Published in final edited form as:
Mol Microbiol. 2004 May ; 52(3): 847–860.

Identification of AbrB-regulated genes involved in biofilm formation by *Bacillus subtilis*

Mélanie A. Hamon[1], **Nicola R. Stanley**[1], **Robert A. Britton**[2,3], **Alan. D. Grossman**[2], and **Beth A. Lazazzera**[1,*]

1 *Department of Microbiology, Immunology and Molecular Genetics University of California Los Angeles 1602 Molecular Sciences Building 405 Hilgard Avenue Los Angeles, CA 90095, USA*

2 *Department of Biology Massachusetts Institute of Technology Cambridge, MA 02139, USA*

Abstract

Bacillus subtilis is a ubiquitous soil bacterium that forms biofilms in a process that is negatively controlled by the transcription factor AbrB. To identify the AbrB-regulated genes required for biofilm formation by *B. subtilis*, genome-wide expression profiling studies of biofilms formed by *spo0A abrB* and *sigH abrB* mutant strains were performed. These data, in concert with previously published DNA microarray analysis of *spo0A* and *sigH* mutant strains, led to the identification of 39 operons that appear to be repressed by AbrB. Eight of these operons had previously been shown to be repressed by AbrB, and we confirmed AbrB repression for a further 6 operons by reverse transcription-PCR. The AbrB-repressed genes identified in this study are involved in processes known to be regulated by AbrB, such as extracellular degradative enzyme production and amino acid metabolism, and processes not previously known to be regulated by AbrB, such as membrane bioenergetics and cell wall functions. To determine whether any of these AbrB-regulated genes had a role in biofilm formation, we tested 23 mutants, each with a disruption in a different AbrB-regulated operon, for the ability to form biofilms. Two mutants had a greater than two-fold defect in biofilm formation. A *yoaW* mutant exhibited a biofilm structure with reduced depth, and a *sipW* mutant exhibited only surface-attached cells and did not form a mature biofilm. YoaW is a putative secreted protein, and SipW is a signal peptidase. This is the first evidence that secreted proteins have a role in biofilm formation by *Bacillus subtilis*.

Keywords

biofilms; AbrB; stationary phase; DNA microarrays; Spo0A; sigma-H; *Bacillus subtilis*

Introduction

Many bacteria exhibit an attached biofilm mode of growth, in which cells grow as an organized three-dimensional community adherent to a surface and encased in a self-produced, polymeric matrix (Costerton *et al*., 1995; Davey and O'Toole G, 2000). It has been proposed that most bacteria in nature are found in a biofilm mode of growth (Costerton *et al*., 1995). Biofilms are also found in medical and industrial settings, where they can be problematic due to the increased resistance of biofilm cells to antimicrobial agents (Kuchma and O'Toole, 2000). Relatively few genes have been identified that are required for bacteria to form biofilms, and little is known about how bacteria coordinate biofilm formation with other phenotypic states that bacteria exhibit.

*For correspondence. E-mailbethl@microbio.ucla.edu; Tel (+1) 310 794 4804; Fax (+1) 310 206 5231.
[3]Current Address: Department of Microbiology and Molecular Genetics Michigan State University, East Lansing, MI 48823, USA

Bacillus subtilis, a Gram-positive soil bacterium, provides a model system to study the molecular mechanisms controlling biofilm formation. At least three global regulatory proteins, AbrB, Spo0A, and Sigma-H, affect biofilm formation by *B. subtilis* (Hamon and Lazazzera, 2001; Branda *et al.*, 2001). Spo0A is required for surface-attached cells to transition to a three-dimensional biofilm structure (Hamon and Lazazzera, 2001; Branda *et al.*, 2001). The role of Spo0A in this process is to repress expression of AbrB (Figure 1) (Hamon and Lazazzera, 2001). Spo0A binds and directly represses the *abrB* promoter (Strauch *et al.*, 1990), and an *abrB* mutation restores biofilm formation to a *spo0A* mutant strain (Hamon and Lazazzera, 2001). Thus, AbrB is a negative regulator of the initiation of biofilm formation. Sigma-H may indirectly repress AbrB expression and stimulate the initiation of biofilm formation, as Sigma-H is known to activate expression of *spo0A* (Figure 1) (Predich *et al.*, 1992). However, Sigma-H is not essential for the initiation of biofilm formation, as mutants defective for Sigma-H do not have an obvious defect in the initiation of biofilm formation but, rather, have a defect in the formation of fruiting bodies on the surface of biofilms formed by wild isolates of *B. subtilis* (Branda *et al.*, 2001). Consistent with Spo0A and Sigma-H stimulating the initiation pathway for biofilm formation, many genes that were shown to be regulated by Spo0A and Sigma-H in genome-wide expression profiling studies were also found to be differentially expressed as cells transitioned from a planktonic state to a biofilm state (Stanley *et al.*, 2003). Which of the Spo0A and Sigma-H regulated genes are regulated by AbrB is not fully known, and the genes repressed by AbrB that are required for biofilm formation are unknown.

AbrB, Spo0A, and Sigma-H are global regulatory proteins that control many processes that occur during the transition of *B. subtilis* from exponential growth to stationary phase. In addition to biofilm formation, these transcription factors regulate differentiation of a sub-population of cells into genetically competent cells capable of taking up exogenous DNA, formation of environmentally resistant spores, and acquisition of new food sources through the production of degradative enzymes and antibiotics (Phillips and Strauch, 2002). This raises the interesting question of how *B. subtilis* coordinates the decision to enter these different phenotypic states. AbrB has a significant role in this decision making process, as the role of Spo0A and Sigma-H in degradative enzyme production, antibiotic production, and genetic competence is due to their role in repressing AbrB expression (Phillips and Strauch, 2002). Unlike for the biofilm formation pathway, at least some of the genes required for the formation of these other phenotypic states that are repressed by AbrB have been identified. However, there have been no studies to generate a complete picture of the genes and, thus, the physiological processes regulated by AbrB.

Here, we present the identification of AbrB-regulated genes that are induced under biofilm formation condition. We identified 57 genes encoded in 39 operons that appear to be repressed by AbrB. More than half of these genes are of unknown function, and many of the genes of known function are involved in metabolism and energy generation. To assess the role of some of these AbrB-regulated genes in biofilm formation, we disrupted 23 of the 39 operons and tested these mutants for their ability to support biofilm formation. Two genes were identified, *sipW*, which encodes a signal peptidase, and *yoaW*, which encodes a secreted protein, that are required for normal biofilm formation.

Results

Identification of genes regulated by AbrB

A profile of the genes differentially expressed during biofilm formation by *B. subtilis* has recently been published (Stanley *et al.*, 2003). 70 of the genes that were differentially expressed after 24 hours incubation under biofilm formation conditions had previously been shown to be activated by Spo0A and/or Sigma-H (Fawcett *et al.*, 2000; Britton *et al.*, 2002). These 70 genes may be regulated by Spo0A or Sigma-H directly or indirectly through Sigma-H and Spo0A

repression of *abrB* (Figure 1). To determine which of these 70 genes are regulated by AbrB, we compared the gene expression profile of *spo0A abrB* or *sigH abrB* mutant strains to a wild-type strain under biofilm formation conditions. AbrB-repressed genes should be those of the 70 genes that are not differentially expressed or have increased expression in the *spo0A abrB* or *sigH abrB* mutant, as AbrB is not present in the mutant strains and is similarly depleted in the wild-type strain due to repression of *abrB* by Spo0A. In contrast, genes that are positively regulated by Spo0A or Sigma-H independently of AbrB should be those of the 70 genes that have decreased expression in the *spo0A abrB* or *sigH abrB* mutant strains.

DNA microarrays comprised of 4074 of the 4100 open reading frames of the *B. subtilis* genome were used to monitor the differences in mRNA levels between by the wild-type strain and either a *spo0A abrB* or *sigH abrB* mutant strain after growth of the cells under biofilm formation conditions for 24 hours (see Materials and Methods). The RNA from the wild-type and the mutant strains was fluorescently labeled with Cy5 and Cy3, respectively, through the generation of cDNA. The DNA microarrays were simultaneously hybridised with the wild-type cDNA and one of the mutant cDNA samples to determine the ratio of gene expression. Those genes that had highly variable expression ratios were eliminated from further analysis as previously described (Stanley *et al.*, 2003).

From the wild-type *versus spo0A abrB* DNA microarray experiments, approximately 60% of the genes had reproducible expression ratios. As much as a 5.1-fold difference in the expression level for a gene was observed. Iterative outlier analysis was applied to those genes that had reproducible ratios to determine which genes had significantly different expression between the two strains. 100 genes were identified through this approach as differentially expressed between the wild-type and *spo0A abrB* strains (see supplementary Table 1).

From the wild-type *versus sigH abrB* DNA microarray experiments, approximately 54% of the genes gave reproducible expression ratios. As much as a 7.1-fold difference in the expression level for a gene was observed. Iterative outlier analysis identified 140 genes as differentially expressed between the wild-type and *sigH abrB* strains (see supplementary Table 2).

