.05; in a few cases some genes did not meet these criteria and are listed below as up-regulated. Including the P value criterion ensured the reliability of the induced-repressed gene list. P values were calculated with log-transformed, normalized intensities. Normalization was relative to the median total fluorescence intensity per slide per channel. The gene functions were obtained from the database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

RNA dot blotting. DNA probes for four representative genes, *clpC*, *ctsR*, *groES*, and *pyrF*, were synthesized by using a PCR DIG probe synthesis kit (Roche Applied Science, Mannheim, Germany). Most of the probes were 400 bp long; the only exception was the *groES* probe, which was only 276 bp long due to the small size of the *groES* genes. Total RNA (1.25, 2.5, or 5 μg for each sample) from independent experiments (which were different from the experiments used to harvest RNA for the DNA microarrays) was blotted on positively charged nylon membranes (Boehringer Ingelheim, Ridgefield, Conn.) by using a Bio-Dot microfiltration apparatus (Bio-Rad, Richmond, Calif.). RNA was fixed by baking the membranes for 2 h at 80°C, and about 400 ng of excess DNA probes was denatured in boiling water for 5 min before hybridization to RNA (serial dilutions of RNA samples were tested in each blot to ensure that there was an excess of the DNA probes). Hybridization (50°C, 16 h) and washes were conducted by following the protocol for DIG labeling and detection (Roche Applied Science). To detect the signal, disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3.3.1.1,7] decan}-4-yl) phenyl phosphate (Roche Applied Science) was used as a substrate to give chemiluminescence, and the light was recorded with Biomax X-ray film (Kodak, Rochester, N.Y.).

Sensitivity of *B. subtilis* to furanone due to the *clpC* mutation. To each 250-ml shake flask, 25 ml of LB medium and 0, 5, 20, or 40 μg of furanone (14.9 mg/ml in 95% ethanol) per ml were added. Appropriate amounts of ethanol were added to eliminate the effect of solvent. The cultures were inoculated directly from glycerol stocks (15 μl for wild-type *B. subtilis* 168 and 30 μl for the *clpC* mutant *B. subtilis* QB4756 because the density of the mutant in stocks was lower due to its defect in stationary-phase growth [21]). The growth of both strains with

different concentrations of furanone was monitored by measuring the OD_{600}, and the specific growth rates were calculated. These experiments were conducted in duplicate.

Epifluorescence microscopy. Ten milliliters of LB broth in a 100-ml shake flask was inoculated with 100 μl of an overnight culture of *B. subtilis* 168. Growth at 37°C was monitored, and when the OD_{600} reached 0.2, either no furanone or 40 μg of furanone per ml was added (the same amount of ethanol was added to both samples). The fixation method was based on the method of Amann et al. (1). Aliquots (250 μl) were removed from the culture flasks 30 and 60 min after the addition of furanone and then mixed immediately with 750 μl of 4% paraformaldehyde–phosphate-buffered saline (130 mM sodium chloride, 10 mM sodium phosphate buffer; pH 7.2) and incubated at 4°C for 3 h. Cells were centrifuged and washed once with phosphate-buffered saline. Fixed cells were resuspended in a phosphate-buffered saline–100% ethanol solution (1:1, vol/vol) and stored at −20°C. Fixed cells were stained with 1 μg of 4′,6-diamidino-2-phenylindole (DAPI) per ml in 50% glycerol.

DAPI-stained cells were viewed by epifluorescence microscopy by using a DMLB microscope with an N PLAN 100×/1.25 numerical aperture oil immersion objective (Leica Microsystems, Wetzlar, Germany); the light source for UV excitation (450 to 490 nm) was a 100-W mercury lamp. Images were captured with a DC100 digital camera by utilizing the IM50 software package (version 1.20, release 9; Leica Microsystems). Twenty fields were analyzed for each treatment.

