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# Galactose Metabolism Plays a Crucial Role in Biofilm Formation by *Bacillus subtilis*

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**ABSTRACT** Galactose is a common monosaccharide that can be utilized by all living organisms via the activities of three main enzymes that make up the Leloir pathway: GalK, GalT, and GalE. In *Bacillus subtilis*, the absence of GalE causes sensitivity to exogenous galactose, leading to rapid cell lysis. This effect can be attributed to the accumulation of toxic galactose metabolites, since the *galE* mutant is blocked in the final step of galactose catabolism. In a screen for suppressor mutants restoring viability to a *galE* null mutant in the presence of galactose, we identified mutations in *sinR*, which is the major biofilm repressor gene. These mutations caused an increase in the production of the exopolysaccharide (EPS) component of the biofilm matrix. We propose that UDP-galactose is the toxic galactose metabolite and that it is used in the synthesis of EPS. Thus, EPS production can function as a shunt mechanism for this toxic molecule. Additionally, we demonstrated that galactose metabolism genes play an essential role in *B. subtilis* biofilm formation and that the expressions of both the *gal* and *eps* genes are interrelated. Finally, we propose that *B. subtilis* and other members of the *Bacillus* genus may have evolved to utilize naturally occurring polymers of galactose, such as galactan, as carbon sources.

**IMPORTANCE** Bacteria switch from unicellular to multicellular states by producing extracellular matrices that contain exopolysaccharides. In such aggregates, known as biofilms, bacteria are more resistant to antibiotics. This makes biofilms a serious problem in clinical settings. The resilience of biofilms makes them very useful in industrial settings. Thus, understanding the production of biofilm matrices is an important problem in microbiology. In studying the synthesis of the biofilm matrix of *Bacillus subtilis*, we provide further understanding of a long-standing microbiological observation that certain mutants defective in the utilization of galactose became sensitive to it. In this work, we show that the toxicity observed before was because cells were grown under conditions that were not propitious to produce the exopolysaccharide component of the matrix. When cells are grown under conditions that favor matrix production, the toxicity of galactose is relieved. This allowed us to demonstrate that galactose metabolism is essential for the synthesis of the extracellular matrix.

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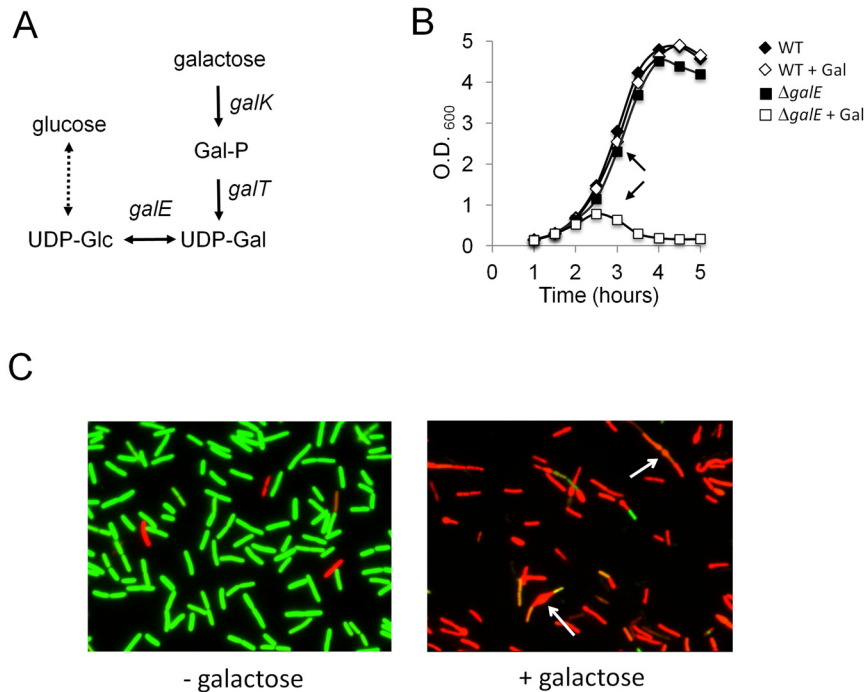
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The Gram-positive bacterium *Bacillus subtilis* forms surface-attached matrix-enclosed multicellular communities known as biofilms (1). The matrix, which provides structure to the biofilm, is composed of two very different components: an amyloid-like protein, TasA, and an exopolysaccharide (EPS) (2, 3). The presence of EPS is a nearly universal feature of biofilms and in some cases accounts for 90% of the colony mass (4). In *B. subtilis*, a 15-gene operon, *epsA* to *epsO* (*epsA-O*), encodes the proteins responsible for the synthesis of this polysaccharide (3, 5). While the structural role of the EPS in the biofilm has been well established, the precursor metabolites used in its synthesis and its final composition are not known (2).

In many bacterial species, sugar nucleotides, such as UDP-glucose and UDP-galactose, are essential for exopolysaccharide biosynthesis. Thus, deletion of the genes involved in the metabolic pathways for these nucleotide sugars often leads to the inability to produce exopolysaccharides (6). One of the major sugar metabo-

lism pathways is the Leloir pathway, which allows the catabolism of galactose via conversion to UDP-galactose (Fig. 1A) (7). In the first step of the pathway, galactose is phosphorylated by the galactokinase GalK. The resulting product, galactose-1-phosphate (Gal-P), is then converted to UDP-galactose by the galactose-1-phosphate uridylyltransferase GalT, which exchanges the glucose-1-phosphate (Glu-P) moiety of UDP-glucose with Gal-P, thereby producing UDP-galactose (UDP-Gal) and releasing glucose-1-phosphate. Glucose-1-phosphate (via conversion to glucose-6-phosphate) can then be catabolized by entering glycolysis. The final step is performed by GalE, a UDP-galactose-4-epimerase, which catalyzes the reversible conversion between UDP-Gal and UDP-glucose (UDP-Glc) (Fig. 1A), thereby regenerating UDP-glucose. In addition, *B. subtilis* GalE is capable of interconverting UDP-*N*-acetylglucosamine (GlcNAc) and UDP-*N*-acetylgalactosamine (GalNAc) (8).