Of the 70 genes previously shown to be activated by Spo0A or Sigma-H and induced under biofilm formation conditions (Stanley *et al.*, 2003), 13 had lower expression in either the *spo0A abrB* or *sigH abrB* mutant strain (see supplementary Table 3). This suggests that Spo0A or Sigma-H, independently of AbrB, activated these 13 genes. These data further suggest that the remaining 57 genes may be regulated by Spo0A or Sigma-H through AbrB (Table 1). These 57 genes are part of 39 known or putative operons. Many genes that are part of these operons were not identified as differentially expressed. However, these genes have similar expression profiles to the genes that were identified as differentially expressed, except their variances were high in the Stanley et al. (2003) study. These genes are listed in Table 1 in parentheses as they are likely to be AbrB regulated. Consistent with the operons in Table 1 being regulated by AbrB, eight of the operons have previously been shown to be regulated by AbrB under planktonic conditions: *aprE* (Jan *et al.*, 2000; Ferrari *et al.*, 1988), *dppABCDE* (Slack *et al.*, 1991), *hutPHUIGM* (Fisher *et al.*, 1994), *phrE* (McQuade *et al.*, 2001), *qcrABC* (Yu *et al.*, 1995), *sdpABC* (Gonzalez-Pastor *et al.*, 2003), *spoVG* (Robertson *et al.*, 1989), and *yqxM sipW tasA* (Stover and Driks, 1999a).

To confirm *via* a second method that the genes identified as regulated by AbrB under planktonic conditions were indeed regulated by AbrB under biofilm formation conditions, we monitored the expression of the *yqxM sipW tasA* operon. This was achieved by measuring the levels of β-galactosidase activity from wild-type, *spo0A* and *spo0A abrB* strains containing a *sipW-lacZ* transcriptional fusion. *sipW* is the second gene in the three gene operon (Stover and Driks,

1999a). The levels of β-galactosidase activity in these strains were measured after growth under planktonic conditions and after growth for 16 hours under biofilm formation conditions.

The *sipW-lacZ* fusion exhibited a 21-fold higher expression under biofilm formation conditions *versus* planktonic conditions in the wild-type strain (data not shown). The expression of *sipW-lacZ* in the *spo0A* mutant strain was near the detection threshold under biofilm formation conditions (0.11 units of activity for the *spo0A* mutant strain *versus* 28 units for the wild-type strain), indicating that *sipW* is under the positive transcriptional control of Spo0A under biofilm formation conditions. In contrast, for the *spo0A abrB* mutant strain, 98 units of activity were observed under biofilm formation conditions, indicating that AbrB inhibits *sipW* transcription in a *spo0A* mutant strain. These data confirm that the expression of the *sipW* operon is regulated in a similar manner under biofilm formation conditions as under planktonic conditions (Stover and Driks, 1999a).

To determine whether any of the genes listed in Table 1, which were not previously determined to be regulated by AbrB, were indeed AbrB-regulated, reverse transcription-PCR (RT-PCR) was utilised. This allowed the expression of a gene to be monitored and expressed as a ratio with respect to a control gene, *veg*, that was known not to be differentially expressed ((Gilman and Chamberlin, 1983;Ollington *et al.*, 1981;Ollington and Losick, 1981) and see Materials and Methods). We examined the expression profile of 7 genes from 7 different operons: *yqxM* (the first gene in the operon containing *sipW*), *ycnK, yoaW, yocH, yvqH, yxbB,* and *yxbC* in wild-type, *spo0A* and *spo0A abrB* mutant strains grown for 24 hours under biofilm formation conditions. The gene *yqxM* was used as a positive control. It was expressed at levels 2-fold higher in the wild-type strain than in the *spo0A* mutant strain (Table 2), indicating that *yqxM* is under the positive transcriptional control of Spo0A. Expression of *yqxM* in the *spo0A abrB* mutant strain was 3.5-fold higher than in *spo0A* mutant strain (Table 2), indicating that AbrB mediated the repression of *yqxM* seen in the *spo0A* strain. Similar expression profiles were seen for *ycnK, yoaW, yocH, yvqH, yxbB,* and *yxbC* (Table 2). These data support the microarray analysis that identified these genes as repressed by AbrB under biofilm formation conditions.

Microtiter plate analysis of biofilm formation by mutants defective for AbrB-regulated genes

To determine whether any of the 39 operons identified as AbrB repressed are involved in biofilm formation, we disrupted 23 of the operons by disrupting the first gene of the operon (Table 3). These 23 operons represent 17 of the 21 operons of unknown function and 6 of the 18 operons of known function. These 23 mutants were tested for their ability to form biofilms in the microtiter plate assay (Table 3). 9 of the 23 mutants exhibited significantly ($p<0.01$) altered levels of biofilm formation . Six mutants, *tagE*, *ymfJ*, *ywqH*, *yqxI*, *yqxM*, and *yoaW*, formed reduced levels of biofilms, and 3 mutants, *yhjM*, *ydeH*, and *era*, formed increased levels compared to the wild-type strain. A growth defect did not appear to be responsible for the altered levels of biofilm formation by all but one of these mutants; only the *tagE* mutant grew slower than wild type in microtiter plates (data not shown). We chose to further study the effect of the *yqxM* and the *yoaW* operons on biofilm formation as only these mutant strains exhibited a greater than 2-fold difference in biofilm formation, .

The biofilm defect of *yqxM* and *yoaW* mutant strains could be due to the loss of YqxM or YoaW proteins, respectively, or loss of another protein encoded in the same operon as *yqxM* or *yoaW*. Analysis of the DNA sequence surrounding *yoaW* indicated that this gene is unlikely to be co-transcribed with another gene. The sequence of a transcription terminator can be found both up and downstream of *yoaW*, and downstream of *yoaW* there is a gene transcribed in the opposite orientation (Figure 2). Thus, the phenotype of *yoaW* mutant cells is likely to be due to loss of YoaW.

In contrast, *yqxM* is known to be the first gene in a three gene operon containing *sipW* and *tasA* (Figure 2) (Stover and Driks, 1999a;Serrano *et al.*, 1999). Thus, we sought to determine whether the defect in biofilm formation observed in the microtiter plate assay for the *yqxM* mutant could be due to a defect in either *tasA* or *sipW* expression. Mutants with non-polar mutations in *yqxM* or *tasA*, or a mutation in *sipW*, which is polar on *tasA*, were tested for biofilm formation using the microtiter plate assay. As shown in Figure 3, the *yqxM* and the *tasA* deletion strains formed biofilms at levels that were statistically indistinguishable from the wild-type strain. In contrast, the *sipW* deletion strain showed a 7.5-fold decrease in biofilm formation ($p<0.001$), which is comparable to the level observed for the *yqxM* operon mutant strain. These data indicate that neither *yqxM* nor *tasA* individually are required for biofilm formation and that the lack of SipW is largely responsible for the biofilm defect of a *yqxM* operon mutant.

SipW is a signal peptidase that is only known to process TasA and YqxM (Stover and Driks, 1999a; b; Serrano *et al.*, 1999). Thus, the biofilm defect of a *sipW* mutant could have been due to the inability of a *sipW* mutant strain to process both TasA and YqxM. To determine whether *tasA* and *yqxM* have redundant functions in biofilm formation, a double *tasA yqxM* mutant, which should still produce SipW (Stover and Driks, 1999c), was tested for biofilm formation using the microtiter plate assay. As shown in Figure 3, the double mutant showed a 2.6-fold increase ($p<0.001$) in the level of biofilm formation compared to the wild-type strain. The reason high levels of biofilm formation were observed for the *tasA yqxM* mutant is not known. These data support the conclusion that neither TasA nor YqxM are required for biofilm formation and indicate that SipW processes at least one protein other than TasA or YqxM that is required for biofilm formation.

Microscopic analysis of surface-adhered sipW and yoaW mutant cells

To determine the step in biofilm formation at which the *yoaW* and the *sipW* mutants were arrested, surface adhered *yoaW* and *sipW* mutant cells were analyzed by confocal scanning laser microscopy (CSLM). A view of the surface in the X-Y plane (i.e. perpendicular to the surface) showed that, similar to the wild-type cells, *yoaW* cells were able to adhere to the surface of the glass slide (compare Figure 4D to 4A). Cross sections through the structure formed by the *yoaW* mutant cells, in the X-Z plane, showed a structure with reduced depth compared to the structure formed by the wild-type cells (compare Figure 4D to 4A). The structure formed by the *yoaW* mutant strain showed an average depth of 8.0±0.5 μm. This is in contrast to the wild-type strain, which formed three-dimensional structures with average depth of 14±0.7 μm. The fold difference in the depth of the biofilm formed by the wild-type and the *yoaW* mutant strains as seen by CSLM is similar to the fold difference in the level of biofilm formation observed in the microtiter plate assay.

A view of surface adhered *sipW* mutant cells by CSLM in the X-Y plane showed that *sipW* mutant cells were able to adhere to the surface of the glass slide (Figure 4C). However, these cells appear unable to cluster together to form microcolonies. Cross-sections through the field, in the X-Z plane, indicate that the *sipW* mutant cells adhere in a monolayer, as CSLM images showed a structure with a depth ≤4 μm (Figure 4C).

To further confirm that *sipW* was the only gene in the *yqxM* operon with a role in biofilm formation, the *tasA yqxM* mutant strain that expresses SipW was analyzed by CSLM. As shown in Figure 4B, the structure formed by the *yqxM tasA* double mutant strain showed a similar biofilm morphology to the wild-type strain, both in the X-Y and the X-Z sections. The average depth the biofilm formed by the mutant strain was 10±1.0 μm, which was not considered significantly different from the wild-type structure as determined by a standard t-test. These data indicate that *tasA* and *yqxM* have no apparent role in biofilm formation.