Confocal laser scanning microscopy. As described above for epifluorescence microscopy, when the OD_{600} reached 0.2, no furanone or 20 μg of furanone per ml was added to a *B. subtilis* 168 culture (the same amount of ethanol was added to both samples). Aliquots (330 μl) were removed from the culture flasks 15 min after the addition of furanone, and the cells were stained with a LIVE/DEAD *Bac*Light bacterial viability kit (Molecular Probes, Eugene, Oreg.) by adding 1 μl of SYTO 9 dye (from a 3.34 mM SYTO 9 stock solution in dimethyl sulfoxide) and 1 μl of propidium iodide (from a 20 mM propidium iodide stock solution in dimethyl sulfoxide). The mixture was then incubated for 15 min.

The stained cells were viewed by confocal laser scanning microscopy by using a DM IRB microscope with a PL APO 100×/1.4 numerical aperture oil immersion objective (true confocal system; TCS SP; Leica Microsystems). The excitation wavelength was 488 nm (argon laser), and fluorescence signals were collected in two photomultiplier tubes set at collection ranges of 497 to 532 nm for SYTO 9 (green) and 608 to 655 nm for propidium iodide (red).

RESULTS

Furanone caused changes in gene expression in *B. subtilis*. To study growth inhibition of gram-positive bacteria caused by furanone, DNA microarrays were used to analyze the gene expression profiles of the model gram-positive species *B. subtilis* with and without furanone treatment. *B. subtilis* cells at the late exponential phase (OD_{600}, 0.8) were incubated with and without 5 μg of furanone per ml for 15 min; this concentration of furanone was chosen because it inhibited cell growth approximately twofold without killing the cells (see below). Under these conditions furanone induced 92 genes (Table 1) and repressed 15 genes (Table 2) more than fivefold. Genes in the same operons were induced together (e.g., of the 30 induced genes with known functions, 6 operons were induced, including the *rbsABCDKR* genes that were individually induced 12- to 46-fold) or repressed together (e.g., *pyrABDDIIEF* were all repressed 7- to 50-fold), indicating that the RNA was of good quality and the hybridization was efficient.

Since the labeling efficiencies of Cy3 and Cy5 can be different, as reported previously (40), a dye-swapping experiment was performed to eliminate any artificial effect in labeling, as well as to provide an independent means to verify the results for the first chip. The data from the dye-swapping experiments in this study agreed very well, indicating that the two dyes labeled consistently. For example, for the fatty acid biosynthesis gene *yjaY* (Table 1) (also known as *fabF*), the original with-furanone/without-furanone ratios for the level of tran-

scription were determined to be 18.1/1.5 and 21.6/1.6 (average transcription induction level, 12.8-fold) with the Cy3-labeled no-furanone sample and the Cy5-label furanone-containing sample, respectively, and 33.5/3.1 and 15.8/2.0 (average transcription level, 9.4-fold) with the Cy3-labeled furanone-containing sample and the Cy5-labeled no-furanone sample, respectively.

Several stress response genes were also induced by furanone (Table 1). The most noticeable among these genes were some class III heat shock genes, including *clpP*, *clpE*, *ctsR*, *yacH*, *yacI*, and *clpC* (30). From the microarray results, it was found that furanone induced five of the genes (*clpC*, 13.2-fold; *clpE*, 89.4-fold; *ctsR*, 15.8-fold; *yacH*, 9.0-fold; and *yacI*, 19.8-fold) and up-regulated *clpP* (3.5-fold, less than the 5-fold cutoff ratio). In addition to their roles in the heat shock response (*clpC* and *clpP* mutants grow at 37°C but not at 54°C [21, 22]), class III heat shock genes are also involved in general stress responses (6), and the *clpC* and *clpP* gene products interact with two-component systems involved in modulating sporulation, competence, and exocellular enzyme synthesis (20–22). Hence, the induction of class III heat shock genes and the increased sensitivity of the *clpC* mutant to furanone (see below) suggest that the class III heat shock genes may be necessary for cell survival with furanone and that the furanone may act as a stressor directly or indirectly.