The Leloir pathway is highly conserved in all organisms (9). In



**FIG 1** Galactose toxicity-induced cell lysis in the *galE* mutant by galactose metabolites. (A) Leloir pathway for galactose metabolism in bacteria. *galK* encodes a galactose kinase, *galT* encodes a galactose-1-phosphate uridylyltransferase, and *galE* encodes a UDP-galactose epimerase. Glc, glucose; Gal, galactose. (B) Growth of the wild type (3610) (WT) and the  $\Delta galE$  mutant (FC300) in LB (Luria-Bertani) medium in the presence or absence of 0.5% galactose. Arrows indicate the time point at which cell samples were collected for microscopy analyses (results shown in panel C). (C) Live/dead cell staining of the *B. subtilis*  $\Delta galE$  mutant (FC300). Cells were grown in LB medium with or without supplementation of 0.5% galactose and collected 3 h after inoculation (indicated by arrows in panel B). Cells were treated with dyes for live/dead cell staining and examined under fluorescence microscopy. Arrows points to cells showing distinct bulge-like structures. (D) Growth of the wild type (3610) and  $\Delta galE$  mutant (FC300) of *B. subtilis* in LB medium supplemented or not supplemented with 0.5% galactose (Gal) or 0.5% D-oxy-galactose (D-oxy-Gal). (E) Growth of the  $\Delta galETK$  (YC563),  $\Delta galETK amyE::galK$  (YC810), and  $\Delta galETK amyE::galTK$  (YC811) mutants in LB medium supplemented or not supplemented with 0.5% galactose (Gal).

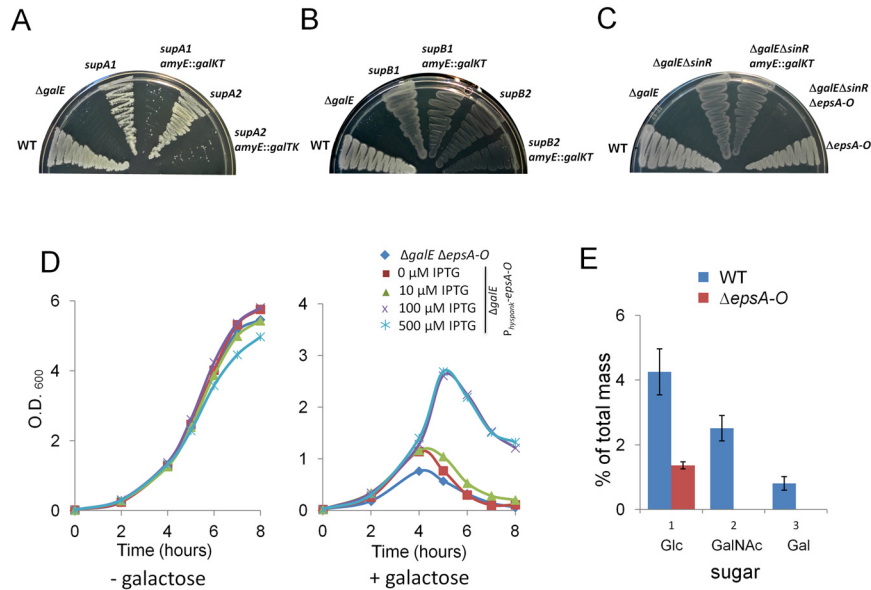
humans, a deficiency in any of the three enzymes of the Leloir pathway leads to a disorder called galactosemia, which results in general organ damage if galactose is ingested. This effect results from the accumulation of a toxic galactose intermediate. In bacteria, *galE* mutants of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium are unable to grow in medium supplemented with galactose (10–12). As with the human disease, this growth inhibition is attributed to accumulation of toxic intermediate metabolites from galactose (12). Similarly, deletion of *galE* in the *B. subtilis* domesticated strain 168 causes sensitivity to exogenous addition of galactose (13).

Here we show that deletion of the *galE* gene in the less domesticated *B. subtilis* strain NCIB3610 also induces sensitivity to exogenous galactose due to accumulation of UDP-galactose, which is toxic under planktonic growth conditions. During biofilm formation, we observed that production of EPS could serve as a shunt pathway for UDP-galactose, thus relieving the galactose toxicity in the *galE* mutant. Additionally, we report that synthesis of UDP-galactose is required for exopolysaccharide biosynthesis and that the transcriptions of the *gal* and *eps* genes are interrelated. Finally, we show that *B. subtilis* is able to acquire galactose through the catabolism of galactan, a polymer of galactose often found in natural settings.

## RESULTS

**Galactose induces rapid cell lysis in the *galE* mutant of *B. subtilis* by accumulation of toxic metabolites.** A *galE* deletion mutant of

*B. subtilis* strain NCIB3610 displayed a growth curve similar to that of the wild type when grown in LB (Luria-Bertani) medium. Galactose requires the presence of arabinose to be taken up by *B. subtilis*; therefore, the medium was simultaneously supplemented with 0.01% arabinose in this work to facilitate the uptake of galactose (14). When cultivated in LB medium supplemented with 0.5% (wt/vol) galactose and 0.01% arabinose, the *galE* mutant displayed a severe growth defect (Fig. 1B). In fact, concentrations of galactose as low as 0.005% impaired the growth of the *galE* mutant (see Fig. S1 in the supplemental material). The *galE* mutant was able to grow for a few hours in the presence of galactose, but the optical density at 600 nm ( $OD_{600}$ ) decreased after reaching about 0.8, suggesting that cell lysis had occurred (open squares in Fig. 1B). To test whether lysis was occurring, samples of  $\Delta galE$  mutant cultures were taken after 3 h of growth in LB medium or in LB medium that had been supplemented with galactose. The cells were then treated with live/dead stain (Invitrogen). Fluorescence microscopy imaging showed that in the absence of galactose, the majority of  $\Delta galE$  mutant cells were alive (Fig. 1C, falsely colored green) and that there was high abundance of dead cells in the presence of galactose (Fig. 1C, falsely colored red). Additionally, these dying cells displayed abnormal bulges at one end or in the middle of the cell, suggesting that they were undergoing lysis (arrows in Fig. 1C). These results confirmed that galactose causes toxicity, which leads to cell death in the  $\Delta galE$  mutant of *B. subtilis*. This observation is also in agreement with a previous report dem-



**FIG 2** Producing EPS (exopolysaccharide) as a shunt pathway for toxic metabolites from galactose. (A to C) Characterization of *galE* suppressor mutants resistant to galactose toxicity. Cells were inoculated on LB agar plates supplemented with 0.5% galactose. (D) Overexpression of the *epsA-O* operon suppressed galactose toxicity in the *galE* mutant. *galE::tet P<sub>hyspank</sub>-epsA-O* cells (YC776) were grown in MSgg media in the absence (left) or presence (right) of 0.1% galactose and with various amounts of IPTG (see the key). The *galE epsA-O* double mutant is also included as a control. (E) Sugar composition analysis of EPS from wild-type (3610) or  $\Delta epsA-O$  mutant (YC771) pellicles. Glc, glucose; GalNAc, *N*-acetyl-galactose; Gal, galactose.

onstrating that galactose caused toxicity and cell lysis in a  $\Delta galE$  mutant derived from the domesticated *B. subtilis* strain 168 (13).