Discussion

In this study, we have generated a list of genes that appear to be activated during biofilm formation due to relief of AbrB repression. This was done by comparing the data from DNA microarray experiments performed in this study, which determined the gene expression profile of *spo0A abrB* mutant and *sigH abrB* mutant cells under biofilm formation conditions, to three previously published DNA microarray experiments (Britton *et al.*, 2002; Fawcett *et al.*, 2000; Stanley *et al.*, 2003). Genes regulated by AbrB are of interest as they are candidate genes for having a role in biofilm formation as well as other processes that occur during the transition to stationary phase. We have tested mutants defective for 23 of the 39 operons identified as AbrB-regulated for their ability to form biofilms. From this analysis, we have identified two genes, *sipW* and *yoaW*, which are necessary for *B. subtilis* to form a biofilm with a three-dimensional structure similar to a wild-type strain.

Comparative analysis of independent DNA microarray experiments led to the identification of AbrB-regulated genes

In this study, we aimed to identify AbrB-regulated genes differentially expressed under biofilm formation conditions by comparing the results from DNA microarray experiments performed in different laboratories under different growth conditions. Stanley *et al.* 2003 previously identified Spo0A- or Sigma-H-regulated genes that were differentially expressed under biofilm formation conditions, by comparing the transcription profile of wild-type cells grown under biofilm formation conditions to the transcription profile of *spo0A* or *sigH* mutant cells under planktonic conditions (Stanley *et al.*, 2003; Fawcett *et al.*, 2000; Britton *et al.*, 2002). As a subset of the Spo0A and Sigma-H-regulated genes should have been controlled by AbrB, we compared the results of the analysis performed by Stanley *et al.* 2003 to the transcription profile of *spo0A abrB* and *sigH abrB* mutant cells under biofilm formation conditions. Using this method, we have identified 39 operons as AbrB repressed.

To our knowledge, this is the first report of a comparative analysis of DNA microarray experiments to study a bacterial system. This type of analysis is powerful at generating new information quickly, as it relies on previously existing data. However, as it compares DNA microarray analyses with cells grown under different growth conditions, it can only provide a partial list of the genes controlled by a transcription factor. Such a partial list may be sufficient in cases such as that presented here, where a partial list of genes regulated by AbrB was sufficient to identify genes required for biofilm formation.

Large-scale analyses such as DNA microarray analyses will inherently generate some level of false positive results (Murphy, 2002; Hatfield *et al.*, 2003). Any comparative DNA microarray analyses will compound the false positive error rate inherit in each DNA microarray experiment. Thus, it is important to consider where false positives might arise. One place is from our assumption that genes, known to be activated by Spo0A or Sigma-H under planktonic conditions and known to be induced under biofilm formation conditions, were indeed induced under biofilm formation conditions due to the activity of Spo0A or Sigma-H, and not due to the activity of another transcription factor. If these genes were differentially expressed under biofilm formation conditions due to the activity of another transcription factor, they should not be differentially expressed in the *spo0A abrB* or *sigH abrB* mutant cells and would be falsely identified as AbrB-regulated genes. At least six genes, *ctaC, D, F, sspB, tagE,* and *bcd*, identified as AbrB-regulated in this study are known to be regulated by other transcription factors (Liu and Taber, 1998; Nicholson *et al.*, 1989; Liu *et al.*, 1998; Debarbouille *et al.*, 1999). Further work will be necessary to determine whether these genes are induced under biofilm formation conditions due to relief of AbrB repression or another transcription factor.

False positive results may also have been generated by the inclusion of genes for which the expression profile data was incomplete. For example, expression data was not obtained for *ctaO* from the *spo0A* DNA microarray experiment and for the *sigH abrB* DNA microarray experiment. In this instance, we used data from the *sigH* microarray analysis and data from the *spo0A abrB* microarray analysis to deduce AbrB regulation. Although this is an indirect method of identifying *ctaO* as AbrB-regulated, we choose not to exclude genes with similarly incomplete expression data, as of the five genes, *spoVG*, *sspB*, *yfhD*, *yhjM*, and *yvdJ*, with similarly missing data one, *spoVG*, is known to be AbrB-regulated (Robertson *et al.*, 1989).

Despite the limitations of this study, the level of false positives appears to be low, as AbrB regulation for 14 of the 39 operons (36%) identified in this study has been confirmed. Eight of these operons have been shown in previous studies to be regulated by AbrB. An additional 6 operons, chosen at random, were shown via RT-PCR to be regulated by AbrB (Table 2). The finding that 100% of those operons chosen for further analysis were AbrB regulated indicates that the incidence of false positives is likely to be low.

Two AbrB regulated genes, sipW and yoaW, are essential for forming a mature biofilm

Previous data had indicated that AbrB repressed at least one gene required for biofilm formation (Hamon and Lazazzera, 2001). In this study, we showed that, of the 23 operons identified as AbrB-regulated and tested for a role in biofilm formation, 9 had a significant effect on biofilm formation under the conditions tested in this study. The effect of 7 of these operons was small and, therefore, not studied further. The effect of 2 operons, the *yqxM* and the *yoaW* operons, was larger and was further studied by CSLM. Both *yqxM* and *yoaW* operon mutants showed defects in forming a mature biofilm structure.

The YoaW protein is required at a late stage of biofilm formation, as a *yoaW* mutant formed a structure with reduced depth compared to a wild-type strain. A mechanism that has been proposed to explain why an *Escherichia coli wcaF* mutant strain exhibits biofilms with reduced depth is that this mutant forms fragile biofilm structures that loose depth upon rinsing for microscopic examination (Danese *et al.*, 2000). WcaF is required for extracellular polysaccharide production, which is presumably part of the extracellular matrix of *E. coli* biofilms (Danese *et al.*, 2000). As the phenotype of an *E. coli wcaF* mutant resembles that of a *B. subtilis yoaW* mutant, YoaW could have a similar role to WcaF in forming the extracellular matrix required for a mature biofilm.

In contrast to the *yoaW* operon that appears to be required at a late stage in the biofilm formation, one gene in the *yqxM* operon is required at an early stage in biofilm formation. Microscopic images of surface attached *sipW* mutant cells indicated that these cells were able to attach to the surface of a glass slide, but were unable to organize into microcolonies. The stage in the biofilm formation pathway that is blocked in a *sipW* mutant is the same stage in biofilm formation that was previously shown to be repressed by AbrB (Hamon and Lazazzera, 2001). Thus, one of the major functions of AbrB in repressing biofilm formation is to repress *sipW*.

As SipW is a signal peptidase, its role in biofilm formation is most likely to process a secreted protein that has a role in allowing surface-adhered cells to form a mature biofilm. Different functions have been shown to enable surface-attached cells to transition to a mature biofilm in different bacterial systems. Synthesis of a polysaccharide intercellular adhesin is required by *Staphylococcus epidermidis*, twitching motility is required under some conditions by *Pseudomonas aeruginosa*, and flagellar motility is required by *E. coli* (Cucarella *et al.*, 2001; O'Toole and Kolter, 1998; Pratt and Kolter, 1998). These observations suggest that the SipW-processed protein may have a role in producing an intercellular adhesin or a motility structure in *B. subtilis*. Identifying the protein(s) processed by SipW that is required for biofilm formation would distinguish these possibilities.

It is interesting to note that neither of the known SipW-processed proteins, YqxM or TasA, were required for biofilm formation. It is tempting to speculate that the production of the SipW-processed protein that is required for biofilm formation is also AbrB regulated. Seven genes identified as AbrB regulated, *aprE*, *qcrA*, *sdpA*, *xynD*, *yocH*, *yoaW*, *yqxI*, *yraI*, encode proteins that have a putative signal sequence and, therefore, could be processed by SipW. Five of these genes were tested for a role in biofilm formation, including *yoaW*, but mutations in these genes did not result in a phenotype similar to *sipW*. Preliminary experiments to detect processing of YoaW by SipW were unsuccessful due to our inability to detect secreted YoaW (data not shown). It is possible that genes encoding multiple SipW-processed proteins will need to be deleted before a phenotype similar to SipW is observed.

Potential role for AbrB-regulated genes under biofilm formation conditions

Many of the AbrB-regulated genes identified in this study may contribute to the metabolic capacity of biofilm cells. AbrB has previously been shown to regulate the expression of extracellular degradative enzymes, nitrogen utilization enzymes, amino acid metabolism enzymes and transporters, all of which would expand the range of potential nutrient sources used by *B. subtilis* (Phillips and Strauch, 2002). In this study, we identified genes that encode enzymes involved in all of these functions. In addition, we identified genes involved in carbon source utilization, nucleotide metabolism, membrane bioenergetics, antibiotic resistance, and delay of sporulation. The altered metabolic capacity conferred by increased expression of these genes may reflect the type of nutrients found in the environment where *B. subtilis* typically forms biofilms as well as the general physiology of cells in a biofilm.

Two extracellular degradative enzymes were identified in this study, XynD and AprE, which are involved in xylan and protein degradation, respectively (Stahl and Ferrari, 1984; Wong *et al.*, 1984; Gosalbes *et al.*, 1991). Production of these extracellular enzymes may be advantageous to *B. subtilis* during biofilm formation, as these enzymes and consequently the nutrients released may be less likely to diffuse away from the cells in a biofilm. Xylan is a plant material, and the induction of a xylan-degrading enzyme during biofilm formation may indicate that decaying plant material is a common surface on which *B. subtilis* forms biofilms. Similarly, the induction of a maltodextran utilizing operon, the *yvdF* operon (Cho *et al.*, 2000), may indicate that starch, a component of plants, is a common nutrient encountered in the environment where *B. subtilis* forms biofilms.