In addition to class III heat shock genes, some other heat shock genes were also up-regulated (the differential expression was less than the 5-fold cutoff ratio); these genes included *dnaK* (2.8-fold), *grpE* (3.3-fold), *groES* (7.0-fold), *groEL* (4.8-fold), and *dnaJ* (2.1-fold). Since the RNA was isolated only 15 min after furanone was added, the induction of these genes took place in less than one doubling time (17.4 min for these conditions). The rapid induction of these stress response genes may help refold lightly damaged proteins and degrade seriously damaged proteins, but how these gene products could allow cells to respond to furanone treatment is not clear (23, 30).

Furanone also greatly induced four genes whose products are involved in fatty acid biosynthesis (Table 1): *fabD* (8.4-fold), *fabHA* (8.5-fold), *fabHB* (74-fold), and *fabF* (11-fold). *fabHA* and *fabF* are likely to belong to the same operon (9), which is consistent with their levels of induction by furanone. The fatty acid biosynthesis pathway is necessary for cell viability and is the target of the antibiotics isoniazid, triclosan, and cerulenin (9).

Furanone at a concentration of 5 μg/ml was also found to repress 15 genes more than fivefold (Table 2). The most noticeable among these genes are the *pyr* genes for pyrimidine biosynthesis, including *pyrAB* (−7.1-fold), *pyrD* (−6.7-fold), *pyrDII* (−7.7-fold), *pyrE* (−33.3-fold), and *pyrF* (−50.0-fold). This may have been due to the repression of the regulatory gene *pyrR* (−2-fold) (data not shown), which encodes a factor that can cause termination of transcription of the *pyr* operon (15). It is also possible that furanone, which contains a furan ring, binds to PyrR and thus directly leads to premature termination of transcription of the *pyr* operon mRNA. It should be noted that the *divIVA* genes that function in initiation of cell division were down-regulated 2.3-fold by 5 μg of furanone per ml, supporting the twofold inhibition of growth seen with furanone. However, these genes were found to be down-regulated