It was further reported that the toxic effect of galactose on the *B. subtilis galE* mutant was due to accumulation of toxic intermediate metabolites, such as phosphorylated galactose (Gal-P) and UDP-galactose (UDP-Gal) (Fig. 1A) (13). To evaluate which one of these two metabolites caused cell lysis, we tested the effect of D-oxy-galactose on the wild type and the *galE* mutant. GalK can phosphorylate this compound, but the product cannot be further converted by GalT, leading to an artificial increase of D-oxy-Gal-P in the cells. -D-oxy-Gal-P has been shown to be toxic to *E. coli* and other bacteria (15). Since the addition of 0.5% of D-oxy-galactose in LB medium did not cause a significant growth defect in a *galE* mutant or in wild-type cells, it is unlikely that Gal-P is toxic for *B. subtilis* (Fig. 1D, circles).

In a different approach, we deleted the *galkT* genes in a *galE* background. If the toxic metabolite is produced by the GalK and/or GalT enzymes, deleting both genes should block galactose utilization and should restore viability to  $\Delta galE$  cells in the presence of galactose. Indeed, the  $\Delta galE \Delta galKT$  triple mutant withstood the presence of exogenous galactose in the medium (Fig. 1E, diamonds). We investigated whether the toxic metabolite was Gal-P (the product of GalK) or UDP-Gal (the product of GalT) by restoring to the  $\Delta galE \Delta galKT$  triple mutant strain either *galk* alone or both *galT* and *galk* at the chromosomal *amyE* locus. As shown in Fig. 1E, the strain to which only *galk* was restored was still resistant to galactose toxicity, but the presence of both *galk* and *galT* at the *amyE* locus restored sensitivity to galactose (Fig. 1E). This result again supported the idea that UDP-Gal, but not Gal-P, is the toxic metabolite in *B. subtilis*.

**Characterization of the galactose-resistant suppressor of the  $\Delta galE$  mutant.** As expected, the  $\Delta galE$  mutant was unable to form colonies on LB agar plates supplemented with 0.5% galactose (Fig.

2A). This allowed us to isolate colonies containing suppressor mutations that allowed the  $\Delta galE$  mutant to grow in the presence of galactose. Using the genetic approaches described in Materials and Methods, we mapped the mutations in order to understand how they help cells cope with galactose toxicity. As might be expected, strains with mutations in two of these suppressors, *supA1* and *supA2* (Fig. 2A), were found to bear mutations in *galk*. The *supA1* mutant contained a frameshift mutation at nucleotide position 70 of the *galk* open reading frame, resulting in a truncated GalK protein, and the *supA2* strain had a point mutation in the codon for a highly conserved alanine at residue 24 (A24P) (Fig. S2). Both mutations likely caused loss of GalK enzymatic activity, thus impairing the accumulation of the toxic UDP-Gal metabolites by blocking the utilization of galactose (see Table S1 in the supplemental material). Accordingly, reintroduction of functional *galkT* genes at the *amyE* locus of the *supA1* and *supA2* suppressor mutants restored sensitivity to galactose toxicity (Fig. 2A).

To reduce the probability of obtaining suppressor mutations in *galkT*, a second copy of these genes was introduced into the  $\Delta galE$  strain at the *amyE* locus. The resulting strain was similarly sensitive to exogenous galactose (data not shown) but still occasionally gave rise to resistant colonies on LB medium-0.5% galactose plates, suggesting that another unknown mechanism(s) may confer resistance to galactose toxicity in *galE* mutant cells. The suppressor phenotype of these resistant colonies was verified, and suppressor mutations in two such suppressor strains (*supB1* and *supB2* mutants) were mapped. Both the *supB1* and *supB2* suppressor strains were found to harbor mutations in the *sinR* gene, which encodes a master transcriptional regulator that represses genes involved in biofilm formation (Fig. 2B; see also Table S1 in the supplemental material). The *supB1* mutant had a nonsense mutation at codon 78 of the gene, whereas the *supB2* mutant had a point

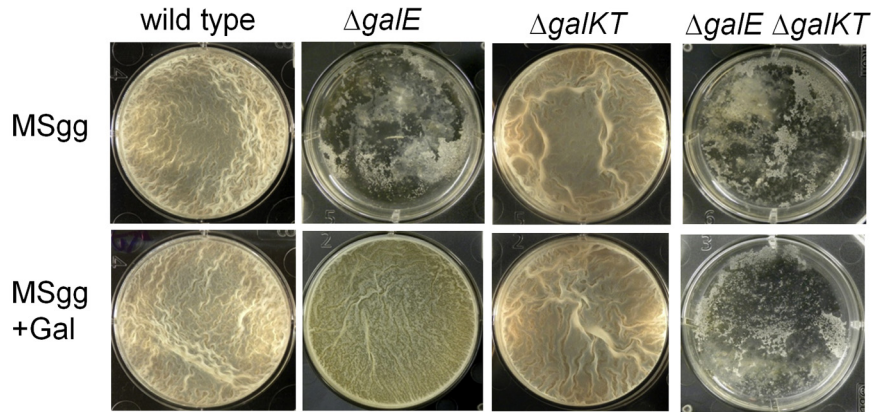


FIG 3 The *gal* genes are important for biofilm formation. Top-down view of pellicle formation of the wild type (3610) and the  $\Delta galE$  (FC300),  $\Delta galTK$  (YC532), and  $\Delta galE \Delta galTK$  (YC563) mutants in MSgg medium with or without 0.5% galactose.