Regulation of genes involved in utilizing amino acids as nitrogen sources, the *bkdR* and *hutP* operons (Debarbouille *et al.*, 1999; Kimhi and Magasanik, 1970), and in transport of dipeptides, the *dppA* operon (Cheggour *et al.*, 2000), may reflect the utilization of substrates released by the extracellular protease AprE. Conversely, the increased expression of genes involved in the synthesis of the amino acids asparagine (*asnH* – part of the *yxbB* operon (Yoshida *et al.*, 1999)), leucine (the *ilvB* operon (Grandoni *et al.*, 1992)), and threonine (the *hom* operon (Parsot and Cohen, 1988)), may reflect the lack of these amino acids in the environment in which *B. subtilis* forms biofilms.

Three operons involved in membrane bioenergetics, *ctaC*, *ctaO*, and *qcrA*, were identified as regulated by AbrB under biofilm formation conditions. The *ctaC* and *qcrA* operons encode enzymes that form the cytochrome oxidase pathway for shuttling electrons from menaquinone to O_2, one of four pathways that *B. subtilis* possesses for reducing O_2 (Saraste *et al.*, 1991; Yu *et al.*, 1995; Winstedt and von Wachenfeldt, 2000). The differential regulation of these pathways suggests that they have different functions (Winstedt and von Wachenfeldt, 2000). The terminal oxidases of *E. coli* also exhibit differential regulation, such that the oxidase with a higher affinity for O_2 is expressed maximally at low O_2 concentrations (Govantes *et al.*, 2000). It is possible that the O_2-reducing pathway that has increased expression under biofilm

formation conditions has a higher affinity for oxygen, as it is well documented that O_2 concentrations are severely limited in the depths of biofilms (Costerton *et al.*, 1995).

The increased expression of CtaO may be important for the activity of the cytochrome oxidase pathway. Heme A is found in the terminal oxidase of this pathway. CtaO has heme O synthesis activity, which is proposed to be a precursor for Heme A synthesis (Mogi *et al.*, 1994. Similarly, the increased expression of a putative copper import protein, YcnJ, may be important for the cytochrome oxidase pathway. The terminal oxidase of this pathway is a heme-copper oxidase (Winstedt and von Wachenfeldt, 2000).

Several genes involved in cell wall functions exhibited increased expression under biofilm formation conditions. Alterations in the cell wall are interesting as they may change how the cells interact with surfaces. TagE, which is involved in teichoic acid biosynthesis in *B. subtilis*, was identified in our studies (Mauel *et al.*, 1991). Teichoic acids have been shown to play a role in adherence of *Staphylococcus aureus* cells to surfaces (Gross *et al.*, 2001). Mutants lacking *tagE* exhibited decreased levels of biofilm formation. However, they also exhibited a slow growth rate, making it difficult to discern the role of teichoic acids in biofilm formation by *B. subtilis*.

Other genes involved in cell wall functions are part of the *ykfA* operon, which has been proposed to play a role in recycling cell wall peptides. YkfA has amino acid similarity to LdcA of *E. coli*, a L,D-carboxypeptidase that was shown to be required to recycle cell wall peptides for new cell wall synthesis (Templin *et al.*, 1999). Interestingly, LdcA is essential for survival of *E. coli* in stationary phase, presumably due to the decreased ability to synthesize cell wall peptides *de novo* under stationary phase conditions (Templin *et al.*, 1999). As biofilm formation in *B. subtilis* occurs in stationary phase, YkfA may be important for survival of *B. subtilis* cells under these conditions. YfkC is an endopeptidase that can cleave cell wall peptides, and YkfB has L-Ala-D/L-Glu epimerase activity (Schmidt *et al.*, 2001). These proteins are proposed to have a role in degrading cell wall peptides, which could be useful to cells in biofilms as a source of nutrients.

Biofilm cells exhibit increased resistance to antimicrobial agents. Thus, it is of particular interest that *yxaM* has increased expression in *B. subtilis* under biofilm formation conditions. YxaM is 23% identical and 45% similar to the tetracycline efflux pump, TetA(P), of *Clostridium septicum* and *perfringens* (Sloan *et al.*, 1994). At this time it is not known whether *B. subtilis* cells are more resistant to tetracycline when growing in a biofilm.

As AbrB regulated processes occur during the transition to stationary phase, the question arises as to how *B. subtilis* coordinates these processes. It is therefore interesting to note one operon responsible for delaying sporulation, *sdpA*, has been identified as differentially expressed under biofilm formation conditions. Gonzalez-Pastor *et al*. (2003) postulate that the *sdpA* operon allows cells to wait as long as possible before committing to sporulation, which is a dormant state. Expression of the *sdpA* operon under biofilm formation conditions may be a mechanism to allow biofilm formation to occur under conditions where nutrient concentrations are low, but sufficient to allow metabolic activity.

In summary, we have performed a comparative analysis of DNA microarrays conducted in different laboratories and under different conditions. To our knowledge, this is the first time such an approach has been applied to study gene regulation in a bacterial system. From this analysis, we have successfully identified AbrB-regulated genes that are activated under biofilm formation conditions. These studies have furthered our knowledge of the process regulated by AbrB. We have further shown that 9 of these AbrB-regulated genes have a significant effect on biofilm formation. We analyzed in greater detail the phenotype of two mutants that showed the greatest defect in biofilm formation, *sipW* and *yoaW*. The gene *sipW* encodes a signal

peptidase, which is the first protein that is not a transcription factor that has been shown to have a role in the transition of surface attached *B. subtilis* cells to a biofilm. The gene *yoaW* encodes a secreted protein, which is also the first protein that is not a transcription factor that has been shown to affect the depth of *B. subtilis* biofilms. Understanding how SipW and YoaW contribute to biofilm formation will further our understanding of the mechanisms used by bacteria to form biofilms.

Experimental procedures

Growth media

LB was used for growth of both *E. coli* and *B. subtilis* for routine strain construction and maintenance. For growth of *B. subtilis* under biofilm growth conditions, biofilm growth medium was used (Hamon and Lazazzera, 2001). Antibiotics were used at the following concentrations as appropriate: 100 μg/ml ampicillin, 5 μg/ml chloramphenicol, 5 μg/ml neomycin, 100 μg/ml spectinomycin.

Strain and plasmid construction

The *B. subtilis* strains used in this study are described in Table 4 and were constructed by transformation with chromosomal DNA or plasmids using standard protocols (Cutting and Vander Horn, 1990). All strains are derivatives of the parental strain BAL218 (JH642) and contain *trpC2* and *pheA1* mutations (Perego *et al.*, 1988).

Disruption of 23 operons identified as AbrB-regulated was accomplished by integrating a plasmid containing an internal fragment of the gene into the chromosome of strain BAL218. The internal fragment of the genes were obtained by PCR amplification using primers that amplify from the positions indicated in Supplementary Table 4. The PCR product was blunt cloned into the *Sma*I site of pBL132 (Stanley *et al.*, 2003). The newly constructed plasmid was then integrated into the chromosome at the gene locus using selection for chloramphenicol resistance associated with the plasmid.

DNA microarray experiments

RNA for use in DNA microarray experiments was isolated from biofilm cells of BAL218 (wild-type), BAL678 (*sigH*::cat::spec, *abrB*::Tn*917*), and BAL734 (*spo0AD*56N::spec, *abrB*::Tn*917*) cells after incubation for 24 hours under biofilm formation conditions. The cells were grown under planktonic conditions in biofilm growth medium at 37°C with shaking at 200 rpm to late-exponential phase (OD_{600} = 2.5). The cells were then diluted to an OD_{600} of 0.1 in 20 mls of fresh biofilm growth medium and placed in 250-ml beakers. The beakers were incubated at 37°C without shaking for 24 hours. At this point, the cells had formed an air-medium interface biofilm, and the growth medium below the biofilm was removed using a pipet with minimal disruption to the biofilm. The biofilm cells were washed in 5 ml of wash buffer (15 mM $(NH_4)_2S0_4$, 80 mM K_2HPO_4, 44 mM KH_2PO_4, 3.4 mM sodium citrate, 1 mM $MgSO_4$) and the RNA was extracted from these cells using a Qiagen RNAeasy Maxiprep kit according to the manufacture's instructions. The isolated RNA was treated with DNaseI (Qiagen) to remove any contaminating genomic DNA.

To determine the relative expression level of a gene between wild-type and mutant cells, the wild-type and mutant RNA samples were labeled with Cy3 and Cy5, respectively, as previously described (Britton *et al.*, 2002). The DNA microarray slides were simultaneously hybridized with the wild-type cDNA and a mutant cDNA to determine the ratio of gene expression between the wild-type and mutant strain as described in Britton *et. al.* 2002. For each gene, the ratios from three independent experiments (i.e. independently grown and prepared samples) was

averaged. The genes with a significantly different expression level were identified using iterative outlier analysis as previously described (Stanley *et al.*, 2003).

Microtiter plate assay of B. subtilis biofilm formation

The microtiter plate assay measures the level of cells adhered to the surface of the microtiter plate wells. These assays were performed as described in Hamon and Lazazzera (2000). For one assay, the OD_{570} of between 16 and 24 wells were averaged. The standard error of the mean of these wells was <10%. Background levels of staining (i.e. wells incubated with growth medium lacking cells) were subtracted from the average. This assay was repeated on at least 4 separate occasions, and the average values from each independent assay was averaged to determine the level of biofilm formation for a strain.