TABLE 1. *B. subtilis* genes induced by 5 μg of furanone per ml

Gene	Induction expression ratio	Function
Metabolism		
acoR	192.3	Positive regulation of the acetoin dehydrogenase operon (*acoABCL*)
acuB	7.1	Acetoin utilization
glpF	34.7	Glycerol utilization
glpK	6.3	Glycerol utilization
licH	67.3	Lichenan degradation product utilization
Fatty acid biosynthesis		
fabD	8.4	Malonyl coenzyme A-acyl carrier protein transacylase
fabHA	8.5	Also known as *yjaX*; 3-oxoacyl-acyl-carrier protein synthase III A
fabHB	74.1	Also known as *yhfB*; 3-oxoacyl-acyl-carrier protein synthase III B
fabF	10.9	Also known as *yjaY*; 3-oxoacyl-acyl-carrier protein synthase II
Stress response and drug resistance		
clpC	13.2	Negative regulator of late competence genes; positive regulator of autolysin (LytC and LytD) synthesis; class III stress response-related ATPase
clpE	89.4	ATP-dependent Clp, protease-like
ctsR	15.8	Negative regulation of class III stress genes
groES	7.0	Class I heat shock protein (molecular chaperonin)
mdr	13.4	Resistance to puromycin, nerfloxacin, and tosufloxacin, but not CCCP; multidrug efflux transporter[a]
mmr	22.8	Methylenomycin A resistance protein
Transport		
kapB	22.4	Required for activation of KinB in the initiation of sporulation
kbl	23.3	2-Amino-3-ketobutyrate coenzyme A ligase (glycine acetyl transferase)
licA	9.2	Phosphotransferase system lichenan-specific enzyme IIA component
licB	134.5	Phosphotransferase system lichenan-specific enzyme IIB component
licC	16.9	Phosphotransferase system lichenan-specific enzyme IIC component
rbsA	11.9	Ribose ABC transporter (ATP-binding protein)%[b]
rbsB	24.6	Ribose ABC transporter (ribose-binding protein)
rbsC	12.8	Ribose ABC transporter (permease)
rbsD	46.2	Ribose ABC transporter (membrane protein)
rbsK	22.0	Ribose metabolism
rbsR	28.6	Negative regulation of ribose operon (*rbsRKDACB*); transcriptional regulator (LacI family)
Synthesis and other functions		
resC	8.7	Essential protein similar to cytochrome *c* biogenesis protein
ribB	6.9	Riboflavin synthase (alpha subunit)
ribG	21.9	Riboflavin biosynthesis
ribH	7.8	Riboflavin synthase (beta subunit)
sigY	6.8	RNA polymerase ECF-type sigma factor (sigma-Y)
spoIIE	9.8	Dephosphorylates SpoIIAA-P and overcomes SpoIIAB-mediated inhibition of sigma-F, required for normal formation of the asymmetric septum (stage II sporulation)
tdh	6.2	Threonine catabolism
Unknown functions		
yabQ	17.6	Unknown
yacH	9.0	Unknown
yacI	19.8	Unknown
ycnC	7.7	Unknown, similar to transcriptional regulator (TetR/AcrR family)
ycnD	37.9	Unknown, similar to NADPH-flavin oxidoreductase
ycnE	40.6	Unknown
ydbH	6.1	Unknown, similar to C4-dicarboxylate transport protein
ydbM	6.7	Unknown, similar to butyryl-coenzyme A dehydrogenase
ydeQ	14.1	Unknown, similar to NAD(P)H oxidoreductase
ydfJ	17.7	Unknown, similar to antibiotic transport-associated protein
ydgI	7.7	Unknown, similar to NADH dehydrogenase
ydgJ	9.3	Unknown, similar to transcriptional regulator (MarR family)
ydiM	6.1	Unknown
yesE	6.7	Unknown
yfiE	495.4	Unknown
yfkO	10.1	Unknown, similar to NAD(P)H-flavin oxidoreductase
yhcL	80.7	Unknown, similar to sodium-glutamate symporter
yhgD	6.9	Unknown, similar to transcriptional regulator (TetR/AcrR family)
yhgE	9.8	Unknown, similar to phage infection protein
yitA	6.3	Unknown, similar to sulfate adenylyltransferase
ykcA	13.4	Unknown, similar to ABC transporter (binding protein)
ykoM	9.1	Unknown, similar to transcriptional regulator (MarR family)
ykvM	6.5	Unknown
ylbP	9.7	Unknown
ylnB	11.3	Unknown, similar to sulfate adenylyltransferase
ylnC	59.4	Unknown, similar to adenylylsulfate kinase
ylnD	682.6	Unknown, similar to uroporphyrin-III C-methyltransferase
ylnE	8.9	Unknown
ylnF	43.9	Unknown, similar to uroporphyrin-III C-methyltransferase

TABLE 1—*Continued*

Gene	Induction expression ratio	Function
ymaA	5.7	Unknown, similar to ribonucleoprotein
yocJ	51.6	Unknown, similar to acyl-carrier protein phosphodiesterase
yodC	7.5	Unknown, similar to nitroreductase
yodS	35.9	Unknown, similar to 3-oxoadipate coenzyme A transferase
yosP	8.2	Unknown, similar to ribonucleoside-diphosphate reductase (beta subunit)
yozB	10.5	Unknown
yqjM	15.6	Unknown, similar to NADH-dependent flavin oxidoreductase
yrhA	71.7	Unknown, similar to cysteine synthase
yrhB	17.0	Unknown, similar to cystathionine gamma-synthase
yrkF	10.1	Unknown
yrkI	15.8	Unknown
yrrT	54.1	Unknown
ysbA	28.6	Unknown
ysxA	7.6	Unknown, similar to DNA repair protein
yusR	13.5	Unknown, similar to 3-oxoacyl-acyl-carrier protein reductase
yvbT	8.8	Unknown, similar to alkanal monooxygenase
yvgW	9.8	Unknown, similar to heavy metal-transporting ATPase
yvrD	32.7	Unknown, similar to ketoacyl-carrier protein reductase
ywgB	35.8	Unknown
ywjE	12.4	Unknown, similar to cardiolipin synthetase
ywnA	66.4	Unknown
ywnB	2,907.2	Unknown
ywnF	8.9	Unknown
ywsB	13.5	Unknown
yxeI	6.6	Unknown, similar to penicillin amidase
yxeK	6.9	Unknown
yxeL	7.6	Unknown
yxeM	17.3	Unknown, similar to amino acid ABC transporter (binding protein)
yxeN	15.9	Unknown, similar to amino acid ABC transporter (permease)
yxeO	6.2	Unknown, similar to amino acid ABC transporter (ATP-binding protein)

[a] CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.
[b] ABC, ATP binding cassette.

only by furanone and were not repressed (so they are not shown in Table 2), because the cutoff ratio was fivefold.