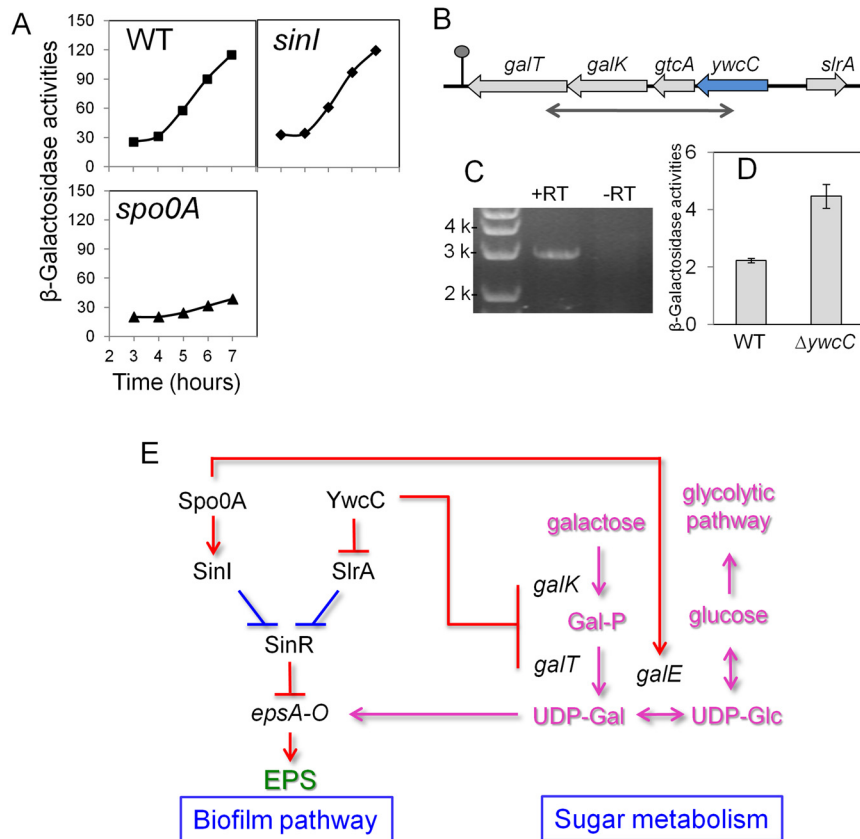
mutation in the codon for the leucine residue at position 74 (L74S). As SinR is known to repress many biofilm genes, including the 15-gene *epsA-O* operon, which produces the matrix exopolysaccharide, we decided to analyze the effect of these point mutations on the expression of the *eps* genes (3). Using the strains harboring a *P<sub>epsA</sub>-lacZ* reporter construct, we showed that both *sinR* suppressor mutations greatly impaired the repressing capacity of SinR, as the expression of the reporter was drastically elevated in both mutants compared to expression in the wild type (Fig. S3). Second, a double mutant with both *galE* and *sinR* deleted was constructed and displayed a much higher resistance to galactose toxicity than the *galE* single mutant (Fig. 2C), further supporting the hypothesis that *sinR* inactivation was the explanation for the suppressor phenotypes of the mutants obtained.

**Resistance of the *galE sinR* double mutant to galactose toxicity depends on the production of exopolysaccharide.** SinR does not influence *galT* or *galK* expression (data not shown), thus pointing to a unique mechanism for the loss of galactose toxicity in *galE sinR* cells. As the *eps* genes were highly induced in the suppressor mutants, we assayed whether a deletion of the *epsA-O* operon could reverse the resistance phenotype of the *galE sinR* double mutant. Indeed, the *galE sinR epsA-O* triple mutant was unable to grow in the presence of galactose (Fig. 2C). Importantly, the  $\Delta epsA-O$  mutation itself in an otherwise wild-type background did not affect galactose toxicity (Fig. 2C) likely because GalE converts UDP-galactose to UDP-glucose, thus avoiding accumulation of the toxic UDP-galactose. Furthermore, deleting other SinR-regulated genes, such as the *tapA-sipW-tasA* operon or the *lutABC* operon, did not show the same effect (data not shown) (16, 17). These results suggest that suppression of galactose toxicity by SinR inactivation requires an increased expression of the *epsA-O* operon. If this is the case, then overexpression of *epsA-O* should have the same effect as a *sinR* mutation and thus restore the viability of *galE* mutant cells in the presence of galactose. We tested this hypothesis by replacing the native *epsA-O* promoter with an IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside)-inducible *hyspank* promoter (*epsA-O* $\Omega P_{hyspank}$ -*epsA-O*) in the *galE* mutant. The resulting cells were grown in MSgg medium (18) supplemented with various amounts of IPTG. In the presence of 0.1% galactose, cells with maximal *epsA-O* gene induction grew much better than those with little or low levels of *epsA-O* induction,

although in the end, significant cell lysis still occurred (Fig. 2D). As a control, in the absence of galactose, IPTG addition did not affect cell growth (Fig. 2D). A *galE epsA-O* mutant was also used as a control in this experiment. Thus, the production of EPS by expression of the *epsA-O* operon is able to restore growth to a *galE* mutant in the presence of galactose.

**Producing exopolysaccharides as a shunt pathway for galactose toxicity.** The above observations led us to hypothesize that a null mutation of *sinR* or overexpression of *epsA-O* can relieve the toxic effect of galactose by shunting its toxic metabolite (UDP-Gal) toward incorporation in the EPS. Since sugar composition of the *B. subtilis* EPS is not known, we decided to characterize it to determine if galactose or its derivatives were indeed present in EPS. To this end, we purified EPS from wild-type and *epsA-O* mutant biofilms and analyzed their sugar compositions as described in Materials and Methods. As shown in Fig. 2E, EPS prepared from the wild-type biofilms was rich in glucose (Glc), *N*-acetyl-galactose (GalNAc), and galactose (Gal), whereas the material extracted from the *epsA-O* mutant completely lacked *N*-acetyl-galactose and galactose (Fig. 2E). These results confirmed that galactose and/or its derivatives are constituents of the biofilm EPS in *B. subtilis*. We conclude that in *B. subtilis*, producing EPS can function as a shunt mechanism for a toxic metabolite(s) from galactose.

**The *gal* genes are important for biofilm formation in *B. subtilis*.** Exopolysaccharide is a key component of the biofilm matrix in *B. subtilis* since mutants deficient in EPS production are unable to form biofilms (2, 3). As galactose is present in purified EPS, we examined the importance of this sugar for biofilm formation. We took advantage of the fact that the *galE* mutant cannot convert UDP-glucose to UDP-galactose, and therefore, it relies entirely on exogenous galactose to synthesize UDP-Gal (Fig. 1A). If this nucleotide sugar is required for EPS biosynthesis, then in the absence of an exogenous source of galactose, a *galE* mutant should be defective in EPS biosynthesis and thus impaired in biofilm formation. As observed in Fig. 3, wild-type cells form floating biofilms (pellicles) that appear white in a top-down view when inoculated into a biofilm-inducing, liquid medium lacking galactose (MSgg). In contrast, the  $\Delta galE$  mutants form only a broken pellicle that strikingly resembles that of the *epsA-O* mutant (see Fig. S4 in the supplemental material). As predicted, addition of exogenous ga-



**FIG 4** Regulation of the *gal* genes in *B. subtilis*. (A)  $\beta$ -Galactosidase activities of the wild type (YC777), the *sinI* mutant (YC778), and the *spo0A* mutant (YC779), which harbor the  $P_{galE}$ -*lacZ* reporter at the *amyE* locus on the chromosome. Cells were grown in MSgg shaking culture, and hour 5 postinoculation was approximately the start of the stationary phase. (B) Genetic organization of the *ywcC*-*gtcA*-*galK*-*galT* gene cluster in *B. subtilis*. (C) Results of the reverse transcription-PCR (RT-PCR) using primers that amplify part of the mRNA transcript of the *ywcC*-*gtcA*-*galK*-*galT* gene cluster. The amplified region (about 3 kb) is indicated by the horizontal double-headed arrow in panel B. PCR amplification without reverse transcription (–RT) was used as a negative control. (D)  $\beta$ -Galactosidase activities of the wild type (YC783) and the *ywcC* mutant (YC784), which contain the  $P_{ywcC}$ -*lacZ* reporter at the chromosomal *amyE* locus. (E) Proposed model for the concerted regulation of the *epsA*-O operon and the *gal* genes in *B. subtilis*. Under normal biofilm-inducing conditions, the master regulator Spo0A activates both *sinI*, which leads to derepression of the *epsA*-O operon, and *galE*, whose protein product generates UDP-Gal as a key nucleotide-sugar substrate for EPS biosynthesis. Under alternative conditions, a TetR-like regulator, YwcC, derepresses both *slrA*, which also leads to derepression of the *epsA*-O operon and *galK* and *galT* in the same operon, whose protein products convert exogenous galactose to UDP-Gal.