Confocal scanning laser microscopy

Confocal scanning laser microscopy images were obtained as described (Hamon and Lazazzera, 2001). Briefly, samples were observed using a Leica TCS-SP confocal laser microscope equipped with an argon ion laser. Samples were viewed using 488 nm as the excitation wavelength. Sections through the X-Y plane and the X-Z plane (Z section) were obtained using the TCS-NT computer program. Each strain was examined on at least 3 separate occasions, and the average depth of the biofilm was determined for each strain using 6 to 10 independent Z section measurements.

β-galactosidase Assays

Measurement of β-galactosidase specific activity under both planktonic and biofilm formation conditions was as described previously (Stanley *et al.*, 2003), except that strains were grown under biofilm formation conditions for 16 hours. The β-galactosidase specific activity of strains BAL881 (*sipW-lacZ* (cat)), BAL1293 (*spo0A*D56N-cat::spec, *abrB*::Tn*917*, *sipW-lacZ* (cat)), and BAL1300 (*spo0A*D56N-cat::spec, *sipW-lacZ* (cat)) was measured.

Reverse transcription-PCR analysis

The cDNA templates used for RT-PCR analysis were generated through reverse transcription of total RNA isolated from cells grown under biofilm formation conditions for 24 hours in microtiter plates. The cells were grown in an identical manner to that for the microtiter plate assay. The complete contents of 10 microtiter plate wells were collected by pipetting and combined. The cells were vortexed to disrupt any cell aggregates, and the OD_{600} measured. RNA was then isolated from ~10^7 cells using an RNAeasy miniprep kit according to the manufacture's instructions (Qiagen). This RNA was digested with DNaseI (Qiagen) until complete digestion of any contaminating genomic DNA was achieved. This was determined by the absence of a PCR product when using the total RNA as a template in a PCR reaction (data not shown). 100 ng of the total RNA was used in a reverse transcription reaction using random hexamers in accordance with the manufacture's instructions (Invitrogen First Strand cDNA synthesis Kit). 1 μl of the resulting cDNA was used in a 20 μl RT-PCR reaction.

RT-PCR analysis (Wall and Edwards, 2002; Wong *et al.*, 1994) is a process where the relative expression of a gene is compared to that of an internal control, in this case *veg*, whose expression is known not to vary under different growth conditions (Gilman and Chamberlin, 1983; Ollington *et al.*, 1981; Ollington and Losick, 1981). The number of PCR amplification cycles required for a linear increase in the PCR product for both the internal control gene *veg* and the gene of interest was determined for the cDNA generated from the wild-type sample. This was determined by following the PCR reaction over a number of cycles and assessing the amount of resulting product by agarose gel electrophoreses and ethidium bromide staining. The fluorescence level from the PCR product was measured using a Chemimager™ 4400

(Alpha Innotech Corporation). Once the number of cycles required for a linear amplification was calculated for *veg* and for the gene of interest with cDNA generated from the wild-type strain, a PCR reaction was performed with cDNA generated from BAL679 (*spo0A*D56N-cat::spec) and BAL734 (*abrB*::Tn*917*, *spo0A*D56N-cat::spec) to monitor the relative level of gene expression between the three strains. The PCR reactions conditions were 1x PCR reaction buffer, 1x Q-solution (Qiagen), 3.5 mM $MgCl_2$, 0.5 μM each primer, 0.2 μM each dNTP, 0.8 units *Taq* DNA polymerase per 20 μl reaction (Qiagen). The sequence of the primers used is listed in Table 4. Between 24 and 32 cycles of amplification were used with 30 seconds at 95° C, 30 seconds at 48°C (except for *yocH* where 54°C was used) and 30 seconds at 72°C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We are grateful for strains AGS157, AGS175 and AGS207, and plasmid pLGW201 kindly provided by Dr. Adam Driks. We would like to thank Dr. Mathew Schibler for advice and help with the confocal scanning laser microscopy performed at the UCLA Brain Research Institute confocal microscope facility. Thank you to Cynthia Lee for her help with strain construction. This work was supported in part by the University of California Academic Senate Council on Research of the Los Angeles Division, the UCLA foundation and a Frontiers of Science-Seed grant form the Howard Hughes Medical Institute (B.A.L.), and by the Public Health Services Grant AI48616 to B.A.L. and GM50895 to A.D.G.. N.R.S. was supported by a long-term postdoctoral fellowship awarded by the EMBO. M.A.H. was supported in part by the Microbial Pathogenesis Training Grant (T32-AI07323) from the National Institute of Health and in part by the Warsaw fellowship.

References

Branda SS, Gonzalez-Pastor JE, Ben-Yehuda S, Losick R, Kolter R. Fruiting body formation by *Bacillus subtilis.* Proc Natl Acad Sci U S A 2001;98:11621–11626. [PubMed: 11572999]

Britton RA, Eichenberger P, Gonzalez-Pastor JE, Fawcett P, Monson R, Losick R, Grossman AD. Genome-wide analysis of the stationary-phase sigma factor (sigma-H) regulon of *Bacillus subtilis.* J Bacteriol 2002;184:4881–4890. [PubMed: 12169614]

Cheggour A, Fanuel L, Duez C, Joris B, Bouillenne F, Devreese B, Van Driessche G, Van Beeumen J, Frere JM, Goffin C. The *dppA* gene of *Bacillus subtilis* encodes a new D-aminopeptidase. Mol Microbiol 2000;38:504–513. [PubMed: 11069674]

Cho HY, Kim YW, Kim TJ, Lee HS, Kim DY, Kim JW, Lee YW, Leed S, Park KH. Molecular characterization of a dimeric intracellular maltogenic amylase of *Bacillus subtilis* SUH4-2. Biochim Biophys Acta 2000;1478:333–340. [PubMed: 10825545]

Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. Annu Rev Microbiol 1995;49:711–745. [PubMed: 8561477]

Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penades JR. Bap, a Staphylococcus aureus surface protein involved in biofilm formation. J Bacteriol 2001;183:2888–2896. [PubMed: 11292810]

Cutting, S.M., and Vander Horn, P.B. (1990) *Molecular biological methods for Bacillus.* Chichester; New York; Toronto; Singapore: John Wiley &Sons Ltd.

Danese PN, Pratt LA, Kolter R. Exopolysaccharide production is required for development of *Escherichia coli* K-12 biofilm architecture. J Bacteriol 2000;182:3593–3596. [PubMed: 10852895]

Davey ME, O'Toole G A. Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev 2000;64:847–867. [PubMed: 11104821]

Debarbouille M, Gardan R, Arnaud M, Rapoport G. Role of bkdR, a transcriptional activator of the sigL-dependent isoleucine and valine degradation pathway in *Bacillus subtilis.* J Bacteriol 1999;181:2059–2066. [PubMed: 10094682]

Fawcett P, Eichenberger P, Losick R, Youngman P. The transcriptional profile of early to middle sporulation in *Bacillus subtilis.* Proc Natl Acad Sci U S A 2000;97:8063–8068. [PubMed: 10869437]

Ferrari E, Henner DJ, Perego M, Hoch JA. Transcription of *Bacillus subtilis* subtilisin and expression of subtilisin in sporulation mutants. J Bacteriol 1988;170:289–295. [PubMed: 2447062]

Fisher SH, Strauch MA, Atkinson MR, Wray LV Jr. Modulation of *Bacillus subtilis* catabolite repression by transition state regulatory protein AbrB. J Bacteriol 1994;176:1903–1912. [PubMed: 8144456]

Gilman MZ, Chamberlin MJ. Developmental and genetic regulation of *Bacillus subtilis* genes transcribed by sigma 28-RNA polymerase. Cell 1983;35:285–293. [PubMed: 6313226]

Gonzalez-Pastor JE, Hobbs EC, Losick R. Cannibalism by sporulating bacteria. Science 2003;301:510–513. [PubMed: 12817086]

Gosalbes MJ, Perez-Gonzalez JA, Gonzalez R, Navarro A. Two beta-glycanase genes are clustered in *Bacillus polymyxa*: molecular cloning, expression, and sequence analysis of genes encoding a xylanase and an endo-beta-(1,3)-(1,4)-glucanase. J Bacteriol 1991;173:7705–7710. [PubMed: 1938968]

Govantes F, Orjalo AV, Gunsalus RP. Interplay between three global regulatory proteins mediates oxygen regulation of the Escherichia coli cytochrome d oxidase (cydAB) operon. Mol Microbiol 2000;38:1061–1073. [PubMed: 11123679]

Grandoni JA, Zahler SA, Calvo JM. Transcriptional regulation of the *ilv-leu* operon of *Bacillus subtilis*. J Bacteriol 1992;174:3212–3219. [PubMed: 1577690]

Gross M, Cramton SE, Gotz F, Peschel A. Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces. Infect Immun 2001;69:3423–3426. [PubMed: 11292767]

Hamon MA, Lazazzera BA. The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis*. Mol Microbiol 2001;42:1199–1209. [PubMed: 11886552]

Hatfield GW, Hung SP, Baldi P. Differential analysis of DNA microarray gene expression data. Mol Microbiol 2003;47:871–877. [PubMed: 12581345]

Jan J, Valle F, Bolivar F, Merino E. Characterization of the 5' subtilisin (*aprE*) regulatory region from *Bacillus subtilis*. FEMS Microbiol Lett 2000;183:9–14. [PubMed: 10650195]

Kimhi Y, Magasanik B. Genetic basis of histidine degradation in *Bacillus subtilis*. J Biol Chem 1970;245:3545–3548. [PubMed: 4990471]

Kuchma SL, O'Toole GA. Surface-induced and biofilm-induced changes in gene expression. Curr Opin Biotechnol 2000;11:429–433. [PubMed: 11024358]

