# Gene Expression in *Bacillus subtilis* Surface Biofilms With and Without Sporulation and the Importance of *yveR* for Biofilm Maintenance

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**Abstract:** Five independent DNA microarray experiments were used to study the gene expression profile of a 5-day *Bacillus subtilis* air–liquid interface biofilm relative to planktonic cells. Both wild-type *B. subtilis* and its sporulation mutant (Δ*spoIIGB*::*erm*) were investigated to discern the important biofilm genes (in the presence and absence of sporulation). The microarray results indicated that suspension cells were encountering anaerobic conditions, and the air–liquid interface biofilm was metabolically active. For the statistically significant differential expression ($P < 0.05$), there were 342 genes induced and 248 genes repressed in the wild-type biofilm, whereas 371 genes were induced and 128 genes were repressed in the sporulation mutant biofilm. The microarray results were confirmed with RNA dot blotting. A small portion of cells (1.5%) in the wild-type biofilm formed spores and sporulation genes were highly expressed. In the biofilm formed by the sporulation mutant, competence genes (*comGA*, *srfAA*, *srfAB*, *srfAD*, and *comS*) were induced which indicate a role for quorum sensing (bacterial gene expression controlled by sensing their population) in biofilms. There were 53 genes consistently induced in the biofilms of both the wild-type strain and its *spoIIGB* mutant—those genes have functions for transport, metabolism, antibiotic production—and 26 genes with unknown functions. Besides the large number of genes with known functions induced in the biofilm (121 genes in the wild-type biofilm and 185 genes in the sporulation mutant biofilm), some genes with unknown functions were also induced (221 genes in the wild-type biofilm and 186 genes in the sporulation mutant biofilm), such as the *yve* operon which appears to be involved in polysaccharide synthesis and the *ybc* operon which inhibits the growth of competitors for nutrients. A knockout mutant of *yveR* was constructed, and the mutant showed major defects in biofilm maintenance. Both the wild-type strain and its sporulation mutant formed normal biofilms, suggesting complete sporulation is not necessary for biofilm formation. The expression profiles of these two strains share more repressed genes than induced genes, suggesting that the biofilm cells repress similar pathways in response to starvation and high cell density. © 2004 Wiley Periodicals, Inc.

**Keywords:** biofilm gene expression; surface biofilms; sporulation; *yveR*; biofilm maintenance

## INTRODUCTION

The bacterial biofilm, a sessile community with high cell density, is ubiquitous in natural, medical, and engineering environments (Elvers and Lappin-Scott, 2000; Potera, 1999). Although some beneficial bacteria can form biofilms with potential applications in medicine and engineering, such as the reduction of mild steel corrosion (Jayaraman et al., 1997a) and the inhibition of urinary tract infections (Potera, 1999), there are numerous examples of biofilms formed by pathogenic strains which pose serious problems to human health, such as lung infections, ear and eye infections, dental diseases, and urinary tract infections (Potera, 1999; Singh et al., 2000). Deleterious biofilms are also problematic in industry because they cause corrosion in heat exchangers and pipelines for transferring oil and service water (Elvers and Lappin-Scott, 2000).

With the cells embedded in a polysaccharide matrix, biofilms are highly resistant to antibiotics (Nickel et al., 1985) and have higher genetic transformation frequencies than planktonic cells (Li et al., 2001). Although planktonic cells are undetectable after the treatment of antibiotics, biofilm cells survive and are often responsible for reoccurring

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symptoms and medical treatment failure (Nickel et al., 1985; Potera, 1999). Therefore, understanding the mechanism of biofilm formation is important for finding effective strategies to control deleterious biofilms and for promoting beneficial biofilms.

Gram-positive bacteria are 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). Gram-positive bacteria have developed different strategies for survival in unfavorable environments (Grossman, 1995). Both sporulation and genetic competence have been extensively studied in the Gram-positive soil bacterium *Bacillus subtilis* (Grossman, 1995; Stragier and Losick, 1996). Sporulation is a survival strategy initiated by complex environmental and physiological signals such as starvation, high cell density, and DNA damage (Grossman, 1995; Stragier and Losick, 1996). During the sporulation process, a cell undergoes an asymmetric division that generates a mother cell and a forespore. Then the forespore is engulfed by the mother cell followed by its cortex development and coat formation. Finally, the mother cell is lyzed, and the dormant and environmentally resistant spore is released. Mature spores can remain dormant for years and return to vegetative growth by germination in an appropriate environment (Grossman, 1995; Moat and Foster, 1995; Stragier and Losick, 1996).

In addition to sporulation, *B. subtilis* has another developmental pathway known as competence by which the cells can bind and take up exogenous DNA (Grossman, 1995). Although the sporulation and competence pathways share some regulatory proteins such as Spo0A, Spo0B, Spo0F, Spo0H, and Spo0K, they are mutually exclusive, and the activation of one pathway inhibits the other (Grossman, 1995). To sporulate or to develop competence is decided by the integration of many environmental and physiological signals (Grossman, 1995). One of the factors is cell density (Grossman, 1995) as communicated by the quorum sensing system (Miller and Bassler, 2001).

Quorum sensing exists extensively in both Gram-negative and Gram-positive bacteria (Bassler, 1999; Lazazzera and Grossman, 1998; Miller and Bassler, 2001; Whitehead et al., 2001) and has been found to control many different bacterial phenotypes, such as bioluminescence (Cao and Meighen, 1989), swarming (Eberl et al., 1996), biofilm formation (Davies et al., 1998), siderophore synthesis (Stintzi et al., 1998), and virulence factor production (Zhu et al., 2002). *B. subtilis* has two major quorum-sensing signals, the ComX pheromone and the competence and sporulation factor (CSF), produced during cell growth and secreted into the environment (Bassler, 1999; Grossman, 1995; Lazazzera and Grossman, 1998). When the cell density is high, ComX will activate the histidine protein kinase ComP (sensor of ComX) and then the activated ComP phosphorylates ComA. The phosphorylated ComA will activate the transcription of *comS* and consequently activate the transcription of *comK*, which encodes the transcription factor of competence. Compared to ComX, CSF has more functions. Low cellular concentrations of CSF activate competence development, while high concentrations of CSF inhibit competence and activate sporulation (Bassler, 1999; Lazazzera and Grossman, 1998). In addition to these two quorum-sensing signals, *B. subtilis* also produces and secretes some other signaling peptides that appear to sense the cell density and negatively regulate several sporulation inhibitors (Lazazzera, 2001; Tjalsma et al., 2000). These peptides belong to the Phr family (CSF, encoded by *phrC*, also belongs to this family), including PhrA, PhrE, PhrF, PhrG, PhrI, and PhrK. The import machinery of the Phr peptides is the oligopeptide permease (Opp), which also functions in importing other peptides as food sources (Lazazzera, 2001).

Although the genetic pathways of sporulation and competence have been extensively investigated and a number of genes have been identified [over 125 genes for sporulation (Lazazzera and Grossman, 1998) and 40 genes for competence (Grossman, 1995)], most of the studies were conducted with suspension cultures and little is known about these pathways in sessile communities. Recently, it was reported that the expression of sporulation genes is not homogeneous in biofilms because the top of aerial structures is preferred (Branda et al., 2001).

Compared to the well-documented physical and chemical characterization of biofilm structure, the genetic basis of biofilm formation is poorly understood. Recently, using random insertion mutagenesis and screening, it has been shown that motility and type I pili are important for *Escherichia coli* early biofilm formation (Pratt and Kolter, 1998), and flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* early biofilm formation (O'Toole and Kolter, 1998). Previous studies have also shown that the sporulation gene *spo0A* is important for *B. subtilis* biofilm formation (Hamon and Lazazzera, 2001), and that the quorum-sensing system *luxI/luxR* is important for *P. aeruginosa* biofilm formation (Davies et al., 1998); however, this is controversial as several reports dispute the importance of quorum sensing in biofilm formation for Gram-negative strains (Heydorn et al., 2002; Purevdorj et al., 2002; Sauer and Camper, 2001).

Since biofilm growth is significantly different from that of suspension cells (Prigent-Combaret et al., 1999), studies on a genome-wide scale are necessary. Proteomics has been used successfully to study *Bacillus cereus* biofilm formation, and it was found that 15 proteins were uniquely expressed in 2-h and 7 proteins were uniquely expressed in 18-h biofilms (Oosthuizen et al., 2001, 2002). Moreover, due to the dynamic character of biofilms, in vivo and three-dimensional studies have been conducted using the green fluorescent protein system (Heydorn et al., 2002; Kievit et al., 2001). With this approach, it was found that the *P. aeruginosa* quorum-sensing genes *lasI* and *rhlI* were most expressed at the bottom of a liquid–solid interface biofilm, and the expression of *lasI* decreased with time while *rhlI* was more consistent during biofilm development (Kievit et al., 2001).

DNA microarrays have been used to monitor global gene expression profiles in response to different stimuli (Shoemaker and Linsley, 2002) including heat shock and other stresses (Helmann et al., 2001; Wilson et al., 1999; Zheng et al., 2001), quorum sensing (DeLisa et al., 2001; Sperandio et al., 2001), anaerobic metabolism (Ye et al., 2000), sporulation (Fawcett et al., 2000), and biofilm formation (Ren et al., 2003; Schembri et al., 2003; Stanley et al., 2003; Whiteley et al., 2001). Recently, Stanley et al., 2003 used DNA microarrays to study the gene expression of *B. subtilis* in the early stage of surface biofilm formation (8, 12, and 24 h after inoculation in batch culture), and found 519 genes were differentially expressed in at least one time point (more than 55% of these genes were only differentially expressed at one time point). In the present study, DNA microarrays were used to study gene expression in mature biofilms (5-day biofilms) of wild-type *B. subtilis* and its sporulation mutant. The gene expression pattern found in our study is significantly different from that of the previous report for early biofilm formation (Stanley et al., 2003), which shares only 15% of the differentially expressed genes with the present study (86 of the 590 genes differentially expressed in the wild-type biofilm compared to suspension cells). This is the first report of global gene expression in mature *B. subtilis* biofilms. The candidate biofilm genes were identified, and the interaction among biofilm formation, sporulation, and competence was investigated. Based on the microarray results, a knockout mutation in a biofilm-induced gene, *yveR*, was constructed which leads to formation of a significantly weakened biofilm.

## MATERIALS AND METHODS

### Strains and Medium

Wild-type *B. subtilis* JH642 (*pheA1, trpC2*) was obtained from the *Bacillus* Genetic Stock Center (Columbus, OH). *B. subtilis* JH642 derivatives BAL666 (*pheA1, trpC2,* Δ*spoIIGB*::*erm*) (Kenney and Moran, 1987), BAL373 (*pheA1, trpC2*, Δ*abrB*::*cat*) (Hamon and Lazazzera, 2001), and BAL667 (*pheA1, trpC2, spoIIAC1*) (Piggot, 1973) were obtained from Dr. Beth A. Lazazzera of University of California, Los Angeles. LB medium (Sambrook et al., 1989) containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl was used to grow the strain and develop the biofilm.

### Biofilm Formation and Cell Harvest

Both wild-type and mutants of *B. subtilis* were grown in continuous reactors to develop air–liquid interface biofilms. Each autoclavable reactor consists of a 6.2-cm conical glass cell, a mild steel 1010 plate at the bottom, and a Teflon top (Örnek et al., 2002). Each reactor contained 150 mL of LB medium, and the temperature was controlled at 34°C. Air was filtered and supplied to head space of the reactors at 200 mL/min (air did not bubble through the liquid and disturb the biofilm). The reactors were inoculated with a 1:150 dilution of an overnight culture. Continuous nutrient addition commenced 1 day after inoculation at 8 mL/h of LB medium. Biofilm and suspension cells were sampled 5 days after inoculation by opening the reactor quickly, and harvesting the biofilm with a sterile stainless spoon and sterile forceps, and washing it gently in cold 0.85% NaCl buffer to slow transcription and remove contaminating suspension cells. The biofilm cells were precipitated in cold Mini-BeadBeater vials (catalog number 10832, BioSpec, Bartlesville, OK) by centrifuging (10,000*g*) for 15 s at room temperature, and the cell pellets were frozen immediately by soaking the tubes in a dry ice–ethanol bath. Cell samples were kept at −80°C until RNA isolation. After the biofilm cells were stabilized in cold 0.85% NaCl buffer, a suspension culture sample (50–100 mL) was removed and immediately put on crushed ice to slow transcription. The suspension culture was then centrifuged for 3 min at −2°C (10,000*g*) to precipitate the cells. The cells were resuspended in 3 mL of cold 0.85% NaCl buffer, transferred to cold Mini-Bead-Beater vials, and centrifuged for 15 s (10,000*g*) at room temperature. The cell pellets were frozen immediately by soaking the tubes in a dry ice–ethanol bath. Cell samples were kept at −80°C until RNA isolation.