lactose rescued the biofilm defect of the *galE* mutant, as the required UDP-Gal could then be synthesized via GalK and GalT (Fig. 3). Deletion of *galKT* had no influence on biofilm formation due to the presence of *galE*, but the *galE galKT* triple mutant was unable to form a pellicle even in the presence of galactose, since these cells could no longer synthesize UDP-Gal (Fig. 3). It is also important to note that under biofilm-inducing conditions, when *eps* gene expression is normally high, adding galactose to the *galE* mutant did not inhibit growth, which was readily observed for the same cells under planktonic growth conditions that do not favor matrix production (Fig. 3). This reinforced the idea that *B. subtilis* shunts UDP-Gal into exopolysaccharide synthesis during biofilm formation.

**Regulation of the *gal* genes is interrelated with that of the *epsA*-O operon in *B. subtilis*.** Our findings that the *galEKT* genes are required for biofilm formation (Fig. 3) proved that nucleotide sugar precursors, such as UDP-Gal, are crucial for EPS synthesis. Hence, we wondered whether the *gal* genes were regulated in concert with the *epsA*-O operon during biofilm formation in *B. subtilis*. To investigate the regulation of the *galE* gene, we constructed

a reporter fusion in which the promoter region of the *galE* gene was fused to a *lacZ* reporter ( $P_{galE}$ -*lacZ*). This construct was then integrated into the *amyE* locus of the wild type and various mutant strains. We first measured *lacZ* expression in wild-type cells grown in MSgg and observed that  $P_{galE}$ -*lacZ* activity was significantly induced at late times (Fig. 4A), similar to what was shown for expression of the *epsA*-O operon (19). We previously demonstrated that EPS biosynthesis is indirectly controlled by the master regulator Spo0A via the expression of the antirepressor SinI (3). Thus, deletion of either of these genes blocks the expression of the *epsA*-O operon (Fig. 4E). While a *spo0A* deletion impaired *galE* gene induction, a *sinI* mutant had no effect (Fig. 4A). This result suggests a coordinated regulation of the *galE* gene and the *epsA*-O operon mediated by Spo0A, but independent of *sinI* activation, during biofilm formation. It is notable that the basal level of *galE* gene expression was quite significant (Fig. 4A). This is consistent with the idea that GalE is also important in making UDP-Gal for other cellular processes during normal cell growth when the EPS is not being synthesized (20).

While *galE* is located elsewhere on the chromosome as a mono-

cistronic unit, *galT* and *galK* are clustered with the *gtcA* and *ywcC* genes (Fig. 4B). The *gtcA* gene encodes a putative glycosyltransferase possibly involved in teichoic acid biosynthesis, whereas *ywcC* encodes a TetR-type repressor. In previous studies, we and others have shown that YwcC represses the divergently transcribed gene *slrA*, a SinR antirepressor (Fig. 4B) (21, 22). Therefore, YwcC is a negative regulator for biofilm formation. Although it was previously postulated that *ywcC*, *gtcA*, *galK*, and *galT* might constitute an operon, there was no experimental evidence for this (22). We used reverse transcription-PCR (RT-PCR) with a pair of primers extending across all four genes to investigate if they were cotranscribed. As shown in Fig. 4C, a fragment of about 3 kb was amplified in the RT-PCR (with reverse transcriptase), confirming that *ywcC*, *gtcA*, *galK*, and *galT* are cotranscribed as a single unit. Whether or not *galK* and *galT* are exclusively transcribed from the promoter upstream of *ywcC* will need further investigation. The negative control, without reverse transcriptase, did not produce a detectable fragment. We then tested whether this operon was autoregulated by the YwcC repressor using a transcriptional fusion of the regulatory region upstream of *ywcC* and *lacZ* ( $P_{ywcC}$ -*lacZ*). The activity of the  $P_{ywcC}$ -*lacZ* reporter was found to be about 2-fold higher in the *ywcC* mutant than in the wild type (Fig. 4D), indicating that the whole operon is negatively autoregulated by YwcC.

Based on our findings, we propose a model in which regulation of the *epsA-O* operon (genes for EPS biosynthesis) and that of the *gal* genes (genes in the UDP-Gal metabolism pathway) are coordinated (Fig. 4E). This model proposes two different pathways leading to biofilm formation (Fig. 4E). Under biofilm-inducing conditions, such as growth in MSgg medium, the master regulator Spo0A induces *eps* gene transcription via production of the SinI antirepressor. At the same time, Spo0A stimulates *galE* transcription, leading to an increased production of UDP-Gal and thus providing sufficient amounts of this key nucleotide sugar as a substrate for EPS biosynthesis. A second pathway for biofilm formation involves YwcC, which upon receiving an as-yet-unknown signal, derepresses both the *galKT* genes and the paralogous anti-repressor gene *slrA*, leading to UDP-Gal biosynthesis and derepression of the *epsA-O* operon. Since galactose is required for UDP-Gal synthesis by GalK and GalT, one might predict that YwcC directly or indirectly senses galactose as a signal. Efforts to detect such a response have, however, been unsuccessful. Alternatively, perhaps YwcC recognizes and responds to a galactose-containing oligosaccharide or other breakdown product of a galactose-rich polysaccharide.

**A conserved metabolic pathway for utilization of galactan in *B. subtilis*.** In the soil and the rhizosphere, the natural habitat of *B. subtilis*, galactose is often found as part of polymers such as galactan, an abundant molecule in the cell wall of plants and of certain bacteria (23, 24). Thus, galactan may constitute an important source of galactose for *B. subtilis* in settings where it interacts with plant roots and other soil microorganisms. A previous report found that the *cycB-ganB* operon in *B. subtilis* might be involved in galactan degradation (25). Within that gene cluster, *cycB*, *ganP*, and *ganQ* encode a putative ABC transporter system, while *ganA* encodes a  $\beta$ -galactosidase-hydrolyzing galactan and *ganB* encodes an endo- $\beta$ -1 to -4-galactosidase (25). Next to this gene cluster is *lacR*, which encodes a LacI family transcriptional repressor and might regulate the nearby gene cluster (Fig. 5A) (26).