Lazazzera BA, Kurtser IG, McQuade RS, Grossman AD. An autoregulatory circuit affecting peptide signaling in *Bacillus subtilis*. J Bacteriol 1999;181:5193–5200. [PubMed: 10464187]

Liu W, Eder S, Hulett FM. Analysis of *Bacillus subtilis tagAB* and *tagDEF* expression during phosphate starvation identifies a repressor role for PhoP-P. J Bacteriol 1998;180:753–758. [PubMed: 9457886]

Liu X, Taber HW. Catabolite regulation of the *Bacillus subtilis ctaBCDE*F gene cluster. J Bacteriol 1998;180:6154–6163. [PubMed: 9829923]

Mauel C, Young M, Karamata D. Genes concerned with synthesis of poly(glycerol phosphate), the essential teichoic acid in *Bacillus subtilis* strain 168, are organized in two divergent transcription units. J Gen Microbiol 1991;137:929–941. [PubMed: 1906926]

McQuade RS, Comella N, Grossman AD. Control of a family of phosphatase regulatory genes (*phr*) by the alternate sigma factor sigma-H of *Bacillus subtilis*. J Bacteriol 2001;183:4905–4909. [PubMed: 11466295]

Mogi T, Saiki K, Anraku Y. Biosynthesis and functional role of *haem O* and *haem A*. Mol Microbiol 1994;14:391–398. [PubMed: 7885224]

Murphy D. Gene expression studies using microarrays: principles, problems, and prospects. Adv Physiol Educ 2002;26:256–270. [PubMed: 12443997]

Nicholson WL, Sun DX, Setlow B, Setlow P. Promoter specificity of sigma G-containing RNA polymerase from sporulating cells of *Bacillus subtilis*: identification of a group of forespore-specific promoters. J Bacteriol 1989;175:2708–2718. [PubMed: 2468649]

O'Toole GA, Kolter R. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol Microbiol 1998;30:295–304. [PubMed: 9791175]

Ollington JF, Haldenwang WG, Huynh TV, Losick R. Developmentally regulated transcription in a cloned segment of the *Bacillus subtilis* chromosome. J Bacteriol 1981;147:432–442. [PubMed: 6790515]

Ollington JF, Losick R. A cloned gene that is turned on at an intermediate stage of spore formation in *Bacillus subtilis*. J Bacteriol 1981;147:443–451. [PubMed: 6790516]

Parsot C, Cohen GN. Cloning and nucleotide sequence of the *Bacillus subtilis hom* gene coding for homoserine dehydrogenase. Structural and evolutionary relationships with *Escherichia coli* aspartokinases-homoserine dehydrogenases I and II. J Biol Chem 1988;263:14654–14660. [PubMed: 3139660]

Perego M, Spiegelman GB, Hoch JA. Structure of the gene for the transition state regulator, *abrB*: regulator synthesis is controlled by the *spo0A* sporulation gene in *Bacillus subtilis*. Mol Microbiol 1988;2:689–699. [PubMed: 3145384]

Phillips ZE, Strauch MA. *Bacillus subtilis* sporulation and stationary phase gene expression. Cell Mol Life Sci 2002;59:392–402. [PubMed: 11964117]

Pratt LA, Kolter R. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol Microbiol 1998;30:285–293. [PubMed: 9791174]

Predich M, Nair G, Smith I. *Bacillus subtilis* early sporulation genes *kinA*, *spo0F*, and *spo0A* are transcribed by the RNA polymerase containing sigma H. J Bacteriol 1992;174:2771–2778. [PubMed: 1569009]

Robertson JB, Gocht M, Marahiel MA, Zuber P. AbrB, a regulator of gene expression in *Bacillus*, interacts with the transcription initiation regions of a sporulation gene and an antibiotic biosynthesis gene. Proc Natl Acad Sci U S A 1989;86:8457–8461. [PubMed: 2554317]

Schmidt DM, Hubbard BK, Gerlt JA. Evolution of enzymatic activities in the enolase superfamily: functional assignment of unknown proteins in *Bacillus subtilis* and *Escherichia coli* as L-Ala-D/L-Glu epimerases. Biochemistry 2001;40:15707–15715. [PubMed: 11747447]

Serrano M, Zilhao R, Ricca E, Ozin AJ, Moran CP Jr, Henriques AO. A *Bacillus subtilis* secreted protein with a role in endospore coat assembly and function. J Bacteriol 1999;181:3632–3643. [PubMed: 10368135]

Slack FJ, Mueller JP, Strauch MA, Mathiopoulos C, Sonenshein AL. Transcriptional regulation of a *Bacillus subtilis* dipeptide transport operon. Mol Microbiol 1991;5:1915–1925. [PubMed: 1766371]

Sloan J, McMurry LM, Lyras D, Levy SB, Rood JI. The *Clostridium perfringens* Tet P determinant comprises two overlapping genes: *tetA(P)*, which mediates active tetracycline efflux, and *tetB(P)*, which is related to the ribosomal protection family of tetracycline-resistance determinants. Mol Microbiol 1994;11:403–415. [PubMed: 8170402]

Stahl ML, Ferrari E. Replacement of the *Bacillus subtilis* subtilisin structural gene with an In vitro-derived deletion mutation. J Bacteriol 1984;158:411–418. [PubMed: 6427178]

Stanley NR, Britton RA, Grossman AD, Lazazzera BA. Identification of catabolite repression as a physiological regulator of biofilm formation by *Bacillus subtilis* by use of DNA microarrays. J Bacteriol 2003;185:1951–1957. [PubMed: 12618459]

Stover AG, Driks A. Control of synthesis and secretion of the *Bacillus subtilis* protein YqxM. J Bacteriol 1999a;181:7065–7069. [PubMed: 10559173]

Stover AG, Driks A. Regulation of synthesis of the *Bacillus subtilis* transition-phase, spore-associated antibacterial protein TasA. J Bacteriol 1999b;181:5476–5481. [PubMed: 10464223]

Stover AG, Driks A. Secretion, localization, and antibacterial activity of TasA, a *Bacillus subtilis* spore-associated protein. J Bacteriol 1999c;181:1664–1672. [PubMed: 10049401]

Strauch M, Webb V, Spiegelman G, Hoch JA. The Spo0A protein of *Bacillus subtilis* is a repressor of the *abrB* gene. Proc Natl Acad Sci U S A 1990;87:1801–1805. [PubMed: 2106683]

Templin MF, Ursinus A, Holtje JV. A defect in cell wall recycling triggers autolysis during the stationary growth phase of *Escherichia coli*. EMBO J 1999;18:4108–4117. [PubMed: 10428950]

Tjalsma H, Bolhuis A, van Roosmalen ML, Wiegert T, Schumann W, Broekhuizen CP, Quax WJ, Venema G, Bron S, van Dijl JM. Functional analysis of the secretory precursor processing machinery of *Bacillus subtilis*: identification of a eubacterial homolog of archaeal and eukaryotic signal peptidases. Genes Dev 1998;12:2318–2331. [PubMed: 9694797]

Wall SJ, Edwards DR. Quantitative reverse transcription-polymerase chain reaction (RT-PCR): a comparison of primer-dropping, competitive, and real-time RT-PCRs. Anal Biochem 2002;300:269–273. [PubMed: 11779121]

Winstedt L, von Wachenfeldt C. Terminal oxidases of *Bacillus subtilis* strain 168: one quinol oxidase, cytochrome aa(3) or cytochrome bd, is required for aerobic growth. J Bacteriol 2000;182:6557–6564. [PubMed: 11073895]

Wong H, Anderson WD, Cheng T, Riabowol KT. Monitoring mRNA expression by polymerase chain reaction: the “primer-dropping” method. Anal Biochem 1994;223:251–258. [PubMed: 7887471]

Wong SL, Price CW, Goldfarb DS, Doi RH. The *subtilisin E* gene of *Bacillus subtilis* is transcribed from a sigma 37 promoter in vivo. Proc Natl Acad Sci U S A 1984;81:1184–1188. [PubMed: 6322190]

Yoshida K, Fujita Y, Ehrlich SD. Three asparagine synthetase genes of *Bacillus subtilis.* J Bacteriol 1999;181:6081–6091. [PubMed: 10498721]

Yu J, Hederstedt L, Piggot PJ. The cytochrome bc complex (menaquinone:cytochrome c reductase) in *Bacillus subtilis* has a nontraditional subunit organization. J Bacteriol 1995;177:6751–6760. [PubMed: 7592464]

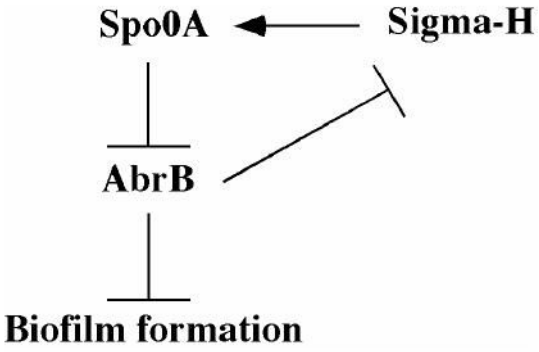

Figure 1.
Model of the genetic network regulating biofilm formation in *Bacillus subtilis*. The arrow indicates positive regulation, and the perpendicular lines indicate negative regulation.