### Total RNA Isolation

To lyze the cells, 1.0 mL of RLT buffer (Qiagen, Inc., Valencia, CA) and 0.2 mL of 0.1 mm zirconia/silica beads (BioSpec) were added to the frozen Bead-Beater vials containing the cell pellets. The tubes were closed tightly and beat for 60 s at the maximum speed in a Mini-Bead-Beater (cat. no. 3110BX, BioSpec). The total RNA was isolated by following the protocol of the RNeasy Mini Kit (Qiagen) including an on-column DNase digestion with RNase-free DNase I (Qiagen). An OD (optical density) reading at 260 nm was used to quantify the RNA yield. $OD_{260}/OD_{280}$ and 23S/16S rRNA were measured to check the purity and integrity of the RNA (RNeasy Mini Handbook, Qiagen).

### DNA Microarrays

Five individual RNA sample sets (each set includes at least two DNA microarrays) were analyzed with DNA microarrays (three sets for the wild-type strain and two sets for the *spoIIGB* mutant). The *B. subtilis* DNA microarrays were prepared as described previously (Ye et al., 2000). Each gene probe was synthesized by PCR and has a size of the full open reading frame (200–2000 nt). The double-strand PCR products were denatured in 50% dimethyl sulfoxide and spotted onto aminosilane slides (Full Moon Biosystems, Sunnyvale, CA) 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* ORFs (Ye et al., 2000). 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 total RNA and 6 μg random hexamer primers (Invitrogen Corp., Carlsbad, CA) were added, and the volume was adjusted to 24 μL with RNase-free water (Invitrogen). The mixture was incubated 10 min at 70°C followed by 10 min at room temperature for annealing. To this mix were added 8 μL of 5× SuperScript II reaction buffer (Invitrogen), 4 μL of 0.1 M dithiothreitol (DTT) (Invitrogen), 1 μL of deoxynucleoside triphosphates (dNTPs) mix (2 mM each of dATP, dGTP, and dTTP and 1 mM dCTP), 1 μL of 0.5 mM Cy3- or Cy5-labeled dCTP (Amersham Biosciences, Piscataway, NJ), and 2 μL of SuperScript II reverse transcriptase (10 U/μL; Invitrogen) to make complementary DNA (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). The efficiency of labeling was checked via absorbance at 260 nm for the cDNA concentration, at 550 nm for Cy3 incorporation, and at 650 nm for Cy5 incorporation.

### Hybridization and Washing

The suspension and the biofilm cDNA samples were each labeled with both Cy3 and Cy5 dyes to remove artifacts related to different labeling efficiencies. Each experiment needed two slides: the Cy3-labeled suspension sample and Cy5-labeled biofilm sample were hybridized on the first slide, and, similarly, the Cy5-labeled suspension sample and Cy3-labeled biofilm sample were hybridized on the second slide. As each gene has two spots on a slide, the two hybridizations generated 8 data points for each gene (4 points for the suspension sample, 4 points for the biofilm sample).

The DNA microarrays were incubated in prehybridization solution [3.5× SSC (Invitrogen), 0.1% SDS (Invitrogen), 0.1% bovine serum albumin (Invitrogen)] at 45°C for 20 min. Then the arrays were rinsed with double-distilled water ($ddH_2O$) and spun dry by centrifugation. Labeled RNA (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, NJ). The hybridization mix was heated to 95°C for 2 min and added to the DNA microarrays; each array was covered with a coverslip (Corning, Big Flats, NY) 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 in room temperature $ddH_2O$ and then spun dry by centrifugation.

### Image and Data Analysis

The hybridized slides were scanned with the Generation III Array Scanner (Molecular Dynamics Corp.), and 570 and 670 nm were used to quantify the probes labeled with Cy3 and Cy5 separately. The signal was quantified with Array Vision 4.0 or 6.0 software (Imaging Research, Toronto, Ontario, Canada). Genes were identified as differentially expressed in the biofilm if the expression ratio was greater than 3-fold (wild-type) or 2.4-fold (*spoIIGB* mutant) and the *P* value (*t*-test) was less than 0.05. Including the *P* value criterion ensures the reliability of the induced/repressed gene list. *P* values were calculated on log-transformed, normalized intensities. Normalization was relative to the median total fluorescent intensity per slide per channel. The gene functions were obtained from the database in National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/).

### RNA Dot-Blotting

DNA probes of 10 representative genes—*amyC*, *yveR*, *cotN*, *yisE*, *yveP, comS*, *cydC*, *qcrB*, *rapA*, and *ybcQ*—were synthesized using the PCR DIG Probe synthesis kit (Roche Applied Science, Mannheim, Germany). Each DNA probe has a length of 400 bp except for *yisE* (141 bp) and *comS* (105 bp) due to the small size of the genes. Total RNA (1.25, 2.5, or 5 μg for each sample) from independent reactor experiments (different from experiments used to harvest RNA for the DNA microarrays) was blotted on positively charged nylon membrane (Boehringer Ingelheim, Ridgefield, CT) using a Bio-Dot microfiltration apparatus (Bio-Rad, Richmond, CA). RNA was fixed by baking for 2 h at 80°C, and about 400 ng of excess DNA probes was denatured in boiling water for 5 min before hybridizing to RNA (serial dilutions of RNA samples were tested in each blot to ensure 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[3.3.1.13,7]decan}-4-yl)phenyl phosphate (Roche Applied Science) was used as a substrate to give chemiluminescence, and the light was recorded by Biomax X-ray film (Kodak, Rochester, NY).

### Spore Assay

This assay was performed as reported previously with slight modifications (Hamon and Lazazzera, 2001). Fifty microliters of biofilm (after washing in 0.85% NaCl buffer) was put in a microcentrifuge tube containing 1 mL of

0.85% NaCl buffer and vortexed for 2 min. The buffer containing cells was then split equally into two parts which were incubated at 80°C or room temperature for 20 min, respectively. The variable cells were quantified by spreading on LB plates after a series of dilution. The percentage of biofilm cells that formed spores was determined from the ratio of heat-resistant colony forming units (CFU) (treated at 80°C) to total CFU (treated at room temperature).

### Construction of the *yveR* Mutant

An 1-kb upstream fragment of the *yveR* gene with flanking *Xba*I and *Eco*RI restriction sites was generated with PCR primers 5′-TTGTGCTCTAGACAGGTCTCCGCTATCGAGTCGGC-3′ (*Xba*I site underlined) and 5′-AGGTGCGAATTCAATGACCGGCTCCTCGTGC-3′ (*Eco*RI site underlined). This fragment was cloned into the *Xba*I and *Eco*RI restriction sites in plasmid of pDG780 (Guerout-Fleury et al., 1995) to create pDG780-yveRup. Similarly, a 0.7-kb fragment downstream of *yveR* gene with flanking *Xho*I and *Kpn*I sites was amplified with PCR primers 5′-TCGTCACTCGAGCATCGGCTGTGTATCAGCGGG-3′ (*Xho*I site underlined) and 5′-TCGCAGGGTACCTCACATAGCGCAGCCTTGACCCGG-3′ (*Kpn*I site underlined), and cloned into the *Xho*I and *Kpn*I restriction sites in plasmid pDG780-yveRup. The resulting plasmid having $Kan^R$ flanked with upstream and downstream fragments of the *yveR* gene (named as pDG780-ΔyveR) was linearized with *Sca*I and transformed into *B. subtilis* JH642 following the method of (Anagnostopoulos and Spizizen, 1961). Possible mutants were selected with 5 μg/mL kanamycin, and the correct mutant with a double crossover was confirmed by a negative PCR result using nested primers 5′-TATCAGAACGTGTCTCGAATCACTG-3′ and 5′-GAATAACGCCTTGATATA GTGCTCC-3′, which amplify a 400-bp internal fragment from wild-type *yveR* gene.

### Biofilm Assay of the *yveR* Mutant

To investigate the effect of the *yveR* deletion on the *B. subtilis* air–liquid interface biofilm, the wild-type strain and its *yveR* mutant were each inoculated in 60 mL of fresh LB medium in a beaker by 1:1,000 dilution of the overnight culture. The beakers were kept at room temperature for 4 days without shaking to form biofilms. The biofilms were imaged every day with a digital camera (Nikon E 950, Tokyo, Japan); representative images are shown in Figure 1.

## RESULTS

### Gene Expression in the Wild-Type *B. subtilis* Biofilm

To find the genes for maintaining *B. subtilis* biofilms at the air–liquid interface, continuous reactors were used in which biofilm and suspension cells were harvested 5 days after inoculation. Three individual sets of wild-type *B. subtilis* microarray experiments (3 pairs of gene chips for 3 independent reactor cultures) were analyzed, and the DNA microarray results were consistent. For example, *yveP* was induced 2.5-, 4-, and 11-fold in the biofilm samples in three data sets, respectively. The data from the

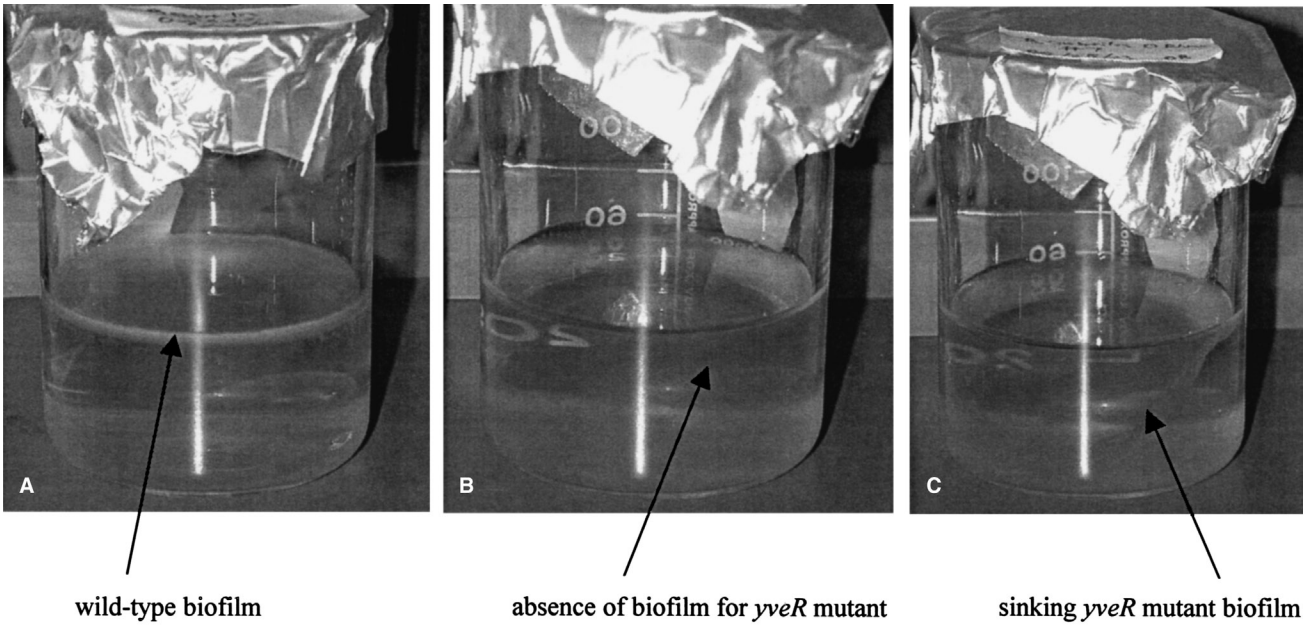


**Figure 1.** Air–liquid interface biofilm formed by wild-type *B. subtilis* and the *yveR* knockout mutant. (A) Air–liquid interface biofilm formed by the wild-type strain (88 h after inoculation). (B) *yveR* mutant lacks the biofilm at the air–liquid interface 88 h after inoculation. (C) The *yveR* mutant forms a fragile biofilm that sinks to the bottom 96 h after inoculation).

set that gave the best hybridization image was used as the representative gene expression profile. Based on this representative microarray result, it was found that 342 genes were induced in the biofilm more than 3-fold, and 248 genes were repressed in biofilm more than 3-fold; hence, 14% of the *B. subtilis* genes were differentially expressed in the biofilm compared to suspension cells.