RNA dot blotting confirmed the microarray results. To validate the gene expression profiles obtained from the microarray hybridizations, total RNA from *B. subtilis* cells grown with and without 5 µg of furanone per ml was isolated in the same way that RNA was isolated in the microarray experiments, and the expression of four genes (randomly chosen as representative induced and repressed genes) was determined by RNA dot blot analysis. Four genes (*clpC*, *ctsR*, *groES*, and *pyrF*) were checked, and the expression ratios of all four agreed with the microarray results (Table 3). For example, *clpC* was induced 13.2-fold in the microarray experiments and was induced 40-fold in the RNA dot blotting experiment.

***clpC* mutant is more sensitive to furanone.** The induction of stress response genes of *B. subtilis* is reasonable given the inhibitory effect of furanone on gram-positive bacteria. Hence, mutation of a stress response gene may cause the cells to be

TABLE 2. *B. subtilis* genes repressed by 5 µg of furanone per ml

Gene	Repression expression ratio	Function
ackA	−6.3	Acetate kinase
ctrA	−9.1	Pyrimidine biosynthesis
hom	−11.1	Threonine/methionine biosynthesis, homoserine dehydrogenase
pyrAB	−7.1	Pyrimidine biosynthesis, carbamoyl-phosphate synthetase (catalytic subunit)
pyrD	−6.7	Pyrimidine biosynthesis, dihydroorotate dehydrogenase
pyrDII	−7.7	Pyrimidine biosynthesis, dihydroorotate dehydrogenase (electron transfer subunit)
pyrE	−33.3	Pyrimidine biosynthesis
pyrF	−50.0	Pyrimidine biosynthesis
spoIVB	−5.9	Intercompartmental signaling of pro-sigma-K processing/activation in the mother cell, essential for spore cortex and coat formation (stage IV sporulation)
thrB	−7.1	Threonine biosynthesis
yfiY	−6.3	Unknown, similar to iron(III) dicitrate transport permease
yhbF	−8.3	Unknown
yvaX	−11.1	Unknown
ywqE	−5.9	Unknown, similar to capsular polysaccharide biosynthesis
yycA	−6.3	Unknown

TABLE 3. Gene expression confirmed by RNA dot blotting

Gene	Function	Expression ratio from DNA microarrays	Expression ratio from RNA dot blotting
clpC	Negative regulator of late competence genes, positive regulator of autolysin (LytC and LytD) synthesis, class III stress response-related ATPase	13.2	40
ctsR	Negative regulation of class III stress genes	15.8	40
groES	Class I heat shock protein (molecular chaperonin)	7.0	15
pyrF	Pyrimidine biosynthesis	−50.0	−20

more sensitive to furanone. To test this hypothesis, wild-type and isogenic *clpC B. subtilis* strains were grown with different concentrations of furanone (0, 5, 20, and 40 μg/ml), and growth was monitored (Fig. 2). Both the wild type and the *clpC* mutant grew well without furanone, and the specific growth rate of the *clpC* mutant was 23% lower than that of the wild type (2.385 ± 0.008 and 1.830 ± 0.001 h^{-1} for the wild type and the *clpC* mutant, respectively). In the presence of furanone, however, the growth rate was reduced more significantly for the *clpC* mutant; 5 μg of furanone per ml decreased the growth rate of the wild-type strain by 43% (to 1.354 ± 0.070 h^{-1}), but no growth was observed after 8 h for the *clpC* mutant. Higher concentrations of furanone (20 and 40 μg/ml) inhibited growth completely over an 8-h period for both the wild type and the *clpC* mutant.