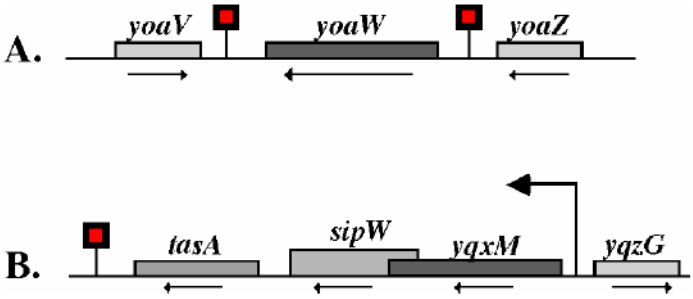

Figure 2.
Diagram of the genetic organization of the chromosomal regions surrounding *yoaW* (A) and *yqxM* (B). Gene direction is indicated by an arrow below the genes, terminators are indicated with boxes, and the *yqxM* promoter is indicated by a large arrow above the gene.

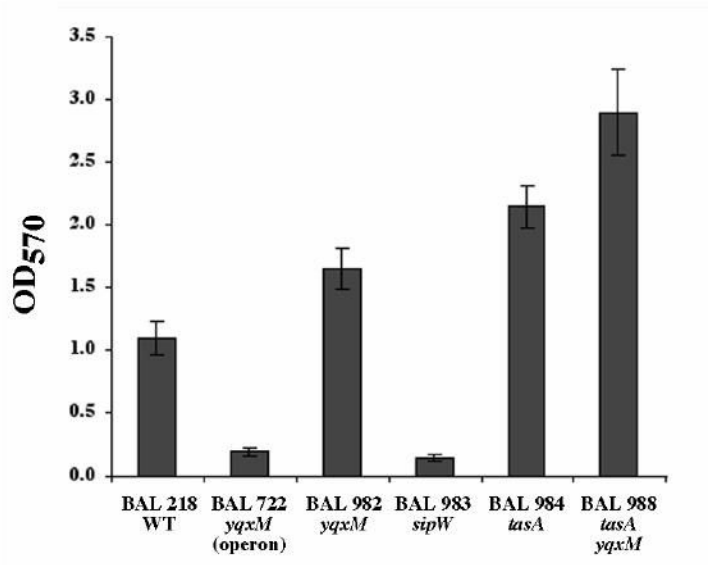

Figure 3.
Microtiter plate assay of biofilm formation by *yqxM* operon mutants. All strains were assayed after 24 hours of growth under biofilm formation conditions. The error bars indicate the standard error of the mean.

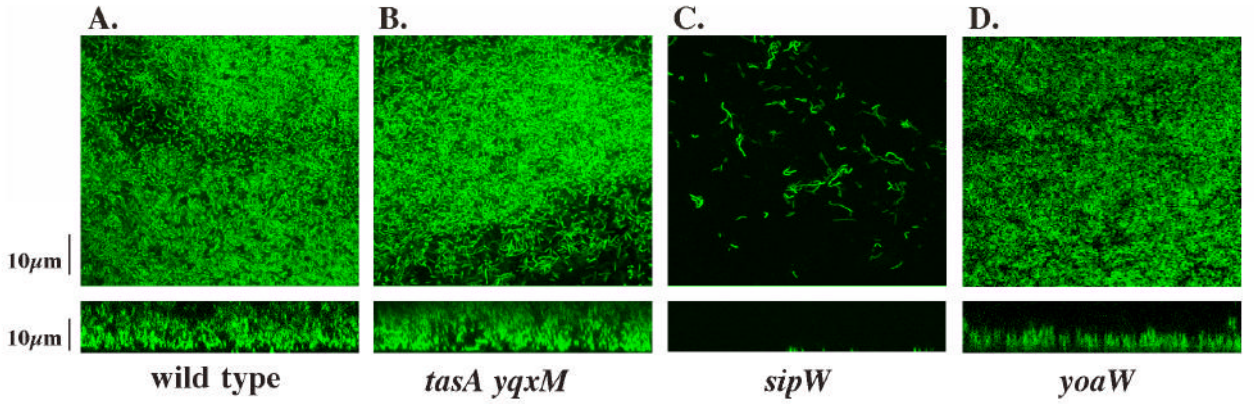

Figure 4.

CSLM analysis of wild-type, *tasA yqxM*, *sipW*, and *yoaW* mutant strains of *B. subtilis*. Biofilms of cells expressing the green fluorescent protein from a chromosomal locus were grown on the surface of glass coverslides and then analyzed by CSLM. Shown are representative images of those obtained on at least three independent occasions. Top images are single sections through the X-Y plane, and the bottom images are single sections through the X-Z plane. Panel A shows BAL835, Panel B shows BAL1062, Panel C shows BAL1061, and Panel D shows BAL1946.

Mol Microbiol. Author manuscript; available in PMC 2006 March 22.

Table 1

Genes repressed by AbrB

Gene[b]	Gene Function	Ratio of Gene Expression[a] B/P[c]	WT/0A[d]	WT/0AabrB[e]	WT/sigH[f]	WT/sigHabrB[g]
aprE *	extracellular protease	8.1	18	1.3	5.3	ND
cdd, era	GTP-binding protein (*era*), cytidine deaminase (*cdd*)	3.0	3.7	1.2	2.0	1.6
(bkdR, ptb), bcd, (buk, lpdv, bkdAA, bkdAB, bkdB)	isoleucine and valine utilization	2.1	14	0.80	ND	0.57
ctaC, D, (E), F, (G)	cytochrome caa3 oxidase	9.4	5.8	0.85	1.9	1.8
ctaO	heme O synthase	3.1	ND	0.83	2.5	ND
dpp(A), B, C, D, (E) *	dipeptide permease	2.9	8.6	0.60	1.9	1.3
hom, (thrC, B)	threonine synthesis	2.6	5.5	1.4	ND	1.7
hut(P), H, (U), I, G (M) *	histidine utilization	8.0	4.3	0.57	ND	1.9
ilvB, (H, C, leuA, B, C, D)	leucine synthesis	2.8	4.4	0.97	ND	0.81
phrE *	RapE regulator	2.5	9.1	0.93	1.6	1.6
qcrA (B,C) *	cytochrome c oxidoreductase	3.7	4.9	0.48	ND	0.86
rapK	response regulator phosphatase	4.0	4.7	1.2	ND	1.0
sdpA, B, (C)	sporulation delaying protein	2.1	14	0.47	5.6	ND
spo0F	regulator of spo0A	3.1	7.9	1.8	3.2	ND
spoVG *	spore cortex synthesis	6.1	ND	0.95	3.0	ND
sspB	small acid-soluble spore protein	9.9	21	ND	ND	0.17
tagE	teichoic acid production	2.1	4.6	1.5	ND	0.77
xynD	xylan degradation	2.0	3.6	1.1	ND	1.3
ycnK, J, I	similar to: transcriptional regulator (*K*), copper import protein (*J*), unknown (*I*)	2.9	12	0.77	1.8	1.4
ydeH	unknown	2.6	3.8	1.1	2.4	0.69
yfhD	unknown	2.6	5.3	ND	ND	1.2
yhjM	similar to transcriptional regulator	3.1	4.0	ND	ND	0.94
yit (N), M	unknown	5.8	8.6	0.99	4.5	ND
ykf (A, B, C), D	cell wall peptide recycling	2.7	4.6	0.92	ND	1.5
ymfJ	unknown	5.1	6.9	1.1	ND	0.65
yoaW	unknown	2.7	ND	1.1	2.3	1.2
yocH	similar to cell wall-binding protein	5.3	6.2	1.9	2.2	0.35
yqcG (F)	unknown	3.0	9.1	1.4	3.4	1.8
yqxI, J	unknown	2.9	14	1.0	3.3	0.90
yqxM, sipW, tasA *	unknown (*yqxM*), signal peptidase (*sipW*), spore component (*tasA*)	2.8	42	0.51	2.3	0.63
yraI, J	unknown	3.8	8.0	0.95	2.8	0.96
yukJ	unknown	3.3	ND	1.0	3.7	1.4
yuxI	unknown	2.9	3.7	1.5	ND	1.8
yvd(F, G, H, I) J (K, malL, pgcM)	similar to maltodextrin utilization system	3.2	ND	1.0	3.8	ND
yvq (I), H,	unknown	2.0	6.44	0.52	2.2	0.47
ywq (H, I), J, K, L	unknown	2.3	6.5	1.1	2.6	1.1
yxbB, A, yxnB, (asnH, yxaM)	unknown (*yxbB, A, yxnB*), asparagine synthetase (*asnH*), similar to tetracycline efflux pump (*yxaM*)	3.6	30	0.37	7.7	0.58
yxbC, (D)	unknown	5.6	51	0.33	5.3	0.87
yydF	unknown	6.4	133	0.49	4.5	ND

[a] Shown is the ratio of gene expression for the first gene in the operon that is not in parenthesis.

[b] * Indicates genes for which previously published data indicate that these genes are regulated by AbrB. Genes in parenthesis are those that appear to be regulated in a similar manner to other genes in the same operon but whose expression value were too variable to be identified as differentially expressed.

[c] Indicated is the ratio reported in Stanley *et al*. (2003) for expression of the indicated gene under biofilm formation conditions (B) divided by the expression of the gene under planktonic conditions (P). A ratio of >1 indicates a higher level of expression in the biofilm formation condition sample.

[d] Indicated is the ratio reported by Fawcett *et al*. (2000) for expression of the gene in the wild-type (WT) strain divided by the expression in the *spo0A* mutant strain (0A). A ratio of >1 represents a higher level of expression in the wild-type strain. ND represents those genes for which no data was available.