### Induction of Sporulation Genes in the Wild-Type *B. subtilis* Biofilm

Among the 342 induced genes in the wild-type biofilm, 121 of them have known functions (NCBI database, http://www.ncbi.nlm.nih.gov/). Interestingly, 74 of these 121 genes (60%) have functions related to different stages of sporulation including genes for septum formation, forespore development, cortex formation, coat formation, and germination (Table I and A1). The biofilm formed at the air–liquid interface had very high cell density, about $5 \times 10^9$ cell/(mL biofilm volume) as estimated by the CFU from a 50-μL sample of biofilm; most of the cells were in the biofilm with relatively few in the suspension culture (which had a optical density at 600 nm of 0.1–0.2). Hence, the high cell density in the biofilm may have caused starvation and therefore induced sporulation. To quantify the extent to which the biofilm cells formed spores, a spore assay was performed, and it was found that a small portion of the biofilm cells formed spores (1.5%, average of 0.9% and 2% from two individual reactors; this may be conservative due to incomplete cell separation during vortexing). Given that our microarray results are a global average of all the biofilm cells without considering their specific locations, we propose that the cells in the biofilm were under a balance of sporulation, germination, and general growth, while only a small portion of the cells have completed sporulation and were heat-resistant.

### Induction of Other Genes in the Wild-Type *B. subtilis* Biofilm

In addition to sporulation genes, genes for transport, biosynthesis, and unknown functions were also induced (Table A2 and A3). Among these genes, the operon *yisCDEFG*, which has homology to the *B. cereus gerP* operon involved in spore germination (Behravan et al., 2000), was induced, and also the *yveMNOPQR* operon was induced, which appears to play a role in polysaccharide synthesis (Branda et al., 2001). *B. subtilis* mutants of *yveR* and *yveQ* have been shown to have defects (very fragile biofilm) in biofilm formation (Branda et al., 2001).

### Repression of Anaerobic Genes in the Wild-Type *B. subtilis* Biofilm

One of the main environmental differences between biofilm cells and suspension cells in the present study was the oxygen concentration. Since the air was added to the headspace (top) of the reactors, the suspension cells were grown under relative anaerobic conditions due to the existence of the thick biofilm (more than 2 mm). In agreement with this, anaerobic genes [such as *narJGK* (Ye et al., 2000)] were induced for the suspension cells and therefore, by comparison, repressed in the biofilm cells (see Table II and Table A4 in the supplementary information for the top 20 repressed genes). Among these genes, the following were repressed: *narGIJ* (encoding nitrate reductase), *nasD* (encoding a subunit of nitrite reductase), *nasF* (encoding uroporphyrin-III C-methyltransferase), *lctE* (encoding L-lactate dehydrogenase), *lctP* (encoding L-lactate permease), *alsD* (encoding α-acetolactate decarboxylase), cydAB (encoding subunit I and II of cytochrome bd ubiquinol oxidase), *cydCD* (encoding an ABC membrane transporter, ATP-binding protein), *ydjL* (encoding 2,3-butanediol dehydrogenase), and *feuA* (encoding an iron-binding protein).

### Sporulation Is Not Necessary for *B. subtilis* Biofilm Formation

As 60% of the genes with known functions induced in the wild-type *B. subtilis* biofilm are sporulation genes, it was investigated if sporulation is necessary for formation of the *B. subtilis* air–liquid interface biofilm. The wild-type strain and sporulation mutants BAL373 (*abrB*), BAL667 (*spoIIAC*), and BAL666 (*spoIIGB*) were grown in the continuous reactors in the same way as in the microarray experiments with the wild-type strain. *abrB* encodes a negative regulator of many genes for sporulation and competence, and *spoIIAC* and *spoIIGB* encode the sporulation sigma factors $\sigma^F$ and $\sigma^E$, respectively (Grossman, 1995). Interestingly, all three of these mutants formed a thick biofilm similar to that of the wild-type after 5 days (about 2 mm, results not shown). Hence, the completion of sporulation is not necessary for air–liquid biofilm formation. To eliminate the interference of the sporulation genes, a spoIIGB mutant was used to form biofilms and was studied with the DNA microarrays.

### Gene Expression in the *spoIIGB* Mutant Biofilm

Two independent sets of *spoIIGB* mutant microarray experiments were analyzed, and the results were consistent. There were 371 genes induced for one set and 231 genes induced in the other data set; and 170 of these genes were induced in both sets. For example, *ybcP* was induced 5.3- and 4.7-fold in the two data sets, respectively. The *spoIIGB* gene encodes the sigma factor $\sigma^E$, and the mutation of this gene blocks sporulation by preventing endospore formation (Kenney and Moran, 1987). As expected, only a few sporulation genes were induced in biofilms of the *spoIIGB* mutant (Table I and A5), and most of them are involved in the initiation of sporulation. The *kinB* gene for initiation of

sporulation was induced 3.5-fold in the wild-type biofilm but was not induced in the *spoIIGB* mutant biofilm (up-regulated slightly, 1.3-fold). Overall, 93% (69 out of 74 genes induced in the wild-type biofilm) of the sporulation genes were successfully removed from the induced gene list by using the *spoIIGB* mutant, and the genes required for biofilm synthesis and maintenance could be studied in the absence of sporulation. From the microarray results (one of the two sets which gave the more complete gene list), 371 genes (186 genes have unknown functions) were induced more than 2.4-fold and 128 genes were repressed more than 2.4-fold in these biofilms compared to suspension cells. Hence, 12% of the genes were differentially expressed in the biofilm compared to suspension cells.

### Induction of Quorum-Sensing and Competence Genes in the *spoIIGB* Mutant Biofilm

The induction of the *phrA*, *phrE*, *phrF*, and *phrK* genes (about 5-fold) and the *oppABCDF* operon (about 5-fold, Table A5), which encode quorum-sensing signaling peptides and oligopeptide permease, respectively (Lazazzera, 2001), is consistent with the high cell density in the biofilm (Elvers and Lappin-Scott, 2000). Consequently, several competence genes were found induced in the biofilm, including *comGA*, *srfAA*, *srfAB*, *srfAD,* and *comS* (*comS* is part of the *srfA* operon), suggesting the cells in the spoIIGB mutant biofilm were competent. Moreover, a sporulation inhibitor, RapA, encoded by *rapA* [same transcript as *phrA*, known to be activated by phosphorylated ComA (Grossman, 1995) and negatively regulated by PhrA when it is imported into the cells] (Lazazzera, 2001), was also induced in the *spoIIGB* mutant biofilm but not in the wild-type biofilm in the present study. This supports the existence of competence and the absence of sporulation in the *spoIIGB* mutant biofilm. Determining the intercellular PhrA concentration may help understand this complex control network.

Compared with the *spoIIGB* mutant, only *phrF* (4.0-fold) and *srfAA* (3.1-fold) were induced in the wild-type biofilm (Table I), although other quorum-sensing and competence genes were also up-regulated, e.g., *oppA* (2.4-fold), *oppC* (2.9-fold), *comS* (1.6-fold), and *srfAC* (1.9-fold). The difference in the expression level of these genes between the wild-type and sporulation mutant are unknown, but it may be due to the induction of different pathways for the formation of their respective biofilms (sporulation in the wild-type and competence development in the *spoIIGB* mutant).

### Induction of Genes for Transport, Metabolism, and Antibiotic Production in the *spoIIGB* Mutant Biofilm

A large number of genes with these functions were induced in the biofilm of the *spoIIGB* mutant (Table A5), suggesting the biofilm cells were metabolically active and changing pathways for survival. First, several operons for transport were induced such as *appABCDF* for oligopeptide transport, *dppABCDE* for dipeptide transport, and *rbsABCD* for ribose transport. Second, many genes for metabolism were induced, such as *acoABCL* and *acuABC* for acetoin utilization, *hutGIU* for histidine utilization, and iolABCEFGHIJ for myo-inositol catabolism. The induction of these genes indicates the cells were starving due to high cell density and the inability of the mutant to sporulate. Without the formation of dormant spores, the cells may need active metabolism to maintain the biofilm. In contrast, the suspension cells had significantly different metabolism with low cell density ($OD_{600}$ = 0.1–0.2) and did not need these genes to be highly expressed to survive. Third, several genes for antibiotic production were induced, such as *pksFJLR* and *ppsADE*; products of these genes may help the biofilm cells to enhance their chances for survival by inhibiting the growth of nutrient competitors.

### Repression of Anaerobic Genes in the *spoIIGB* Mutant Biofilm

Similar to the wild-type microarray results, some anaerobic genes were repressed in the biofilm including *alsD*, *lctEP*, *narGIJK*, *nasDEF*, *feuA*, *hmp*, and *ydjL* (see Table II and Table A6 in the supplementary information). All of the above genes, except for *hmp*, were also repressed in the wild-type biofilm compared to suspension cells, suggesting that each biofilm formed without shaking had a good structure which covered the surface and led to an anaerobic environment for the suspension culture.

According to Fick's second law (Bird et al., 1960), the diffusion of oxygen in the medium can be described as,

$$\frac{\partial C_A}{\partial t} = D_{AB}\frac{\partial^2 C_A}{\partial y^2}$$

in which $C_A$ is the oxygen concentration in the suspension culture, $C_{A0}$ is the oxygen concentration at the surface of the medium, $D_{AB}$ is the oxygen–water diffusion constant which is estimated as 0.00002859 $cm^2/s$ at 34 °C based on the Stokes-Einstein equation (Bird et al., 1960), $y$ is the depth in the suspension culture (cm), and $t$ is the time (s). The analytical solution of this equation is

$$\frac{C_A}{C_{A0}} = 1 - erf\frac{y}{\sqrt{4D_{AB}t}}$$

The numerical solution indicates that $C_A/C_{A0}$ = 0.5 when y = 3.4 cm. Hence, the diffusion of oxygen in the medium is very slow, and the oxygen concentration at 3.4 cm depth of the medium reaches only 50% of that at the surface after 5 days of diffusion. Given the cells had a growth rate of 0.05 $h^{-1}$, the supplement of oxygen may not be enough to support aerobic growth of the whole culture. Furthermore, the diffusion coefficient is expected to be significantly decreased due to the existence of a biofilm and therefore to

cause a further reduction of the oxygen concentration in the suspension culture underneath the biofilm. In addition, the consumption of oxygen in the biofilm with a high density of cells will further decrease the oxygen concentration. The anaerobic condition caused by biofilms is well known and is the basis for corrosion prevention with regenerative biofilms (Jayaraman et al., 1997b). The data in the present study presents some of the first genetic evidence for such effects.