To confirm the involvement of these genes in galactan utilization, we constructed a strain lacking the entire gene cluster (*cycB-*

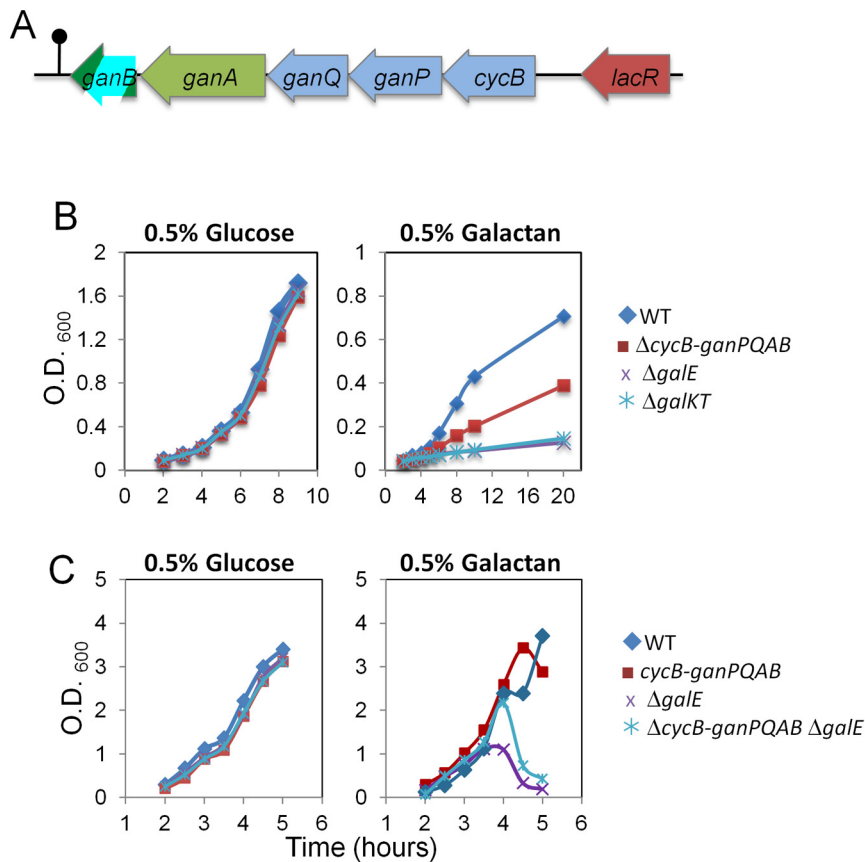
*ganPQAB*). This mutant, together with the wild type, the  $\Delta$ *galE* mutant, and the  $\Delta$ *galKT* mutant, was grown in a modified M9 minimal medium with either 0.5% galactan or 0.5% glucose as the sole carbon source. The growth of all of the mutants was similar to that of the wild type when cells were grown in the presence of 0.5% glucose as the sole carbon source (Fig. 5B). However, compared to the wild type, the *cycB-ganPQAB* mutant showed slightly impaired growth in minimal medium containing galactan as the sole carbon source (Fig. 5B). This limited growth impairment indicates that while the *cycB-ganPQAB* operon appears to be involved in galactan utilization, additional enzymes might contribute to galactan utilization in *B. subtilis*. This is supported by a bioinformatic analysis of the *B. subtilis* genome indicating that other homologs of genes in the *cycB-ganPQAB* operon are also present in the genome of *B. subtilis* (data not shown). In contrast, both the *galKT* and the *galE* mutants were unable to grow in the presence of galactan as the sole carbon source (Fig. 5B). This argues that galactan must be first broken down to galactose and then converted to glucose via the Leloir pathway. This idea was further supported by growing the  $\Delta$ *galE* cells in LB medium supplemented with 0.5% galactan. We found that the *galE* mutant could not grow to a high cell density and displayed a cell lysis phenotype similar to that described above when cells were cultivated with 0.5% galactan (Fig. 5C). Deletion of the *cycB-ganPQAB* operon, encoding the galactan utilization pathway, only partially suppressed the toxicity in the *galE* mutant, once again suggesting a redundant pathway for galactan utilization in this strain (Fig. 5C).

## DISCUSSION

We have shown that galactose metabolism plays a central role in biofilm formation by *B. subtilis*. Whereas accumulation of UDP-galactose can be toxic to *B. subtilis* during planktonic growth, it is required for the biosynthesis of EPS as a nucleotide sugar substrate and thus for matrix production. Consistently with the above, the genes encoding the Leloir pathway for galactose catabolism and the operon responsible for EPS biosynthesis show coordinated transcriptional regulation. Finally, we observed that *B. subtilis* also has the capacity to degrade galactan and thus acquire galactose from this naturally occurring polymer.

Galactose has been shown to be essential for the synthesis of various exopolysaccharides, such as colanic acid in *E. coli* (27). Similarly, enzymes in the Leloir pathway, and in particular the GalE epimerase, have been correlated with production of exopolysaccharide in *Streptococcus thermophilus*, *Porphyromonas gingivalis*, and *Neisseria* strains (6, 28–31). Thus, our observation that UDP-galactose synthesis is necessary for exopolysaccharide biogenesis in *B. subtilis* is consistent with what has been observed for other bacteria.

Prior to this study, little was known about the requirement for nucleotide sugars in the production of *B. subtilis* exopolysaccharide. It had been demonstrated that GtaB, which catalyzes the formation of UDP-glucose from glucose-1-P and UTP, is necessary for biofilm formation in LB medium by the *B. subtilis* domesticated strain 168 (32). The authors of that work suggested that the requirement for this molecule meant that UDP-glucose is either involved as a signaling molecule or as a precursor for synthesis of EPS. Since our analysis of *B. subtilis* exopolysaccharide showed a large amount of glucose and galactose residues, it is reasonable to conclude that UDP-glucose, like UDP-galactose, is required as a precursor for EPS synthesis during biofilm formation.



**FIG 5** Utilization of galactan by *B. subtilis*. (A) Genetic organization of the putative gene cluster for galactan utilization in *B. subtilis*. *ganB* encodes a putative endo-1,4-beta-galactosidase, *ganA* encodes a putative  $\beta$ -galactosidase, *cycB-ganP-ganQ* encodes a sugar transportation system, and *lacR* encodes a LacI family transcription repressor. (B) Growth of the wild type (3610),  $\Delta cycB-ganPQAB$  mutant (YC1063),  $\Delta galE$  mutant (FC300), and  $\Delta galKT$  mutant (YC532) in modified M9 minimal medium with 0.5% galactan as the sole carbon source. As a control, these strains were also grown in M9 minimal medium with 0.5% glucose as the sole carbon source. (C) Strains were grown in LB medium (lower left) or LB medium supplemented with 0.5% galactan (lower right) to test galactan toxicity. Strains are designated as follows: WT is strain 3610,  $\Delta cycB-ganPQAB$  is YC1063,  $\Delta galE$  is FC300, and  $\Delta cycB-ganPQAB \Delta galE$  is YC1095.