[e] Indicated is the ratio of expression of the gene in the wild-type strain (WT) divided by the expression of the gene in the *spo0A abrB* strain (0AabrB). Both stains were grown under biofilm formation conditions for 24 hours. A ratio of >1 represents a higher expression level in the wild-type strain. ND represents those genes for which no reproducible ratio was obtained.

[f] Indicated is the ratio reported by Britton *et al*. (2002) for expression of the gene in the wild-type strain (WT) divided by the expression in the *sigH* mutant strain (sigH). A ratio of >1 represents a higher level of expression in the wild-type strain. ND represents those genes that were not identified as differentially expressed in the study.

[g] Indicated is the ratio of expression of the gene in the wild-type strain (WT) divided by the expression of the gene in the *sigH abrB* strain (sigHabrB). Both stains were grown under biofilm formation conditions for 24 hours. A ratio of > 1 represents a higher expression level in the wild-type strain. ND represents those genes for which no reproducible ratio was obtained.

Table 2

RT-PCR analysis of AbrB-regulated genes

Gene name	Strain[a] wild type	*spo0A*	*spo0A abrB*
yqxM	0.47±0.1	0.23±0.04	0.75±0.1
ycnK	1.1±0.3	0.75±0.1	1.4±0.2
yoaW	1.0±0.1	0.84±0.1	2.3±0.3
yocH	1.4±0.4	1.1±0.2	2.2±0.2
yvqH	0.77±0.2	0.47±0.06	0.86±0.1
yxbB	0.56±0.1	0.38±0.03	1.3±0.07
yxbC	0.68±0.2	0.22±0.05	1.5±0.3

[a]Strains used are as follows; wild type, BAL 218; *spo0A*, BAL679; *spo0A abrB*, BAL734. The numbers represent the ratio of gene expression as a function of *veg* expression and are the average of 3 independent experiments. The errors represent the standard error of the mean.

Table 3

Biofilm formation by mutants defective for AbrB-repressed genes

Strain	Biofilm formation[a]
BAL 218 (WT)	1
BAL 722 (*yqxM*)	0.18*
BAL 1099 (*yoaW*)	0.46*
BAL 1115 (*tagE*)	0.53*
BAL 1106 (*ymfJ*)	0.61*
BAL 1128 (*ywqH*)	0.64*
BAL 1107 (*yqxI*)	0.71*
BAL 1105 (*ykfA*)	0.71
BAL 1129 (*ycnK*)	0.72
BAL 1108 (*aprE*)	0.86
BAL 762 (*yocH*)	0.90
BAL 1114 (*yvdF*)	0.93
BAL 1109 (*yvqH*)	1.0
BAL 1127 (*sdpA*)	1.0
BAL 1102 (*rapK*)	1.2
BAL 1125 (*yukJ*)	1.2
BAL 1101 (*dppA*)	1.2
BAL 1104 (*yitN*)	1.3
BAL 1126 (*yuxI*)	1.4
BAL 1116 (*yqcG*)	1.5
BAL 1117 (*yhjM*)	1.5*
BAL 1103 (*ydeH*)	1.6*
BAL 1100 (*era*)	1.7*
BAL 1124 (*yraI*)	2.0

[a] Biofilm formation was measured using the microtiter plate assay. Numbers shown are the ratio of OD_{570} of mutant to OD_{570} of wild-type.

* Indicates that the ratio is significantly ($p<0.01$) different from wild-type.

Table 4

Bacillus subtilis strains used in this study.

Strain	Relevant genotype	Reference/construction[a]
AGS157	Δ*sipW*::neo	(Stover and Driks, 1999c)
AGS175	*yqxM*::neo	(Stover and Driks, 1999c)
AGS207	Δ*tasA::spec*	(Stover and Driks, 1999c)
BAL216	*abrB::Tn*917 (MLS)	(Perego *et al.*, 1988)
BAL218	*trpC2 pheA1*	(Perego *et al.*, 1988)
BAL364	*sigH::cat::spec*	Laboratory collection
BAL373	Δ*abrB::cat*	Laboratory collection
BAL678	*sigH::cat::spec abrB*::Tn*917* (MLS)	BAL364 → BAL216
BAL679	*spo0A*D56N-*cat::spec*	(Lazazzera *et al.*, 1999)
BAL722	*yqxM*::pBL105 (*cat*)	pBL105 → BAL218
BAL734	*spo0A*D56N -*cat::spec abrB*::Tn*917* (MLS)	BAL679 → BAL216
BAL762	*yocH*::pBL114 (*cat*)	pBL114 → BAL218
BAL835	*amyE::gfp-cat*	(Stanley *et al.*, 2003)
BAL881	*sipW-lacZ* (*cat*)	pLGW201 → BAL218(Tjalsma *et al.*, 1998)
BAL982	Δ*tasA::spec*	AGS207 → BAL218
BAL983	Δ*sipW*::neo	AGS157 → BAL218
BAL984	*yqxM*::neo	AGS175 → BAL218
BAL988	*yqxM*::neo Δ*tasA::spec*	BAL984 → BAL982
BAL1099	*yoaW*::pBL179 (*cat*)	pBL179 → BAL218
BAL1100	*era*::pBL180 (*cat*)	pBL180 → BAL218
BAL1101	*dppA*::pBL181 (*cat*)	pBL181 → BAL218
BAL1102	*rapK*::pBL182 (*cat*)	pBL182 → BAL218
BAL1103	*ydeH*::pBL183 (*cat*)	pBL183 → BAL218
BAL1104	*yitN*::pBL184 (*cat*)	pBL184 → BAL218
BAL1105	*ykfA*::pBL185 (*cat*)	pBL185 → BAL218
BAL1106	*ymfJ*::pBL186 (*cat*)	pBL186 → BAL218
BAL1107	*yqxI*::pBL187 (*cat*)	pBL187 → BAL218
BAL1108	*aprE*::pBL188 (*cat*)	pBL188 → BAL218
BAL1109	*yvqH*::pBL178 (*cat*)	pBL178 → BAL218
BAL1114	*yvdF*::pBL189 (*cat*)	pBL189 → BAL218
BAL1115	*tagE*::pBL190 (*cat*)	pBL190 → BAL218
BAL1116	*yqcG*::pBL411 (*cat*)	pBL411 → BAL218
BAL1117	*yhjM*::pBL412 (*cat*)	pBL412 → BAL218
BAL1124	*yraI*::pBL413 (*cat*)	pBL413 → BAL218
BAL1125	*yukJ*::pBL414 (*cat*)	pBL414 → BAL218
BAL1126	*yuxI*::pBL415 (*cat*)	pBL415 → BAL218
BAL1127	*sdpA*::pBL416 (*cat*)	pBL416 → BAL218
BAL1128	*ywqH*::pBL417 (*cat*)	pBL417 → BAL218
BAL1129	*ycnK*::pBL418 (*cat*)	pBL418 → BAL218
BAL1061	Δ*sipW*::neo *amyE::gfp-cat*	pBL165 → BAL983
BAL1062	Δ*tasA*::spec *yqxM*::neo *amyE::gfp-cat*	pBL165 → BAL 988
BAL1293	*spo0A*D56N-*cat::spec sipW*-lacZ (*cat*) *abrB::Tn917* (MLS)	BAL881 → BAL734
BAL1300	*spo0A*D56N-cat::spec *sipW-lacZ* (*cat*)	BAL679 → BAL881

[a] Strain construction is indicated by an arrow. Chromosomal DNA or plasmid DNA listed at the tail of arrow was used to transform the strains listed at the head of the arrow.

Table 5

Primers used for RT-PCR analysis

Primer	Gene	Sequence 5′ – 3′	Position[a]	Number of Cycles[b]
BL129	*yocH*	GCAAAAGGGTGATACGCTCTGG	+90 - +112	24
BL130	*yocH*	GCTGTGTTTGCTGCTGTTGCCTG	+400 - +377	24
BL388	*ycnK*	GTCAATCAGCTGGTTCAGT	+118 - +136	26
BL389	*ycnK*	GTCTTGAAGCATGTCTTCCAG	+540 - +520	26
BL390	*yvqH*	CGTCAGTTCATGAAGGACTTG	+35 - +55	26
BL511	*yvqH*	CGCTGACTTTGAATAATTCGC	+561 - +541	26
BL468	*veg*	CGAAGACGTTGTCCGATATTAAAAG	+5 - +29	24 (25)
BL469	*veg*	CAACAGTCTCAGTCAAAATATCAGC	+217 - +193	24 (25)
BL482	*yqxM*	AGTCTGACTGCCGCAATATG	+82 - +101	29
BL483	*yqxM*	CGCATTTTGCAAGCCTCAT	+565 - +547	29
BL484	*yxbC*	GATTTACAAGCATACCGGGGT	+116 - +136	26
BL485	*yxbC*	TGGCGGATCTCCTTTCCAAT	+594 - +575	26
BL486	*yoaW*	GGCTTTGACTATCCATGTAGGG	+39 - +60	31
BL487	*yoaW*	GATCAAACTGAAAACCGTATAGACC	+398 - +394	31
BL512	*yxbB*	GGCACAGGTCCAGGTTAT	+130 - +148	26
BL513	*yxbB*	CTGAACAAACTGGTGAATGTC	+532 - +552	26

[a]The position of the primers is given, with +1 being the ‘A’ of the ATG start codon.

[b]Represented is the number of cycles used in the RT-PCR analysis. The number in parenthesis represents the number of cycles used at an annealing temperature of 54°C for analysis with the *yocH* primers.