**Table I.** Genes consistently induced in both the wild-type and *spoIIGB* mutant biofilms.

| Gene | Induction expression ratio (Wild type) | Induction expression ratio (*spoIIGB* mutant) | Description |
|---|---|---|---|
| *acoA* | 3.0 | 6.9 | Acetoin dehydrogenase E1 component (TPP-dependent alpha subunit) |
| *acoC* | 4.2 | 10.1 | Acetoin dehydrogenase E2 component (dihydrolipoamide acetyltransferase) |
| *ald* | 3.3 | 4.9 | L-Alanine dehydrogenase |
| *appB* | 3.8 | 4.8 | Oligopeptide ABC transporter (permease), oligopeptide transport |
| *appC* | 4.5 | 6.1 | Oligopeptide ABC transporter (permease) |
| *appF* | 4.3 | 7.0 | Oligopeptide ABC transporter (ATP-binding protein) |
| *cotN* | 5.7 | 4.1 | Spore coat-associated protein |
| *csaA* | 45.2 | 4.7 | Involved in protein secretion, molecular chaperonin |
| *ctaC* | 3.6 | 5.3 | Cytochrome caa3 oxidase (subunit II) |
| *dppD* | 3.0 | 6.3 | Dipeptide ABC transporter (ATP-binding protein) |
| *glgA* | 4.8 | 3.8 | Starch (bacterial glycogen) synthase |
| *glgP* | 4.5 | 3.9 | Degrades starch and glycogen by phosphorylation (glycogen metabolism) |
| *leuC* | 3.5 | 5.7 | Leucine biosynthesis |
| *lonB* | 3.6 | 4.3 | Lon-like ATP-dependent protease |
| *mpr* | 5.8 | 5.4 | Extracellular metalloprotease |
| *msmE* | 3.0 | 4.5 | Multiple sugar-binding protein |
| *phrF* | 4.0 | 7.5 | Regulator of the activity of phosphatase RapF |
| *qcrA* | 3.2 | 4.0 | Menaquinone oxidase |
| *qcrB* | 3.4 | 4.3 | Menaquinol:cytochrome c oxidoreductase (cytochrome b subunit) |
| *qcrC* | 3.7 | 4.3 | Menaquinol:cytochrome c oxidoreductase (cytochrome b/c subunit) |
| *rocE* | 3.0 | 3.8 | Arginine and ornithine utilization |
| *spoIIAA* | 3.0 | 4.4 | Binding to SpoIIAB in the presence of ADP selectively in the forespore |
| *spoIIAB* | 3.0 | 5.5 | Binding to sigma-F (SpoIIAC) in the presence of ATP before septation, phosphorylation of SpoIIAA (stage II sporulation) |
| *spoIIB* | 4.0 | 5.6 | Endospore development (stage II sporulation) |
| *spoIIGA* | 27.1 | 5.8 | Processing of pro-sigma-E (SpoIIGB) to active sigma-E (stage II sporulation) |
| *srfAA* | 3.1 | 4.6 | Surfactin production and competence, surfactin synthetase |
| *wprA* | 3.1 | 6.5 | Cell wall-associated protein precursor (CWBP23, CWBP52) |
| *yabS* | 3.7 | 6.0 | Unknown |
| *ybcO* | 3.4 | 8.6 | Unknown |
| *ybcP* | 4.2 | 5.3 | Unknown |
| *ybcQ* | 3.3 | 5.3 | Unknown |
| *ybcS* | 4.3 | 4.4 | Unknown |
| *ybdA* | 3.6 | 5.8 | Unknown, similar to ABC transporter (binding protein) |
| *ybdB* | 3.8 | 5.4 | Unknown, similar to ABC transporter (permease) |
| *ycgM* | 4.3 | 5.0 | Unknown, similar to proline oxidase |
| *ycgN* | 3.2 | 5.2 | Unknown, similar to 1-pyrroline-5-carboxylate dehydrogenase |
| *ydjP* | 3.5 | 2.5 | Unknown, similar to arylesterase |
| *yhaA* | 5.3 | 4.4 | Unknown, similar to aminoacylase |
| *yisS* | 3.5 | 4.0 | Unknown, myo-inositol 2-dehydrogenase |
| *yjdK* | 3.4 | 4.9 | Unknown, similar to cytochrome c oxidase assembly factor |
| *ykfA* | 4.4 | 3.9 | Unknown, similar to chloromuconate cycloisomerase |
| *yknV* | 3.6 | 2.5 | Unknown, similar to ABC transporter (ATP-binding protein) |
| *ykrQ* | 3.1 | 4.5 | Unknown, similar to two-component sensor histidine kinase |
| *ykuU* | 3.1 | 2.7 | Unknown, similar to 2-cys peroxiredoxin |
| *ykuV* | 3.4 | 3.2 | Unknown |
| *yoaW* | 3.1 | 2.9 | Unknown |
| *yppD* | 4.2 | 3.8 | Unknown |
| *ytfJ* | 4.0 | 4.5 | Unknown |
| *yuiB* | 3.3 | 2.7 | Unknown |
| *yvaX* | 3.3 | 6.4 | Unknown |
| *yxnB* | 3.2 | 6.6 | Unknown |
| *yydG* | 4.6 | 6.4 | Unknown |
| *yydJ* | 3.0 | 2.5 | Unknown |

**Table II.** Genes consistently repressed in both the wild-type and *spoIIGB* mutant biofilms.

| Gene | Repression expression ratio (Wild type) | Repression expression ratio (*spoIIGB* mutant) | Description |
|---|---|---|---|
| *abrB* | −7 | −4 | Transcriptional regulator |
| *alsD* | −100 | −50 | Acetoin biosynthesis |
| *alsSx* | −50 | −100 | Unknown |
| *cwlA* | −4 | −3 | Cell wall hydrolase (minor autolysin) |
| *cydA* | −8 | −11 | Cytochrome bd ubiquinol oxidase (subunit I) |
| *cydBx* | −6 | −10 | Unknown |
| *cydC* | −9 | −10 | ABC membrane transporter (ATP-binding protein) |
| *cydD* | −9 | −11 | ABC membrane transporter (ATP-binding protein) |
| *dhbA* | −20 | −6 | Siderophore 2,3-dihydroxybenzoate (DHB) synthesis |
| *dhbBx* | −20 | −6 | Unknown |
| *dhbC* | −7 | −3 | Siderophore 2,3-dihydroxybenzoate (DHB) synthesis |
| *dhbE* | −8 | −7 | Siderophore 2,3-dihydroxybenzoate (DHB) synthesis |
| *dhbF* | −13 | −6 | Involved in siderophore 2,3-dihydroxybenzoate (DHB) synthesis |
| *drm* | −4 | −3 | Conversion of ribose-1-P/deoxyribose-1-P to ribose-5-P/deoxyribose-5-P (purine nucleoside salvage) |
| *eno* | −9 | −5 | Glycolysis |
| *feuA* | −25 | −8 | Component of iron-uptake system |
| *feuB* | −14 | −6 | Component of iron-uptake system |
| *feuC* | −17 | −8 | Component of iron-uptake system |
| *fhuD* | −25 | −5 | Ferrichrome ABC transporter (ferrichrome-binding protein) |
| *fruB* | −5 | −3 | Fructose-1-phosphate kinase |
| *fruR* | −4 | −3 | Negative regulation of the fructose operon (fruRBA) |
| *gap* | −14 | −8 | Glycolysis, glyceraldehyde-3-phosphate dehydrogenase |
| *glpD* | −10 | −14 | Glycerol utilization |
| *hisH* | −13 | −3 | Histidine biosynthesis,imidazoleglycerol-phosphate dehydratase |
| *lctE* | −33 | −33 | L-Lactate dehydrogenase |
| *lctP* | −50 | −33 | L-Lactate permease |
| *med* | −3 | −3 | Positive regulator of comK |
| *mtlD* | −10 | −6 | Mannitol-1-phosphate dehydrogenase |
| *narG* | −50 | −33 | Nitrate reductase (alpha subunit) |
| *narI* | −50 | −33 | Nitrate reductase (gamma subunit) |
| *narJ* | −50 | −33 | Nitrate reductase (protein J) |
| *narK* | −25 | −25 | Nitrate extrusion protein |
| *nasD* | −6 | −8 | Assimilatory nitrate reductase (subunit) |
| *nasE* | −5 | −7 | Assimilatory nitrate reductase (subunit) |
| *nasF* | −5 | −4 | Porphyrin biosynthesis |
| *pgk* | −8 | −5 | Glycolysis, phosphoglycerate kinase |
| *pgm* | −8 | −5 | Glycolysis |
| *pnp* | −4 | −3 | Purine nucleoside phosphorylase |
| *pta* | −4 | −3 | Phosphotransacetylase |
| *pyrC* | −10 | −3 | Pyrimidine biosynthesis |
| *tpi* | −7 | −5 | Glycolysis, triose phosphate isomerase |
| *treA* | −5 | −8 | Trehalose-6-phosphate hydrolase |
| *treP* | −6 | −11 | Phosphotransferase system (PTS) trehalose-specific enzyme IIBC component |
| *treR* | −4 | −4 | Negative regulation of the trehalose operon (trePAR) |
| *ybfS* | −8 | −4 | Unknown, similar to phosphotransferase system enzyme II |
| *ycgT* | −5 | −3 | Unknown, similar to thioredoxin reductase |
| *yclJ* | −4 | −5 | Unknown, similar to two-component response regulator [YclK] |
| *yclK* | −4 | −4 | Unknown, similar to two-cpmponent sensor histidine kinase [YclJ] |
| *yclN* | −6 | −5 | Unknown, similar to ferrichrome ABC transporter (permease) |
| *yclO* | −6 | −5 | Unknown, similar to ferrichrome ABC transporter (permease) |
| *yclQ* | −9 | −4 | Unknown, similar to ferrichrome ABC transporter (binding protein) |
| *ydbN* | −13 | −4 | Unknown |
| *ydhL* | −3 | −4 | Unknown, similar to chloramphenicol resistance protein |
| *ydjL* | −13 | −10 | Unknown, similar to L-iditol 2-dehydrogenase |
| *yetG* | −6 | −3 | Unknown |
| *yhbI* | −9 | −5 | Unknown, similar to transcriptional regulator (MarR family) |
| *yhgD* | −20 | −11 | Unknown, similar to transcriptional regulator (TetR/AcrR family) |
| *yhxD* | −4 | −3 | Unknown, similar to ribitol dehydrogenase |
| *yjdD* | −50 | −20 | Unknown, similar to fructose phosphotransferase system enzyme II |
| *yjdE* | −50 | −20 | Unknown, similar to mannose-6-phosphate isomerase |
| *yjdF* | −10 | −8 | Unknown |

*(continued)*

**Table II.** (*continued*).

| Gene | Repression expression ratio (Wild type) | Repression expression ratio (*spoIIGB* mutant) | Description |
|---|---|---|---|
| *yjeA* | −5 | −3 | Unknown, similar to endo-1,4-beta-xylanase |
| *ykuO* | −14 | −3 | Unknown |
| *ykuP* | −20 | −3 | Unknown, similar to sulfite reductase |
| *ykwC* | −3 | −3 | Unknown, similar to 3-hydroxyisobutyrate dehydrogenase |
| *yoeB* | −6 | −7 | Unknown |
| *yrhD* | −4 | −4 | Unknown |
| *yrhE* | −4 | −4 | Unknown, similar to formate dehydrogenase |
| *ytxM* | −3 | −3 | Unknown, similar to prolyl aminopeptidase |
| *yuiI* | −13 | −3 | Unknown |
| *yvdT* | −4 | −3 | Unknown, similar to transcriptional regulator (TetR/AcrR family) |
| *yvgK* | −5 | −3 | Unknown, similar to mercuric transport protein |
| *yvmA* | −9 | −4 | Unknown, similar to transporter |
| *yvmB* | −20 | −6 | Unknown |
| *yvpB* | −7 | −6 | Unknown |
| *yvqA* | −4 | −2 | Unknown, similar to two-component response regulator [YvqB] |
| *ywaC* | −13 | −3 | Unknown, similar to GTP-pyrophosphokinase |
| *ywbL* | −3 | −3 | Unknown |
| *ywbM* | −10 | −3 | Unknown |
| *ywcJ* | −17 | −17 | Unknown, similar to nitrite transporter |
| *ywkA* | −4 | −3 | Unknown, similar to malate dehydrogenase |
| *yxcC* | −17 | −3 | Unknown, similar to metabolite transport protein |
| *yxeB* | −8 | −6 | Unknown |
| *yxiE* | −4 | −4 | Unknown |
| *yxlC* | −4 | −10 | Unknown |
| *yxlD* | −6 | −13 | Unknown |
| *yxlE* | −7 | −11 | Unknown |
| *yxlF* | −6 | −8 | Unknown, similar to ABC transporter (ATP-binding protein) |
| *yxlG* | −3 | −5 | Unknown |
| *yxlH* | −3 | −3 | Unknown, similar to multidrug-efflux transporter |
| *yybP* | −3 | −3 | Unknown |

### Comparison of the Gene Expression Profile Between the Wild-Type and *spoIIGB* Mutant Biofilm

Because the mutation in *spoIIGB* blocked sporulation (Kenney and Moran, 1987), the induced gene list was significantly different between the wild-type and the *spoIIGB* mutant. While there were 74 sporulation genes induced in the wild-type biofilm, only 5 of them were induced in the *spoIIGB* mutant biofilm (*cotN*, *spoIIAA, spoIIAB*, *spoIIB*, *spoIIGA*, Table I). In agreement with this, the spore assay found there were no spores in the *spoIIGB* mutant biofilm (<0.0001%).

Although there was significant difference in gene expression between the wild-type and the *spoIIGB* mutant biofilms, a number of genes were both consistently induced (53 genes) and repressed (92 genes) in these two types of biofilms. Generally, the biofilm cells were metabolically active relative to the suspension cells with many genes for transport and metabolism induced (Table I).