Furanone is bactericidal for *B. subtilis*. Since furanone at concentrations of 20 and 40 μg/ml completely inhibited the growth of both wild-type *B. subtilis* 168 and the *clpC* mutant (*B. subtilis* QB4756), we investigated whether furanone was bacteriostatic or bactericidal. After treatment with 20 μg of furanone per ml, the viability assay (Fig. 3) indicated that a significant portion of the furanone-treated cells were dead (no dead cells were seen with the sample containing no furanone); hence, this concentration of furanone kills *Bacillus* cells. Similar results were obtained for *B. subtilis* JH642 (data not shown). In addition, DAPI staining of untreated cells showed that these cells uniformly had clearly defined nucleoids, while DAPI-stained cells treated with 40 μg/ml showed a diffuse staining pattern and most of the treated cells lacked a clearly defined nucleoid (Fig. 4).

DISCUSSION

Natural furanone inhibits the growth of *B. subtilis* significantly, as found in this study and previously (25); 5 μg of furanone per ml decreased the growth rate of the wild-type strain by 43%, and 20 μg of furanone per ml inhibited growth completely. In contrast, furanone has no effect on the growth rate of gram-negative bacteria (26), while it inhibits the multicellular behavior of these organisms, including swarming (10, 26), bioluminescence (17), and biofilm formation (12, 26). The mechanism of this inhibition for gram-negative bacteria was delineated with DNA microarrays, which indicated that these phenotypes were the result of interference with both the AI-1 and AI-2 signaling systems. Hentzer et al. (13) found that the synthetic compound 4-bromo-5-(bromomethylene)-2(5H)-furanone in *P. aeruginosa* PAO1 repressed 80% of the genes that were induced by AI-1 quorum sensing, and the natural furanone in the present study repressed 79% of the genes that were induced by AI-2 in *Escherichia coli* (26a).

Here, the gene expression pattern of *B. subtilis* was identified by using DNA microarrays, which showed that a sublethal furanone concentration (5 μg/ml) rapidly leads to the induction of specific genes, a significant number of which are related to the stress response (rather than the genes related to signaling, as seen with gram-negative strains). However, general growth genes (such as the *divIVA* genes discussed above) were not identified as repressed due to the fivefold cutoff ratio for the microarrays and the limited growth inhibition (twofold) caused by the addition of 5 μg of furanone per ml. *B. subtilis* has four classes of heat shock genes (6), but furanone induced only the class I and III genes (no class II or class IV heat shock genes were induced) at nonbactericidal concentrations. It is also interesting that furanone induced *ctsR* (15.8-fold), since this gene encodes a negative regulator of class III heat shock genes (6). Since all of the class III heat shock genes were induced by furanone (Table 1), there might be some additional mechanism that controls the expression of these class III heat shock genes.

Besides the induction of the stress response genes noted above and 59 genes with unknown functions, the ribose transport and metabolism genes were also induced by furanone; *rbsA* (11.9-fold), *rbsB* (24.6-fold), *rbsC* (12.8-fold), *rbsD* (46.2-fold), and *rbsK* (22.0-fold) were all induced (Table 1). The reason for induction of these genes is not clear. However, the existence of a ribose-like ring in furanone suggests that furanone might have some direct effects on the proteins encoded by these genes. It would be interesting to examine the furanone
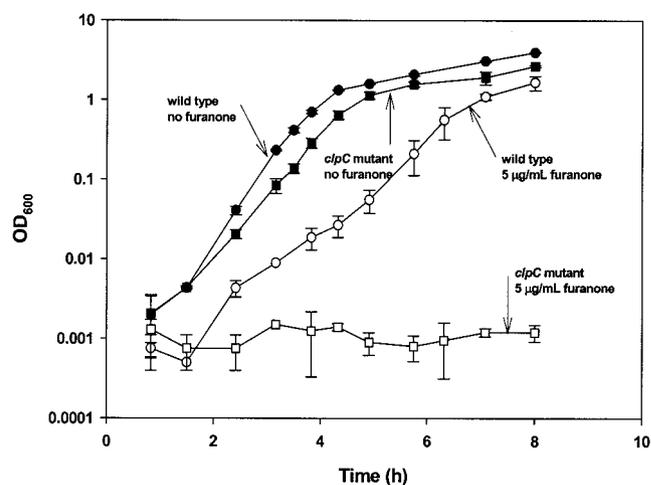