We propose a working model (Fig. 6A) for the catabolism of galactan by *B. subtilis* and for the utilization of galactose either as a carbon source via the Leloir pathway or as a nucleotide-sugar substrate for EPS biosynthesis during biofilm formation. Interestingly, the *cycB-ganPQAB* operon is present not only in *B. subtilis* but also in several related bacterial species (Fig. 6B). In two plant-associated *Bacillus* species, *B. licheniformis* and *B. amyloliquefaciens*, these genes are not only conserved and clustered but also grouped with the *gal* genes (Fig. 6B). A similar genetic organization of these genes was also found in a gut-colonizing bacterium, *Lactobacillus acidophilus*. This gene cluster was indeed recently proposed to be involved in the utilization of host-released polymers of galactose by *L. acidophilus* (33). Our study thus provides an interesting example of how bacteria have adapted to utilize host-derived sugars not only as a carbon source, but also for the establishment of a close symbiotic relationship between the bacterium and the host.

## MATERIALS AND METHODS

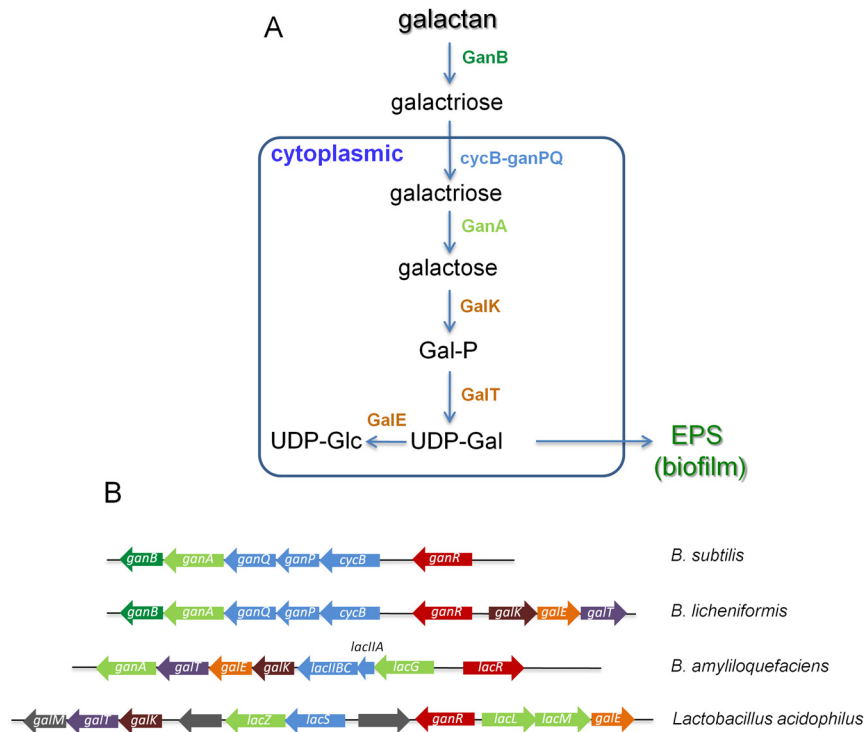
**Bacterial strains, media, and reagents.** For general purposes, *B. subtilis* strains PY79, NCIB 3610, and their derivatives were grown in LB medium (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter of broth) at 37°C. For assays of biofilm formation, MSgg medium was used and cells were

incubated at 23°C. The recipe for MSgg medium was described previously (18). *E. coli* strains were grown in LB medium at 37°C, and strain DH5 $\alpha$  was used for molecular cloning. A list of strains, plasmids, and oligonucleotides used in this work are summarized in Tables S2 and S3 in the supplemental material. Antibiotics were added to the media at the following concentrations for *B. subtilis* strains: 10  $\mu\text{g ml}^{-1}$  tetracycline, 100  $\mu\text{g ml}^{-1}$  spectinomycin, 10  $\mu\text{g ml}^{-1}$  kanamycin, 5  $\mu\text{g ml}^{-1}$  chloramphenicol, and 1  $\mu\text{g ml}^{-1}$  erythromycin. For *E. coli* strains, ampicillin was added at 100  $\mu\text{g ml}^{-1}$ . Galactose, deoxygalactose, arabinose, xylose, galactan, and other chemicals were purchased from Sigma.

**Strain construction.** General methods for molecular cloning followed published protocols (34). SPP1 phage-mediated transduction was used to transfer antibiotic-marked DNA fragments among different strains (35). Long flanking PCR mutagenesis was applied to generate insertional deletion mutations (36).

The insertional mutations in *galK-galT*, *epsA-O*, and *cycB-ganPQAB* gene clusters were generated by long flanking PCR mutagenesis by using primers *galTK*-P1, -P2, -P3, -P4, *eps*-P1, -P2, -P3, -P4, and *gan*-P1, -P2, -P3, -P4 (see Table S3), respectively. To construct the inducible *galK-galT* fusion (*P<sub>hyspank</sub>-galTK*), the coding region of *galK-galT* was amplified by PCR using 3610 genomic DNA as the template and the primers *galTK*-F1 and *galTK*-R1. The PCR products were cloned into NheI and HindIII sites of the vector pDR111 (gift of D. Rudner). The resulting recombinant plasmid (pYC245) was first introduced into PY79 by transformation. The





**FIG 6** Galactan metabolism in *B. subtilis* and other bacteria. (A) Proposed pathway in *B. subtilis* for utilization of naturally occurring galactan. Galactan is first broken down to galactotriose by GanB outside the cell. Galactotriose is then taken up by the permease composed of CycB-GanP-GanQ and further broken down to galactose by GanA. Galactose is eventually converted to glucose by the enzymes in the Leloir pathway encoded by the *gal* genes or becomes a sugar substrate of EPS during biofilm formation. (B) Genetic arrangements of the putative gene clusters for utilization of galactan in various bacteria. In two plant-associated *Bacillus* species, *B. licheniformis* and *B. amyloliquefaciens*, as well as in a gut-associated bacterium, *Lactobacillus acidophilus*, the homologous (to *cycB-ganPQAB* in *B. subtilis*) genes whose protein products are predicted to function in the breakdown of galactan to galactose are not only clustered but also grouped with the *gal* genes whose protein products are involved in further metabolism of galactose. Conservation of this genetic arrangement of the above-described genes in various bacteria implies that these bacteria adapted to utilize host-derived sugar polymers. In *L. acidophilus*, the gene cluster contains three *ganA* paralogs (light green) and two unknown phage-like genes (gray). In *B. amyloliquefaciens*, the two permease-like genes (blue) encode a putative PtlI sugar uptake system, whereas in *L. acidophilus*, a single *lacS* gene (blue) encodes a putative sugar permease.

reporter fusion at the *amyE* locus was then introduced into other 3610 derivatives by SPP1 phage-mediated transduction.