Among the 53 biofilm genes consistently induced for both the wild-type and *spoIIGB* mutant, 26 of them have unknown functions (Table I). Recently, it was reported that *ybcO*, *ybcS*, and *ybcPQ* encode a peptide which inhibits the growth of the rice pathogen *Xanthomonas orzae* pv. *oryzae* (*Xoo*) (Lin et al., 2001). In the present study, we found the *ybcOPQS* operon was induced in the sporulation mutant and wild-type biofilms, which is in agreement with previous reports that Gram-positive strains including *B. subtilis* produce antimicrobial peptides in late log phase and early stationary phase to inhibit the growth of competitors (Kleerebezem and Quadri, 2001). Hence, the induction of the *ybc* operon may help the biofilm cells to survive in the competitive environment. In addition, a recent study by González-Pastor et al. (2003) found that sporulating *B. subtilis* produces the sporulation killing factor (encoded by *ybcOPST* and *ybdABDE*) to lyze sister cells when nutrients become available again in the environment. All the *ybccOPST* and *ybdABDE* genes were induced in both the wild-type and *spoIIGB* mutant biofilms in the present study except for the *ybcT* and *ybdE* genes of the wile-type which were up-regulated (about 2-fold). Given this strategy of *B. subtilis* to avoid the disadvantage of continuing sporulation when the environment has changed to become rich in nutrients (González-Pastor et al., 2003), the induction of the *ybc* and *ybd* operons in the biofilm, along with the continuous addition of nutrients to the reactor, may explain the low number of spores found in the mature wild-type biofilm in our study.

Although the induced gene list is very different between wild-type and *spoIIGB* mutant biofilms, 92 genes are con-

sistently repressed in the biofilm of both strains (Table II). Several of the consistently repressed genes are those for anaerobic growth, such as *narGIJ*, *nasDF*, *alsD*, *cydC*, *ydjL*, and *lctEP,* and those for iron-uptake, such as *feuABC* (therefore these genes are induced for the suspension cells). The glycolysis pathway was also repressed (repression of *eno*, *gap*, *pgk*, *pgm*, and *tpi*, Table II), which may be caused by starvation. Importantly, *abrB* was repressed 4- to 7-fold in the biofilm of both the sporulation mutant and wild-type strains (Table II); AbrB is a negative regulator of sporulation and competence. Hence, the repression of *abrB* ensures the appropriate response to high cell density (Grossman, 1995).

## RNA Dot-Blotting to Confirm the Microarray Results

To validate the gene expression profiles obtained from the five sets of DNA microarray hybridizations, total RNA from the biofilm and suspension cells (for both the wild-type and the *spoIIGB* mutant) were isolated in the same way as in the microarray experiments (using independent reactors), and the resulting mRNA concentrations were quantified with RNA dot-blotting. Five genes of the wild-type strain (*amyC*, *yveR*, *cotN*, *yisE*, and *yveP*) and seven genes of the *spoIIGB* mutant (*comS*, *cygD*, *rapA*, *qcrB*, *ybcQ*, *yveP*, and *yveR*) were checked, and the expression ratios (biofilm cells/suspension cells) of all these 12 genes agree with the microarray results (Table III). For example, *amyC* was induced 8.3-fold in the wild-type biofilm in the microarray experiments and induced 6-fold in the RNA dot-blotting experiment.

Interestingly, the *yveP* and *yveR* genes, which appear to be involved in polysaccharide synthesis (Branda et al., 2001), were induced in the wild-type biofilm (about 4-fold); however, they were only consistently up-regulated, but not consistently induced (about 1.5-fold), in duplicate experiments of the *spoIIGB* mutant biofilm relative to suspension cells (*yveR* was up-regulated 1.4-fold in first data set and induced 2.5-fold in the second data set; *yveR* was up-regulated 1.6-fold in the first data set and up-regulated 2-fold in the second data set). RNA dot-blotting result shows both of these two genes were induced about 5-fold in the *spoIIGB* mutant

**Table III.** Gene expression confirmed by RNA dot-blotting.

| Strains | Gene | Primers used for probe synthesis | Expression ratio from DNA microarrays | Expression ratio from RNA dot-blotting |
|---|---|---|---|---|
| Wild-type | *amyC* | 5′-GGATCATTACGCTTCTTGCAGCCAT-3′<br>5′-ATGAAAATGGCGATTGCGTGTGTAT-3′ | +8.3 | +6 |
| | *yveR* | 5′-TATCAGAACGTGTCTCGAATCACTG-3′<br>5′-GAATAACGCCTTGATATAGTGCTCC-3′ | +4.3 | +15 |
| | *cotN* | 5′-AGAAATTGAGTTTAGGAGTTGCTTC-3′<br>5′-AAGTCTTTAAGGTTCGCATCATCTA-3′ | +5.7 | +15 |
| | *yisE* | 5′-ATGATCTTTACAGTCATCAACCGCA-3′<br>5′-AAGAGATTCAGGCGGTGTATCAAAA-3′ | +8.7 | +25 |
| | *yveP* | 5′-CACGGATTTCCAAACGGGTTTTATC-3′<br>5′-ATGTGCGGCAAGCTTCAACAGGAAC-3′ | +4.0 | +20 |
| *SpoIIGB* mutant | *comS* | 5′-TTGAACCGATCAGCAAGCATCTTA-3′<br>5′-TGTAGCTTGTGTTTGCTTGTCCAAG-3′ | +3.2 | +10 |
| | *cydC* | 5′-TCAGACAGCCGCCATTATTATGCAG-3′<br>5′-GATGGCTGATGTCCGATCCTGAAAA-3′ | −10 | −5 |
| | *qcrB* | 5′-CAAAATTTATGACTGGGTAGACGAG-3′<br>5′-CATAACAAAAAAGATCAGCACACCG-3′ | +4.3 | +8 |
| | *rapA* | 5′-GCAGAAGATGTTTGTCAGTGCCATG-3′<br>5′-CCCAAAGTATTCGGCTGCCTTTTGC-3′ | +6.2 | +8 |
| | *ybcQ* | 5′-ATGCAAAAGCTAGCAGCCCATGGAG-3′<br>5′-CTGCACGAAAGATAAAAGCACCCGC-3′ | +5.3 | +6 |
| | *yveP* | 5′-CACGGATTTCCAAACGGGTTTTATC-3′<br>5′-ATGTGCGGCAAGCTTCAACAGGAAC-3′ | +1.4 | +5 |
| | *yveR* | 5′-TATCAGAACGTGTCTCGAATCACTG-3′<br>5′-GAATAACGCCTTGATATAGTGCTCC-3′ | +1.6 | +5 |

biofilm relative to suspension cells. Hence, these two genes are significantly expressed in both biofilms.

### *yveR* Mutant Has a Defect in Biofilm Maintenance

The *yve* operon was induced in the wild-type biofilm and up-regulated in the *spoIIGB* mutant biofilm in the present study. However, in a recent study of early stage *B. subtilis* biofilm using DNA microarrays, the *yve* operon was not differentially expressed (Stanley et al., 2003). This suggests the *yve* operon may be important for biofilm maintenance, not formation. To further study the role of the *yve* operon in biofilm maintenance, a *yveR* deletion mutant was constructed and its biofilm was studied. The wild-type strain (*B. subtilis* JH642) started to form a biofilm 36 h after inoculation into a quiescent LB medium. Then the biofilm slowly accumulated with a white film clearly seen on the top of the culture (Fig. 1A). The biofilm looked thicker around the wall and a little thinner in the center. The *yveR* mutant also formed a biofilm, but it was formed about 12 h later than the wild-type. More importantly, the biofilm of the *yveR* mutant was very thin and fragile; it broke and sank to the bottom of the beaker by gravity and was unable to accumulate (see the Fig. 1C for the sinking fragile biofilm). Hence, no clear white biofilm was seen on the surface (Fig. 1B). In a previous study, it was shown that both the wild isolate and its *yveR* mutant formed thick biofilms, but the biofilm of the wild isolate had vein-like structure and the *yveR* mutant biofilm was very fragile (Branda et al., 2001). Similarly, in the present study, the wild-type (*B. subtilis* JH642, a laboratory strain) formed a smooth white biofilm, and the *yveR* mutant did not form a stable biofilm. Consistently, *yveR* appears to be important in maintaining a biofilm for both the wild isolate and the laboratory strain. Although further study is necessary to discern the function of each individual gene, the *yve* operon, as evidenced by the present study (the defects in the *yveR* mutant biofilm) and that of Branda et al. (2001), appears to function in optimizing the polysaccharide synthesis which is used for constructing and maintaining the three dimensional structure of biofilm for transport of nutrients and removal of wastes (Elvers and Lappin-Scott, 2000).

## DISCUSSION

In the present study, five sets of DNA microarrays were successfully used to generate the gene expression profiles of a *B. subtilis* biofilm relative to suspension cells in the same rector, and the results of replicated experiments were consistent. Genes clustered in the same operons were found to be induced/repressed together, indicating the data are sound; for example, 21 *cot* genes were induced in the wild-type biofilm and their expression ratios were similar (around 10-fold, see Table A1). Among the genes with known functions, there were 54 operons (121 genes) induced and 43 operons (103 genes) repressed in the wild-type biofilm vs. the suspension cells. Accordingly, 79 operons (185 genes) were induced and 33 operons (56 genes) were repressed in *spoIIGB* mutant biofilm versus suspension.

Although there are accumulating reports of gene expression in biofilms studied with DNA microarrays (Ren et al., 2003; Schembri et al., 2003; Stanley et al., 2003; Whiteley et al., 2001), technical improvement is still needed for identifying the real biofilm genes. One challenge in studying biofilms with DNA microarrays is the choice of control to compare with the gene expression of the biofilm. Most of the previous studies compared gene expression in biofilm cells with that from an independently grown suspension culture which was controlled (with shaking) to maintain one or more environmental conditions, such as cell density in the *E. coli* study (Schembri et al., 2003). For example, Stanley et al. (2003) mixed the biofilm and suspension cells in the same beaker (without shaking) and compared with another independent shaking culture to study the difference in gene expression. However, the presence/absence of shaking causes significant difference in oxygen and mass transport which could lead to serious artifacts. In addition, the different cell densities in the control suspension culture gave very different results (Schembri et al., 2003 ), Furthermore, when Oosthuizen et al. (2002) studied the *B. cereus* biofilm formation on glass wool in a shaking culture, a difference in protein expression was found even between the suspension cells grown with and without glass wool. Biofilm formation is a dynamic process which includes cell-surface attachment, microcolony formation, biofilm maturation, and cell detachment (Watnick and Kolter, 2000). Therefore, the planktonic cells from the same reactor of biofilm, which are under a balance among planktonic stage, biofilm formation, and biofilm detachment, are more representative compared to the strategy that uses independent suspension sample as a control. In the present *B. subtilis* study, as well as that for our *E. coli* biofilm gene expression study (Ren et al., 2003), the gene expression profiles were successfully obtained from the biofilm and suspension cells from the *same* reactor, and the results are consistent, such as the induction of sporulation genes in the wild-type biofilm, the induction of competence and quorum sensing genes in the *spoIIGB* biofilms, and the induction of anaerobic genes in the suspension cells. However, the potential biofilm genes identified through these DNA microarray are only candidates. No matter how consistent the microarray results are, mutant construction and assay are the best approach to confirm the importance of genes for biofilm formation (such as our study with *yveR* mutant).

*B. subtilis* forms a biofilm at the air–liquid interface, possibly driven by air attraction and high cell density. Biofilm structure can recover in hours after mechanical disturbance (data not shown). This suggests the biofilm state is preferred by the cells under these conditions. In support of this, a number of anaerobic genes (such as *alsD*, *lctEP*, *narGIJK*, *nasDF, feuA,* and *ydjL*) were repressed in both the wild-type and *spoIIGB* mutant biofilms (indicating the genes

were induced for the suspension cells) and *hmp* was repressed 33-fold in the *spoIIGB* mutant biofilm (Table A6). Ye et al. (2000) has studied anaerobic metabolism with DNA microarrays and found a global change in gene expression (several hundreds of genes were differentially expressed) between aerobic growth and anaerobic growth with all the above anaerobic genes of our studies induced during nitrate and nitrite respiration. More recently, Clements et al. (2002) studied *B. subtilis* nitrate respiration and fermentation with proteomics and found that groups of proteins were expressed for nitrate respiration (proteins encoded by *feuA*, *hmp*, and *ytkD*), fermentation (proteins encoded by *pyrR*, *sucD*, *trpC*, and *ywjH*), or both nitrate respiration and fermentation (proteins encoded by *acuB*, *pdhC*, *ydjL*, and *yvyD*). In comparison, the number of anaerobic genes induced in the suspension cells was smaller in our study. This is probably due to the thick biofilm which generated a gradient of oxygen in the biofilm with the result the cells embedded at the bottom of the biofilm were also partially anaerobic.