FIG. 2. *clpC* mutation increases the sensitivity of *B. subtilis* to furanone.
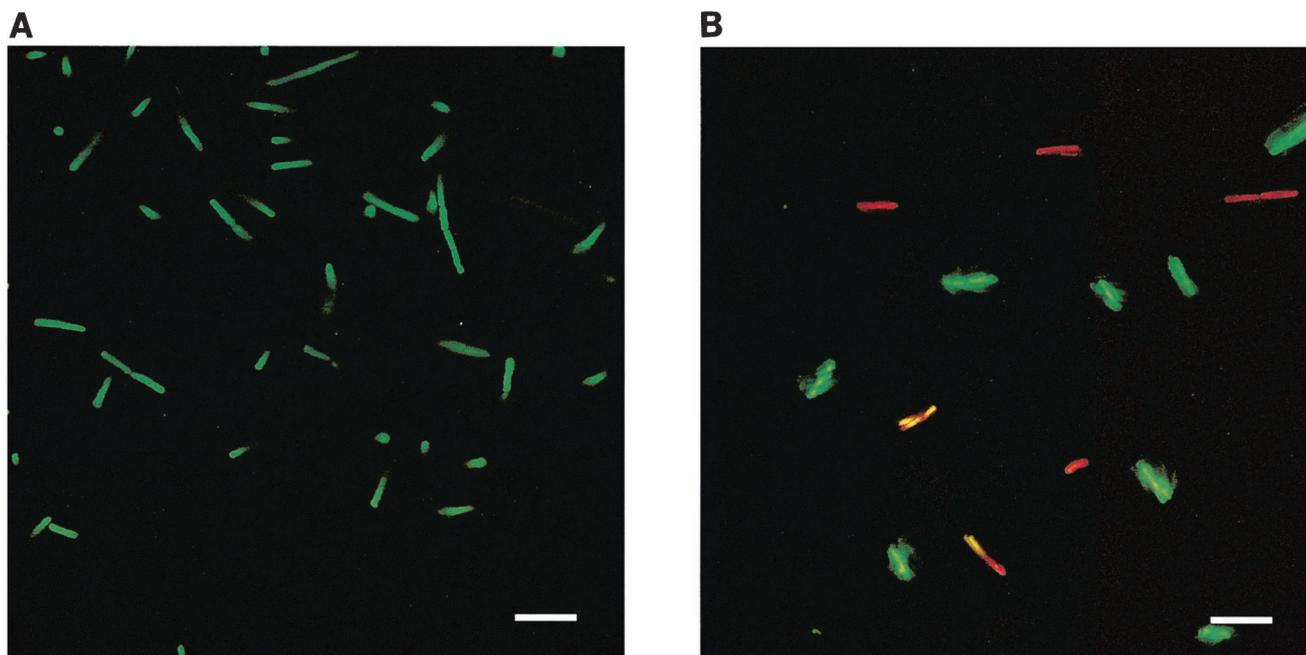
A

B

FIG. 3. *B. subtilis* 168 treated with no furanone (A) or with 20 μg of furanone per ml (B) and stained with the LIVE/DEAD viability stain. Live cells are green, and dead cells are red. Scale bars = 10 μm.

sensitivity of a strain that can neither transport nor catabolize ribose.

The 9- to 165-fold induction of *licABCH* is also interesting (Table 1). These four genes form an operon and encode proteins for importing and utilizing oligomeric β-glucosides (compounds which are probably not present in LB medium) as carbon sources. These oligosaccharides are produced by degrading β-1,3-1,4-glucan (lichenan) with the β-1,3-1,4-endoglucanase encoded by *licS* (29, 35, 36). *licS* (previously named *bglS* [35]) was also found to be induced by furanone in the present study (12.9-fold); however, the *P* value was higher than 0.05 (data not shown).