To construct the inducible *gaK* fusion ( $P_{hyspank}$ -*galK*), the plasmid pYC245 was digested with NheI to remove a large internal fragment of *galT*. After digestion, the plasmid was religated and transformed into DH5 $\alpha$ . The resulting recombinant plasmid contains functional *galK* only under the control of the *hyspank* promoter. This fusion construct was introduced into 3610 derivatives by following steps similar to those described above.

To construct the reporter fusion  $P_{galE}$ -*lacZ*, the promoter region of *galE* was amplified by PCR. PCR products were cloned into EcoRI and BamHI sites of the vector pDG268 (36) to make a promoter-*lacZ* transcriptional fusion. The inducible fusion  $eps\Omega P_{hyspank}$ -*eps* was constructed in FC598, which was a gift of Frances Chu. The construct was introduced into 3610 derivatives by SPP1 phage-mediated transduction.

**Live/dead cell staining.** Live/dead staining followed the protocol provided by the manufacturer (Invitrogen, CA). In brief, *galE* (FC300) cells were grown in LB medium in shaking culture in the presence or absence of 0.5% galactose. Three hours after inoculation, cell samples were collected and washed with phosphate-buffered saline (PBS) buffer twice. Cells were resuspended and diluted to about  $5 \times 10^7$  cells per ml in PBS buffer. One microliter of reconstituted fluorescent reactive dye was added to one milliliter of the cell suspension and mixed well. Cell samples were incubated on ice for 30 min and were concentrated for examination by fluorescence microscopy.

**Assays for galactose toxicity.** For plate assays of galactose toxicity, cells were picked from a freshly grown plate and streaked out on an LB

medium plate supplemented with 0.5% galactose and 0.01% arabinose. Arabinose is added to facilitate uptake of galactose by an arabinose-inducible transporter system in *B. subtilis*. The plates were then incubated at 37°C overnight. The next day, pictures of the plates were taken with a Nikon Coolpix 400 camera. The galactose toxicity was visualized by lack of growth on the plate. For assays of galactose toxicity in liquid medium, cells were first grown in LB medium to exponential phase and then inoculated 1:100 into fresh LB liquid medium supplemented with 0.5% galactose and 0.01% arabinose. Cell optical density was measured by an Ultraspec 2000 spectrometer (Pharmacia, NJ) or by a Synergy 2 plate reader (Bio-Tek, Canada).

**Characterization of the suppressor mutations.** Cells susceptible to galactose toxicity were streaked out on LB agar plates supplemented with 0.5% galactose and 0.01% arabinose. Plates were incubated at 37°C for 2 days. Resistant colonies were picked and restreaked on fresh LB medium-galactose plates to confirm the resistance phenotype. A mini-Tn10 transposon bearing plasmid pIC333 (38) was introduced into the suppressor mutants by transformation. Cells were selected for Sp<sup>c</sup> and resistance to macrolides, lincosamides, and streptogramins (MLS<sup>r</sup>) on solid medium at 30°C to verify the presence of the pIC333 plasmid in the suppressor mutants. Resistant colonies were inoculated into LB liquid medium and grown at 37°C overnight to allow for continued cell division and to block replication of pIC333. Following the selection, genomic DNAs were prepared from the above-described cultures and introduced into the *galE* mutant by transformation. Transformants were selected on LB medium-galactose-spectinomycin plates. Resistant colonies were picked and verified for galactose and spectinomycin resistance. The ge-

netic linkage between the *spec* resistance marker and the suppressor mutation that confers galactose resistance was calculated. The flanking region of the mini-Tn10 insertion was then sequenced to confirm the presence of the suppressor mutations.

**$\beta$ -Galactosidase assays.** A detailed protocol for assays of  $\beta$ -galactosidase activities was published previously (17).

**Assays of biofilm formation.** For both colony formation and pellicle development, we followed a protocol that was described previously (17).

**Analysis of the sugar compositions of *B. subtilis* EPS.** Cells of either the wild type or the *epsA-O* mutant were inoculated into 1 liter of MSgg liquid medium in standing culture and left at 30°C for 5 days. Pellicles were harvested, washed with distilled water, and centrifuged. Cell pellets were resuspended in PBS buffer and were mildly sonicated to separate exopolysaccharides from cells. Cells were pelleted, and the soluble fractions with released exopolysaccharides were then precipitated with 5 volumes of isopropanol overnight at 4°C. The pellets were centrifuged, lyophilized, and resuspended in distilled water. The lyophilized resuspension was dialyzed (molecular weight cutoff [MWCO] = 3,500) against distilled water overnight. Glycosyl composition analysis was performed by combined gas chromatography-mass spectrometry (GC/MS) of the per-*O*-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the samples by acidic methanolysis. Methyl glycosides were first prepared from 500  $\mu$ g of the dry sample by methanolysis in 1 M HCl in methanol at 80°C for 18 to 22 h, followed by re-*N*-acetylation with pyridine and acetic anhydride in methanol (for detection of amino sugars). The samples were then per-*O*-trimethylsilylated by treatment with Tri-Sil at 80°C for 30 min. GC/MS analysis of the TMS methyl glycosides was performed on an HP 5890 gas chromatograph interfaced with a model 5970 mass selective detector (MSD), using a Supelco DB-1 fused-silica capillary column (inside diameter, 30 m by 0.25 mm).

**Reverse transcription-PCR.** 3610 cells were grown in LB medium to exponential phase. Cells were harvested, and total RNA was harvested using the RNeasy minikit (Qiagen). Total RNA was used as a template in reverse transcription reactions carried out using SuperScript II reverse transcriptase (Invitrogen). Subsequently, in the regular PCRs, purified cDNA from the above-described experiments was used as the template, and primers *gal*-RT-F1 and *gal*-RT-R1 were also included. PCR products were size fractionated on an agarose gel.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00184-12/-/DCSupplemental>.

- Figure S1, TIF file, 0.2 MB.
- Figure S2, TIF file, 1 MB.
- Figure S3, TIF file, 0.1 MB.
- Figure S4, TIF file, 1.9 MB.
- Table S1, DOCX file, 0.1 MB.
- Table S2, DOCX file, 0.1 MB.
- Table S3, DOCX file, 0.1 MB.

## ACKNOWLEDGMENTS

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