From the DNA microarray results, it was found that the expression of 13% (14% for the wild-type and 12% for the *spoIIGB* mutant) of the genes is significantly different between biofilm and suspension cells. Previously it was reported that up to 38% of total genes are expressed differently in *E. coli* biofilm compared to suspension cells using random insertion mutagenesis (Prigent-Combaret et al., 1999).

Although the sporulation mutants formed thick biofilms similar to that of the wild-type in the present study (indicating sporulation is not necessary for biofilm formation), sporulation and biofilm formation are related and the top of the aerial structures in a biofilm is the most preferred place for sporulation (Branda et al., 2001). Compared to the large number of sporulation genes induced in the wild-type biofilm, only 1.5% of the biofilm cells were found as heat-resistant spores. Similar biofilms of *B. subtilis* have also been studied in batch culture, and more than 50% of the biofilm cells formed spores 96 h after inoculation (Branda et al., 2001). The lower percentage of spores in the biofilm in the present study was probably caused by continuous addition of nutrients (LB, 8 mL/h), which, although not high enough to eliminate starvation, may delay the completion of sporulation.

Sporulation and competence development are two different strategies for survival under high cell density and other unfavorable conditions. Based on the large number of sporulation genes induced (74 genes) and the inhibition between sporulation and competence development (Grossman, 1995), the main character of the wild-type biofilm is sporulation, although it cannot be ruled out that some cells in the wild-type biofilm are competent. Compared to the wild-type biofilm, the biofilm of the *spoIIGB* mutant had genes for quorum sensing (such as *phrAEFK* and *oppABCDF*) and competence (such as *srfAA*, *srfAB*, *srfAD*, *comS*, and *comGA*) induced. Of these, only *phrF* and *srfAA* were induced in the wild-type biofilm. Since only 5–10% of the cells can be competent in a given culture (Grossman, 1995), the real number of the competence genes induced may be larger.

Previous studies have shown that *spo0A* is necessary for *B. subtilis* biofilm formation in microtiter plates, although sporulation itself is not necessary (Hamon and Lazazzera, 2001). In the present study, the *spoIIGB* mutant also formed thick biofilms like the wild type, suggesting that sporulation is not necessary for biofilm formation. However, the RNA yield from the biofilm cells was lower for the *spoIIGB* mutant compared to the wild type (around 50% less, data not shown). Hence the cells may be less healthy in the *spoIIGB* mutant biofilm and sporulation may be the preferred mode for the cells to survive with high populations accompanied by a depletion of nutrients.

While there is accumulating knowledge about quorum sensing, sporulation, and competence development, most of the previous studies were based on individual cells from suspension cultures (Branda et al., 2001). The cells in natural habitats, however, mostly grow in sessile biofilms. Hence, DNA microarrays have advantages for studying the interconnected networks of biofilm formation, competence, and sporulation under more realistic conditions. The present study identified the gene expression pattern in *B. subtilis* biofilms and found strong links between sporulation, competence development, and biofilm formation.

Recently, (Stanley et al. 2003) used DNA microarrays to study the transition stages of *B. subtilis* biofilm formation, and 519 genes were found differentially expressed at least at one time point (8, 12, and 14 h after inoculation). Only 86 of these 519 genes were differentially expressed in the wild-type biofilm in the present study. For example, there were 17 sporulation genes (*cotJC*, *cotNWXYZ*, *phrAE*, *spoIIAA*, *spoIIAB*, *spoVFB*, *spoVG*, *rapAGH*, *spoVAB*, *spsA*, *ysfA*, *ygaI*, *sspB*, and *sspC*) induced in at least one time point (mostly at 24 h after inoculation) in the results of Stanley et al. (2003), while there were 74 sporulation genes induced in the wild-type biofilm in the present study. It should be noted that the present study used a Bead-Beater to lyze cells which ensures the RNA isolation from all components including spores, while the previous study (Stanley et al., 2003) followed the standard protocol (Qiagen) using lysozyme, which might not be able to lyze the spores. However, because the present study is concerned with mature biofilms and the report of Stanley et al. (2003) studied the early stages of biofilm formation, differences are expected in the number of sporulation genes induced in the biofilm. Interestingly, a larger number (170) of the 519 genes reported by Stanley et al. (2003) were found differentially expressed in our *spoIIGB* biofilm compared to suspension cells (data not shown), which suggests the mutation in *spoIIGB* causes the biofilm (5 day) to have more characteristics of an early-stage biofilm (8–24 h). Given that the *spoIIGB* mutant has the sporulation pathway blocked by the mutation, the early-stage biofilm characteristics may be caused by the inability to sporulate. Hence, sporulation may be preferred by the cells in the mature air–liquid biofilm; further study of the biofilm structure along with live/dead cell counting may provide additional proof of this hypothesis. The differences in the results of the present study and the previous report

(Stanley et al., 2003) reveal the dynamic character of biofilm formation.

The present study focused on gene expression profiles of mature biofilms. Further study on the candidate genes found in the present study may help identify the genes necessary for maintaining biofilms. Hence, the results of the present study have potential for directing the biofilm control on a genetic level. The information from the present study has applications in two areas. First, it may help find the conditions favorable for biofilm formation and therefore improve those applications involving beneficial biofilms, such as those used in corrosion prevention (Jayaraman et al., 1997b). Second, drug screening with these biofilm genes as targets may find new antagonists for biofilm formation and therefore help find novel therapies for patients with biofilm infections.

## APPENDIX-Supplementary Data

**Table A1.** Sporulation genes *induced* in the wild-type *B. subtilis* biofilms.

| Stage | Gene | Induction expression ratio | Description |
|---|---|---|---|
| Stage I and II formation of septum | *kinB* | 3.5 | involved in the initiation of sporulation (activation of the alternate pathway to sporulation) |
| | *sigE* | 3.5 | early mother cell-specific gene expression ''/note='' alternate gene name: spoIIGB |
| | *spoIIAA* | 3.0 | binding to SpoIIAB in the presence of ADP selectively in the forespore (stage II sporulation) |
| | *spoIIAB* | 3.0 | binding to sigma-F (SpoIIAC) in the presence of ATP before septation, phosphorylation of SpoIIAA (stage II sporulation) |
| | *spoIIB* | 4.0 | endospore development (stage II sporulation) |
| | *spoIIEx* | 3.9 | sporulation |
| | *spoIIGA* | 27.1 | processing of pro-sigma-E (SpoIIGB) to active sigma-E (stage II sporulation) |
| Stage III prespore development | *sigG* | 3.3 | late forespore-specific gene expression |
| | *sspA* | 3.5 | small acid-soluble spore protein (alpha-type SASP) |
| | *sspD* | 3.2 | small acid-soluble spore protein (alpha/beta-type SASP) |
| | *sspE* | 4.5 | acid-soluble spore protein (gamma-type SASP) |
| | *sspF* | 5.4 | small acid-soluble spore protein (alpha/beta-type SASP) |
| | *spoIIIAA* | 3.7 | engulfment (stage III sporulation) |
| | *spoIIIAF* | 3.2 | engulfment (stage III sporulation) |
| | *spoIIIAG* | 5.1 | engulfment (stage III sporulation) |
| | *spoIIIAH* | 3.2 | engulfment (stage III sporulation) |
| | *spoIIIC* | 4.7 | late mother cell-specific gene expression (stage III/IV sporulation) |
| | *spoIIID* | 5.8 | required for complete dissolution of the asymmetric septum (stage II sporulation) |
| | *spoIIP* | 4.6 | required for dissolution of the septal cell wall (stage II sporulation) |
| Stage IV cortex formation | *cotA* | 8.6 | spore coat protein (outer) |
| | *cotB* | 9.2 | spore coat protein (outer) |
| | *cotD* | 9.0 | spore coat protein (inner) |
| | *cotE* | 6.3 | outer coat assembly |
| | *cotF* | 8.6 | spore coat protein |
| | *cotG* | 8.5 | required for the incorporation of CotB into the coat |
| | *cotH* | 12.4 | involved in the assembly of several proteins in the outer layer of the coat |
| | *cotJA* | 5.5 | polypeptide composition of the spore coat, required for the assembly of CotJC |
| | *cotJB* | 6.2 | polypeptide composition of the spore coat |
| | *cotJC* | 5.9 | polypeptide composition of the spore coat |
| | *cotK* | 4.7 | spore coat protein |
| | *cotL* | 4.8 | spore coat protein |
| | *cotM* | 7.3 | spore coat protein (outer) |
| | *cotN* | 5.7 | spore coat-associated protein |
| | *cotS* | 9.9 | spore coat protein |
| | *cotT* | 8.5 | spore coat protein (inner) |
| | *cotV* | 7.4 | spore coat protein (insoluble fraction) |
| | *cotW* | 9.1 | spore coat protein (insoluble fraction) |
| | *cotX* | 9.6 | spore coat protein (insoluble fraction) |
| | *cotY* | 7.4 | spore coat protein (insoluble fraction) |
| | *cotZ* | 6.2 | spore coat protein (insoluble fraction) |
| | *spoIVA* | 4.2 | required for proper spore cortex formation and coat assembly (stage IV sporulation) |
| | *spoIVB* | 5.2 | intercompartmental signalling of pro-sigma-K processing/ activation in the mother-cell, essential for spore cortex and coat formation (stage IV sporulation) |
| | *spoIVCA* | 3.5 | sporulation |
| | *spoIVCB* | 6.1 | late mother cell-specific gene expression (stage IV sporulation) |

**Table A1.** *(continued).*

| Stage | Gene | Induction expression ratio | Description |
|---|---|---|---|
| Stage V coat formation | *spoVAA* | 9.7 | mutants lead to the production of immature spores (stage V sporulation) |
| | *spoVAD* | 6.9 | mutants lead to the production of immature spores (stage V sporulation) |
| | *spoVAF* | 4.4 | mutants lead to the production of immature spores (stage V sporulation) |
| | *spoVD* | 3.9 | required for spore morphogenesis (spore cortex) (stage V sporulation) |
| | *spoVFA* | 8.0 | stage V sporulation |
| | *spoVFB* | 9.3 | stage V sporulation |
| | *spoVK* | 3.9 | disruption leads to the production of immature spores (stage V sporulation) |
| | *spoVR* | 4.8 | involved in spore cortex synthesis (stage V sporulation) |
| Other spore genes | *spoVID* | 6.1 | required for assembly of the spore coat (stage VI sporulation) |
| | *cgeA* | 9.6 | involved in maturation of the outermost layer of the spore |
| | *cgeC* | 7.5 | involved in maturation of the outermost layer of the spore |
| | *cgeD* | 6.7 | involved in maturation of the outermost layer of the spore |
| | *cgeE* | 7.4 | involved in maturation of the outermost layer of the spore |
| | *cwlC* | 3.5 | sporulation-specific mother cell wall hydrolase |
| | *dacB* | 6.4 | required for spore cortex synthesis (peptidoglycan biosynthesis) |
| | *gerBC* | 5.9 | germination response to the combination of glucose, fructose, L-asparagine, and KCl |
| | *gerE* | 6.3 | required for the expression of late spore coat genes (germination) |
| | *gerM* | 4.2 | germination (cortex hydrolysis) and sporulation (stage II, multiple polar septa) |
| | *sleB* | 4.5 | spore cortex-lytic enzyme |
| | *spsA* | 9.3 | spore coat polysaccharide synthesis |
| | *spsB* | 9.3 | spore coat polysaccharide synthesis |
| | *spsC* | 8.4 | spore coat polysaccharide synthesis |
| | *spsD* | 8.1 | spore coat polysaccharide synthesis |
| | *spsE* | 7.5 | spore coat polysaccharide synthesis |
| | *spsF* | 10.3 | spore coat polysaccharide synthesis |
| | *spsG* | 9.2 | spore coat polysaccharide synthesis |
| | *spsI* | 8.5 | spore coat polysaccharide synthesis |
| | *spsJ* | 8.4 | spore coat polysaccharide synthesis |
| | *tgl* | 6.2 | cross-links in spore coat proteins (sporulation stages IV and V) |
| | *usd* | 7.7 | required for translation of spoIIID |