The extracytoplasmic factor gene *sigY* was also induced by

furanone. However, none of the other 16 *sigY*-controlled genes (2) was induced by furanone. This might have been because of the short contact time, and more genes might be induced upon longer incubation with furanone. It is also notable that 15 genes with unknown functions, including 5 genes related to pyrimidine biosynthesis, were repressed (Table 2).

Two other sets of gene chip experiments with RNA from cells grown with 5 and 20 μg of furanone per ml were also conducted, but lysozyme rather than the bead beater was used to lyse the cells for RNA isolation (another four microarrays). The results obtained with this RNA were generally similar to the results obtained with bead beater-lysed RNA (there was 80% agreement in the operons that were induced or repressed,
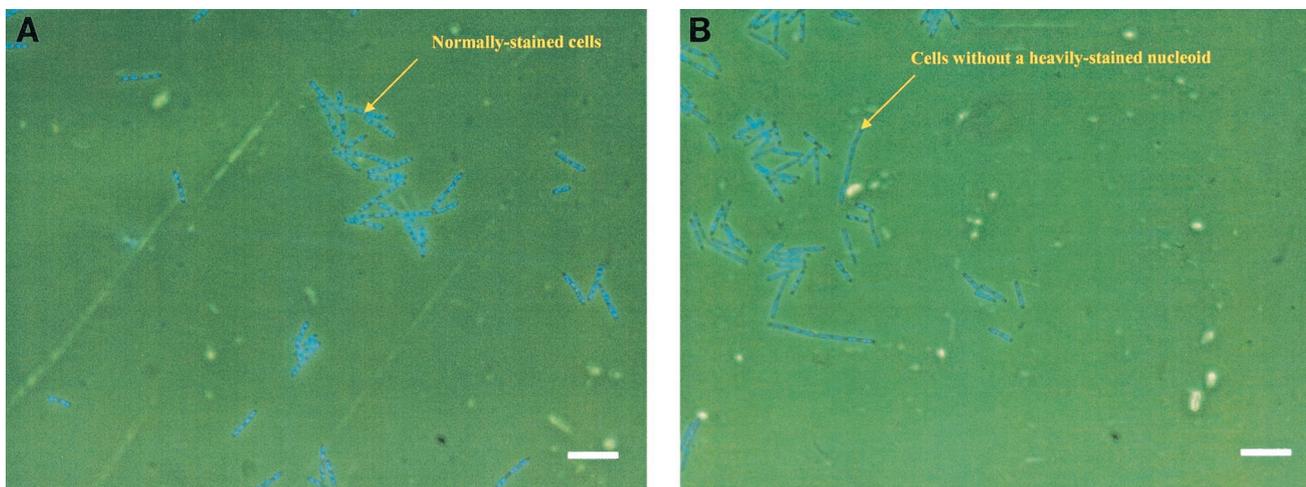
A　Normally-stained cells

B　Cells without a heavily-stained nucleoid

FIG. 4. *B. subtilis* 168 treated with no furanone (A) or with 40 μg of furanone per ml (B) and stained with DAPI to allow observation of nucleoids. DNA is indicated by blue fluorescence. Scale bars = 10 μm.

indicating the reproducibility of these experiments); however, since the bead beater method lyses cells faster (1 min) than the lysozyme method (5 to 10 min), the RNA quality is expected to be better for the bead beater method, and so this set of data was used for our analysis.

Microscopic analysis indicated that furanone acts as a bactericide at higher concentrations. The red cells seen upon viability staining demonstrated that the furanone treatment (30 min) caused a significant portion of the treated cells to become more permeable, presumably as a result of damage to the plasma membrane. DAPI staining demonstrated that the furanone treatment had a significant effect on the nucleoid distribution in the treated cells. It should be noted that the concentrations of furanone used for microscopy were higher than the concentration used for the microarrays since higher concentrations were required to see changes in the population.

Further study of the furanone-induced genes may help us understand the strategies used by gram-positive bacteria for survival under stress. Also, the furanone-induced genes found in the microarrays are candidates for discovering drugs with activity against gram-positive bacteria.

ACKNOWLEDGMENTS

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