**Table A2.** Other genes with known functions induced in the wild-type *B. subtilis* biofilm.

| Gene | Induction expression ratio | Description |
|---|---|---|
| *acoA* | 3.0 | acetoin dehydrogenase E1 component (TPP-dependent alpha subunit) |
| *acoC* | 4.2 | acetoin dehydrogenase E2 component (dihydrolipoamide acetyltransferase) |
| *ald* | 3.3 | L-alanine dehydrogenase |
| *amyC* | 8.3 | maltose transport protein |
| *appB* | 3.8 | oligopeptide transport |
| *appC* | 4.5 | oligopeptide transport |
| *appF* | 4.3 | oligopeptide transport |
| *aprX* | 6.2 | alkaline serine protease |
| *argE* | 4.9 | arginine biosynthesis |
| *asd* | 4.3 | asparate-semialdehyde dehydrogenase |
| *bioA* | 3.5 | biotin biosynthesis |
| *comER* | 5.3 | non-essential gene for competence |
| *csaA* | 45.2 | involved in protein secretion |
| *csfB* | 4.3 | unknown |
| *ctaC* | 3.6 | cytochrome caa3 oxidase (subunit II) |
| *cwlJ* | 4.7 | cell wall hydrolase |
| *dppD* | 3.0 | dipeptide ABC transporter (ATP-binding protein) |
| *gdh* | 6.2 | glucose 1-dehydrogenase |

**Table A2.** *(continued).*

| Gene | Induction expression ratio | Description |
|---|---|---|
| *glgA* | 4.8 | starch (bacterial glycogen) synthase |
| *glgP* | 4.5 | degrades starch and glycogen by phosphorylation (glycogen metabolism) |
| *glnH* | 4.0 | glutamine ABC transporter (glutamine-binding protein) |
| *hutH* | 5.5 | histidine utilization |
| *leuC* | 3.5 | leucine biosynthesis |
| *lonB* | 3.6 | Lon-like ATP-dependent protease |
| *lplD* | 3.4 | lytic enzyme |
| *lytD* | 3.8 | hydrolyses bond between the N-acetyglucosaminyl and the N-acetymuramyl residues in the glycan chain |
| *mmgA* | 4.2 | acetyl-CoA acetyltransferase |
| *mmgD* | 4.0 | citrate synthase III |
| *mpr* | 5.8 | extracellular metalloprotease |
| *msmE* | 3.0 | multiple sugar-binding protein |
| *phoB* | 3.0 | alkaline phosphatase III |
| *phrF* | 4.0 | regulator of the activity of phosphatase RapF |
| *phrI* | 3.5 | regulator of the activity of phosphatase RapI |
| *prkA* | 5.2 | serine protein kinase |
| *qcrA* | 3.2 | menaquinone oxidase |
| *qcrB* | 3.4 | menaquinol:cytochrome c oxidoreductase (cytochrome b subunit) |
| *qcrC* | 3.7 | menaquinol:cytochrome c oxidoreductase (cytochrome b/c subunit) |
| *ribH* | 3.2 | riboflavin biosynthesis |
| *rocE* | 3.0 | arginine and ornithine utilization |
| *sipW* | 3.5 | type I signal peptidase |
| *splB* | 3.4 | repair of UV radiation-induced DNA damage during spore germination |
| *srfAA* | 3.1 | surfactin production and competence |
| *thiA* | 3.4 | biosynthesis of the pyrimidine moiety of thiamin (thiamin biosynthesis) |
| *tlp* | 5.2 | methyl-accepting chemotaxis protein |
| *tuaG* | 3.5 | biosynthesis of teichuronic acid |
| *wprA* | 3.1 | cell wall-associated protein precursor |

**Table A3.** Genes with unknown functions induced in the wild-type *B. subtilis* biofilm (top 20 genes and *ybc, yve* operons shown).

| Gene | Induction expression ratio | Description |
|---|---|---|
| *ysnD* | 55.0 | unknown |
| *yyaC* | 41.8 | unknown |
| *ythA* | 29.0 | unknown, similar to cytochrome d oxidase subunit |
| *ytlA* | 16.1 | unknown |

**Table A3.** *(continued).*

| Gene | Induction expression ratio | Description |
|---|---|---|
| *ytlB* | 14.9 | unknown |
| *ybxH* | 12.8 | unknown |
| *ypuC* | 12.4 | unknown |
| *yjmJ* | 12.2 | unknown, similar to altronate hydrolase |
| *yraE* | 11.1 | unknown, similar to spore coat protein |
| *ycgL* | 11.1 | unknown |
| *ylbD* | 10.7 | unknown |
| *ytcB* | 10.4 | unknown, similar to NDP-sugar epimerase |
| *yodH* | 9.8 | unknown |
| *ytcA* | 9.5 | unknown, similar to NDP-sugar dehydrogenase |
| *yqcI* | 9.2 | unknown |
| *yisC* | 9.1 | unknown |
| *ytxO* | 9.1 | unknown |
| *yisG* | 9.0 | unknown |
| *yhcO* | 9.0 | unknown |
| *yitA* | 8.9 | unknown, similar to sulfate adenylyltransferase |
| *ybcO* | 3.4 | unknown |
| *ybcP* | 4.2 | unknown |
| *ybcQ* | 3.3 | unknown |
| *ybcS* | 4.3 | unknown |
| *yveM* | 3.9 | unknown |
| *yveM* | 3.7 | unknown |
| *yveN* | 3.9 | unknown |
| *yveO* | 3.6 | unknown |
| *yveP* | 4.0 | unknown |
| *yveQ* | 4.3 | unknown |
| *yveR* | 4.3 | unknown |

**Table A4.** Genes repressed in the wild-type *B. subtilis* biofilm (top 20 genes shown).

| Gene | Repression expression ratio | Description |
|---|---|---|
| *alsD* | −86.7 | alpha-acetolactate decarboxylase |
| *yjdE* | −63.5 | unknown, similar to fructokinase |
| *yjdD* | −59.0 | unknown, similar to fructose phosphotransferase system enzyme II |
| *narJ* | −53.8 | nitrate reductase (protein J) |
| *narG* | −46.5 | nitrate reductase (alpha subunit) |
| *lctP* | −46.4 | L-lactate permease |
| *narI* | −45.7 | nitrate reductase (gamma subunit) |
| *alsSx* | −44.5 | unknown |
| *lctE* | −39.0 | L-lactate dehydrogenase |
| *narK* | −28.5 | nitrite extrusion protein |
| *feuA* | −27.0 | component of iron-uptake system |
| *fhuD* | −26.6 | ferrichrome ABC transporter (ferrichrome-binding protein) |
| *ydaD* | −24.0 | unknown, similar to alcohol dehydrogenase |

**Table A4.** *(continued).*

| Gene | Repression expression ratio | Description |
|---|---|---|
| *yxaE* | −23.5 | unknown |
| *dhbA* | −21.4 | siderophore 2,3-dihydroxybenzoate (DHB) synthesis |
| *ykuP* | −21.2 | unknown, similar to sulfite reductase |
| *ykuN* | −20.4 | unknown, similar to flavodoxin |
| *dhbBx* | −20.3 | unknown |
| *yhgD* | −18.5 | unknown, similar to transcriptional regulator (TetR/AcrR family) |
| *yvmB* | −18.3 | unknown |

**Table A5.** Genes induced in the *spoIIGB* mutant biofilm.

| Function | Gene | Induction expression ratio | Description |
|---|---|---|---|
| Competence and sporulation | *comGA* | 2.7 | late competence gene |
| | *comS* | 3.2 | regulation of genetic competence, assembly ink between other regulatory components of the competence signal transduction pathway |
| | *cotN* | 4.1 | spore coat-associated protein |
| | *rapA* | 6.2 | prevents sporulation by dephosphorylating Spo0F-P (and thus the phosphorelay) |
| | *sigF* | 2.7 | early forespore-specific gene expression |
| | *spo0E* | 2.6 | negative sporulation regulatory phosphatase |
| | *spo0F* | 3.4 | initiation of sporulation (stage 0 sporulation) |
| | *spoIIAA* | 4.4 | binding to SpoIIAB in the presence of ADP selectivity in the forespore (stage II sporulation) |
| | *spoIIAB* | 5.5 | binding to sigma-F (SpoIIAC) in the presence of ATP before septation, phosphorylation of SpoIIAA (stage II sporulation) |
| | *spoIIB* | 5.6 | endospore development (stage II sporulation) |
| | *spoIIE* | 4.4 | dephosphorylates SpoIIAA-P and overcomes SpoIIAB-mediated inhibition of sigma-F, required for normal formation of the asymmetric septum (stage II sporulation) |
| | *spoIIGA* | 5.8 | processing of pro-sigma-E (SpoIIGB) to active sigma-E (stage II sporulation) |
| | *spoIIQ* | 3.6 | required for completion of engulfment |
| | *spoVG* | 3.3 | required for spore cortex synthesis (stage V sporulation) |
| | *spoVS* | 2.8 | required for dehydratation of the spore core and assembly of the coat (stage V sporulation) |
| | *srfAA* | 4.6 | surfactin production and competence, surfactin synthetase |
| | *srfAB* | 3.4 | surfactin production and competence, surfactin synthetase |
| | *srfAD* | 3.8 | surfactin production and competence, surfactin synthetase |
| Transport | *amyD* | 3.0 | sugar transport |
| | *appA* | 5.5 | oligopeptide ABC transporter (oligopeptide-binding protein) oligopeptide transport |
| | *appB* | 4.8 | oligopeptide ABC transporter (permease), oligopeptide transport |
| | *appC* | 6.1 | oligopeptide ABC transporter (permease) |
| | *appD* | 9.9 | oligopeptide ABC transporter (ATP-binding protein) |
| | *appF* | 7.0 | oligopeptide ABC transporter (ATP-binding protein) |
| | *blt* | 2.8 | multidrug-efflux transporter |
| | *dppA* | 4.4 | dipeptide ABC transporter |
| | *dppB* | 5.0 | dipeptide ABC transporter (permease) |
| | *dppC* | 7.7 | dipeptide ABC transporter (permease) |
| | *dppD* | 6.3 | dipeptide ABC transporter (ATP-binding protein) |
| | *dppE* | 4.2 | dipeptide ABC transporter (dipeptide-binding protein) |
| | *levE* | 4.7 | phosphotransferase system (PTS) fructose-specific enzyme IIB component |
| Transport | *levF* | 4.3 | phosphotransferase system (PTS) fructose-specific enzyme IIC component |
| | *levG* | 4.3 | phosphotransferase system (PTS) fructose-specific enzyme IID component |
| | *oppA* | 4.8 | required for initiation of sporulation, competence development, and oligopeptide transport (stage 0 sporulation) |

**Table A5.** *(continued).*

| Function | Gene | Induction expression ratio | Description |
|---|---|---|---|
| | *oppB* | 6.2 | required for initiation of sporulation, competence development, and oligopeptide transport (stage 0 sporulation)'' |
| | *oppC* | 4.7 | oligopeptide ABC transporter (permease) |
| | *oppD* | 4.4 | required for initiation of sporulation, competence development, and oligopeptide transport (stage 0 sporulation)'' |
| | *oppF* | 4.8 | required for initiation of sporulation, competence development, and oligopeptide transport (stage 0 sporulation)'' |
| | *rbsA* | 2.5 | ribose transport |
| | *rbsB* | 3.4 | ribose transport |
| | *rbsC* | 2.4 | ribose ABC transporter (permease) |
| | *rbsD* | 2.7 | ribose transport |
| Antibiotic Production | *pksF* | 3.3 | involved in polyketide synthesis |
| | *pksJ* | 4.4 | involved in polyketide synthesis |
| | *pksL* | 3.4 | polyketide synthase of type 1 |
| | *pksR* | 3.7 | polyketide synthase |
| | *pps* | 2.8 | peptide synthetase |
| | *ppsA* | 4.7 | peptide synthetase |
| | *ppsD* | 4.3 | peptide synthetase |
| | *ppsE* | 4.9 | peptide synthetase |
| Synthesis and Metamolism | *abnA* | 3.6 | degradation of plant cell wall polysaccharide (arabinan and arabinose utilization) |
| | *acoA* | 6.9 | acetoin dehydrogenase E1 component (TPP-dependent alpha subunit) |
| | *acoB* | 7.1 | acetoin dehydrogenase E1 component (TPP-dependent alpha subunit) |
| | *acoC* | 10.1 | acetoin dehydrogenase E2 component (dihydrolipoamide dehydrogenase) |
| | *acoL* | 8.6 | acetoin dehydrogenase E3 component (dihydrolipoamide dehydrogenase) |
| | *acoR* | 4.8 | positive regulation of the acetoin dehydrogenase operon (acoABCL) |
| | *acsA* | 4.8 | acetyl-CoA synthetase |
| | *acuA* | 3.5 | acetoin dehydrogenase |
| | *acuB* | 3.9 | acetoin dehydrogenase |
| | *acuC* | 4.1 | acetoin dehydrogenase |
| | *ald* | 4.9 | L-alanine dehydrogenase |
| | *amyE* | 3.1 | alpha-amylase |
| | *aprE* | 6.6 | serine alkaline protease (subtilisin E) |
| | *argB* | 2.8 | arginine biosynthesis |
| | *argJ* | 2.5 | arginine biosynthesis |
| | *bjlS* | 6.3 | lichenan degradation |
| | *bofC* | 2.7 | forespore regulator of the sigma-K checkpoint |
| | *bpr* | 9.6 | bacillopeptidase F |
| | *bsaA* | 2.6 | glutathione peroxidase |
| | *cccA* | 5.1 | cytochrome c550 |
| | *citB* | 4.8 | aconitate hydratase |
| | *citC* | 2.5 | isocitrate dehydrogenase |
| | *citS* | 2.5 | two-component sensor histidine kinase |
| | *citT* | 2.9 | two-component response regulator |
| | *citZ* | 3.4 | citrate synthase II |
| | *csaA* | 4.7 | involved in protein secretion, molecular chaperonin |
| | *cstA* | 4.7 | carbon starvation-induced protein |
| | *ctaC* | 5.3 | cytochrome caa3 oxidase (subunit II) |
| | *ctaDx* | 5.0 | unknown |
| | *ctaE* | 4.9 | cytochrome caa3 oxidase (subunit III) |
| | *ctaF* | 5.6 | cytochrome caa3 oxidase (subunit III) |
| | *ctaG* | 4.3 | unknown |
| | *cypX* | 3.4 | cytochrome P450-like enzyme |
| | *cysH* | 4.3 | cysteine biosynthesis |
| | *dat* | 3.4 | O6-methylguanine DNA alkyltransferase |
| | *dhaS* | 3.1 | aldehyde dehydrogenase |
| | *gabP* | 5.0 | gamma-aminobutyrate (GABA) permease |
| | *gapB* | 9.6 | glycolysis, glyceraldehyde-3-phosphate dehydrogenase |
| | *glgA* | 3.8 | starch (bacterial glycogen) synthase |
| | *glgB* | 3.6 | introduces alpha-1,6-linkages in starch and glycogen (glycogen biosynthesis) |
| | *glgC* | 3.3 | activates glucose-1-phosphate using ATP (glycogen biosynthesis) |
| | *glgP* | 3.9 | degrades starch and glycogen by phosphorylation (glycogen metabolism) |
| | *hom* | 5.1 | threonine/methionine biosynthesis |
| | *hutG* | 5.0 | histidine utilization |
| | *hutI* | 4.0 | histidine utilization |
| | *hutU* | 4.2 | histidine utilization |
| | *ilvA* | 5.2 | isoleucine biosynthesis |
| | *ilvB* | 7.9 | valine/isoleucine biosynthesis |
| | *ilvC* | 3.5 | valine/isoleucine biosynthesis |
| | *ilvD* | 6.4 | valine/isoleucine biosynthesis |
| | *ilvN* | 7.3 | valine/isoleucine biosynthesis |

**Table A5.** *(continued).*

| Function | Gene | Induction expression ratio | Description |
|---|---|---|---|
| | *iolA* | 4.8 | myo-inositol catabolism |
| | *iolB* | 5.4 | myo-inositol catabolism |
| | *iolC* | 4.9 | myo-inositol catabolism |
| | *iolE* | 5.6 | myo-inositol catabolism |
| | *iolF* | 5.4 | myo-inositol catabolism |
| | *iolG* | 7.6 | myo-inositol catabolism |
| | *iolH* | 6.4 | myo-inositol catabolism |
| | *iolI* | 5.3 | myo-inositol catabolism |
| | *iolJ* | 4.6 | myo-inositol catabolism |
| | *ispA* | 3.3 | intracellular serine protese |
| | *katA* | 3.5 | vegatative catalase 1 |
| | *katX* | 3.1 | catalase |
| | *kbl* | 4.8 | 2-amino-3-ketobutyrate CoA ligase (glycine acetyl transferase) |
| | *leuB* | 7.7 | leucine biosynthesis |
| | *leuC* | 5.7 | leucine biosynthesis |
| | *leuD* | 4.0 | leucine biosynthesis |
| | *lonB* | 4.3 | Lon-like ATP-dependent protease |
| | *lytE* | 4.3 | cell wall lytic activity |
| | *melA* | 2.7 | alpha-D-galactoside galactohydrolase |
| | *metC* | 3.3 | methionine biosynthesis |
| | *mpr* | 5.4 | extracellular metalloprotease |
| | *mrgA* | 3.2 | metalloregulation DNA-binding stress protein |
| | *msmE* | 4.5 | multiple sugar-binding protein |
| | *msmX* | 2.7 | multiple sugar-binding transport ATP-binding protein |
| | *ndk* | 3.1 | nucleoside diphosphate kinase |
| | *nprE* | 2.7 | extracellular neutral metalloprotease |
| | *pbp* | 3.0 | peptidoglycan biosynthesis |
| | *pbpE* | 2.6 | penicillin-binding protein 4 |
| | *pckA* | 10.9 | phosphoenolpyruvate carboxykinase |
| | *pel* | 4.0 | pectate lyase |
| | *phrA* | 6.6 | inhibitor of the activity of phosphatase RapA |
| | *phrE* | 2.7 | regulator of the activity of phosphatase RapE |
| | *phrF* | 7.5 | regulator of the activity of phosphatase RapF |
| | *phrK* | 3.7 | regulator of the activity of phosphatase RapK |
| | *purL* | 3.2 | purine biosynthesis |
| | *pycA* | 3.1 | pyruvate carboxylase |
| | *qcrA* | 4.0 | menaquinone oxidase |
| | *qcrB* | 4.3 | menaquinol:cytochrome c oxidoreductase (cytochrome b subunit) |
| | *qcrC* | 4.3 | menaquinol:cytochrome c oxidoreductase (cytochrome b/c subunit) |
| | *racX* | 3.2 | amino acid racemase |
| | *rapC* | 2.6 | response regulator aspartate phosphatase |
| | *rapE* | 3.2 | response regulator aspartate phosphatase |
| | *rapF* | 2.9 | response regulator aspartate phosphatase |
| | *rapH* | 3.5 | response regulator aspartate phosphatase |
| | *rapK* | 3.4 | response regulator aspartate phosphatase |
| | *rbsk* | 2.9 | ribose metabolism |
| | *rbsR* | 2.5 | transcriptional regulator (LacI family) |
| | *rocA* | 3.3 | arginine and ornithine utilization |
| | *rocB* | 5.4 | involved in arginine and ornithine utilization |
| | *rocC* | 7.1 | amino acid permease |
| | *rocD* | 4.2 | arginine and ornithine utilization |
| Synthesis and metabolism | *rocF* | 3.8 | arginine and ornithine utilization |
| | *sacC* | 3.5 | levanase |
| | *sucD* | 2.4 | succinyl-CoA synthetase (alpha subunit) |
| | *tagC* | 2.8 | polyglycerol phosphate assembly and export (teichoic acid biosynthesis) |
| | *tdh* | 3.1 | threonine catabolism |
| | *thrB* | 4.9 | threonine biosynthesis |
| | *thrC* | 4.2 | threonine biosynthesis |
| | *ureC* | 2.9 | urease (alpha subunit) |
| | *vpr* | 6.1 | extracellular serine protease |
| | *wprA* | 6.5 | cell wall-associated protein precursor (CWBP23, CWBP52) |
| Phage-related functions | *xhlA* | 4.2 | involved in cell lysis upon induction of defective prophage PBSX |
| | *xhlB* | 5.2 | hydrolysis of 5-bromo 4-chloroindolyl phosphate (X-phos), involved in cell lysis upon induction of defective prophage PBSX |
| | *xkdE* | 5.2 | PBSX prophage |
| | *xkdF* | 4.0 | PBSX prophage |
| | *xkdG* | 6.2 | PBSX prophage |
| | *xkdI* | 6.4 | PBSX prophage |
| | *xkdK* | 4.7 | PBSX prophage |
| | *xkdM* | 4.5 | PBSX prophage |
| | *xkdN* | 3.5 | PBSX prophage |
| | *xkdO* | 4.9 | PBSX prophage |
| | *xkdQ* | 4.4 | PBSX prophage |
| | *xkdS* | 2.4 | PBSX prophage |
| | *xkdT* | 3.1 | PBSX prophage |
| | *xkdU* | 3.1 | PBSX prophage |
| | *xkdV* | 4.2 | PBSX prophage |
| | *xkdY* | 3.3 | lytic exoenzyme associated with defective prophage PBSX |

**Table A5.** *(continued).*

| Function | Gene | Induction expression ratio | Description |
|---|---|---|---|
| | *xlyA* | 3.6 | major role in defective prophage PBSX-mediated lysis |
| | *xlyB* | 3.4 | involved in defective prophage PBSX-mediated lysis |
| | *xtmB* | 4.2 | PBSX defective prophage terminase (large subunit) |
| genes of unknown function ( 20 genes and *ybc* operon shown) | *yjdB* | 8.9 | unknown |
| | *yydF* | 8.3 | unknown |
| | *yvaY* | 7.9 | unknown |
| | *ywaD* | 7.8 | unknown, similar to aminopeptidase |
| genes of unknown function ( 20 genes and *ybc* operon shown) | *ywkC* | 7.4 | threonine/methionine biosynthesis |
| | *ybdN* | 7.3 | unknown |
| | *yxnB* | 6.6 | unknown |
| | *yraI* | 6.4 | unknown |
| | *yvaX* | 6.4 | unknown |
| | *yydG* | 6.4 | unknown |
| | *yvrK* | 6.3 | unknown |
| | *yvaW* | 6.2 | unknown |
| | *ylqB* | 6.2 | unknown |
| | *yabS* | 6.0 | unknown |
| | *ydbH* | 5.9 | unknown, similar to C4-dicarboxylate transport protein |
| | *yxbB* | 5.9 | unknown, similar to ABC transporter (binding protein) |
| | *ybdA* | 5.8 | unknown |
| | *yxbA* | 5.8 | unknown |
| | *yfmG* | 8.6 | unknown |
| | *ybcP* | 5.3 | unknown |
| | *ybcQ* | 5.3 | unknown |
| | *ybcS* | 4.4 | unknown |
| | *ybcT* | 2.5 | unknown |

**Table A6.** Genes *repressed* in the *spoIIGB* mutant biofilm (top 20 shown).

| Gene | Repression expression ratio | Description |
|---|---|---|
| *alsSx* | −100 | unknown |
| *alsD* | −50 | acetoin biosynthesis |
| *narJ* | −33 | nitrate reductase (protein J) |
| *narG* | −33 | nitrate reductase (alpha subunit) |
| *lctP* | −33 | L-lactase permease |
| *narI* | −33 | nitrate reductase (gamma subunit) |
| *hmp* | −33 | flavohemoglobin |
| *lctE* | −33 | L-lactate dehydrogenase |
| *narK* | −25 | nitrite extrusion protein |

**Table A6.** *(continued).*

| Gene | Repression expression ratio | Description |
|---|---|---|
| *yjdD* | −20 | unknown, similar to fructose phosphotransferase system enzyme II |
| *yjdE* | −20 | unknown, similar to mannose-6-phosphate isomerase |
| *ywcJ* | −17 | unknown, similar to nitrate transporter |
| *pyrAA* | −14 | pyrimidine biosynthesis |
| *glpD* | −14 | glycerol utilization |
| *yxlD* | −13 | unknown |
| *yhgE* | −13 | unknown, similar to phage infection protein |
| *yxlE* | −11 | unknown |
| *cydD* | −11 | ABC membrane transporter (ATP-binding protein) |
| *yhgD* | −11 | unknown, similar to transcriptional regulator (TetR/AcrR family) |
| *treP* | −11 | phosphotransferase system (PTS) trehalose-specific enzyme IIBC component |

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