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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.

IMPORTANTANCE 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.

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 amylloid-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 metabolism 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 (Fig. 1A), thereby regenerating UDP-glucose. In addition, B. subtilis GalE is capable of interconverting UDP-N-acetylglucosamine (GlcNAc) and UDP-N-acetylglucosamine (GlcNAc) (8).

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

$$\text{galE}$$

mutants of 

$$\text{Escherichia coli}$$

and 

$$\text{Salmonella enterica serovar Typhimurium}$$

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 

$$\text{galE}$$

in the 

$$\text{B. subtilis}$$

domesticated strain 168 causes sensitivity to exogenous addition of galactose (13).

Here we show that deletion of the 

$$\text{galE}$$

gene in the less domesticated 

$$\text{B. subtilis}$$

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 

$$\text{galE}$$

mutant. Additionally, we report that synthesis of UDP-galactose is required for exopolysaccharide biosynthesis and that the transcriptions of the 

$$\text{gal}$$

and 

$$\text{eps}$$

genes are interrelated. Finally, we show that 

$$\text{B. subtilis}$$

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 \( \text{galE} \) mutant of \( \text{B. subtilis} \) by accumulation of toxic metabolites.** A \( \text{galE} \) deletion mutant of 

$$\text{B. subtilis}$$

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 

$$\text{B. subtilis}$$

; 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 \( \text{galE} \) mutant displayed a severe growth defect (Fig. 1B). In fact, concentrations of galactose as low as 0.005% impaired the growth of the \( \text{galE} \) mutant (see Fig. S1 in the supplemental material). The \( \text{galE} \) mutant was able to grow for a few hours in the presence of galactose, but the optical density at 600 nm (OD600) 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 \( \text{galE} \) 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 dyes for live/dead cell staining and examined under fluorescence microscopy. Arrows point to cells showing distinct bulge-like structures. (D) Growth of the wild type (3610) and \( \Delta \text{galE} \) mutant (FC300) of 

$$\text{B. subtilis}$$

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 \( \Delta \text{galETK} \) (YC563), \( \Delta \text{galETK amyE::galK} \) (YC810), and \( \Delta \text{galETK amyE::galTK} \) (YC811) mutants in LB medium supplemented or not supplemented with 0.5% galactose (Gal).
onstrating that galactose caused toxicity and cell lysis in a ΔgalE 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 Δ-oxy-galactose on the wild type and the galE mutant (Fig. 1D, circles). We investigated whether the toxic metabolite was produced by the GalK enzyme, and/or GalT enzymes, deleting both genes should block galactose metabolism and should restore viability to a ΔgalKΔgalT triple mutant with-
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_{epsA-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 ΔepsA-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-β-D-thiogalactopyranoside)-inducible hyspank promoter (epsA-O\(_{\text{IPTG-hyspank}}\)-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 preshyspankence 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 ΔgalE 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-
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 galEK 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 (PgalE–lacZ). This construct was then integrated into the amyE locus on the chromosome. We first measured lacZ expression in wild-type cells grown in MSgg and observed that PgalE–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 auto-regulated 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 M59g 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 antirepressor 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, galactan 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 carbon source for B. subtilis in settings where it interacts with plant roots and other soil microorganisms. A previous report found that the cycB-ganPAB 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 β-galactosidase-hydrolyzing galactan and ganB encodes an endo-β-1,4-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-ganPAB). This mutant, together with the wild type, the ΔgalE mutant, and the ΔgalKT 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-ganPAB operon 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-ganPQB 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-ganPQB 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 ΔgalE 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-ganPQB 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 biosynthesis 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.
We propose a working model (Fig. 6A) for the catabolism of galactan by \textit{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 \textit{cycB-ganPQAB} operon is present not only in \textit{B. subtilis} but also in several related bacterial species (Fig. 6B). In two plant-associated \textit{Bacillus} species, \textit{B. licheniformis} and \textit{B. amyloliquefaciens}, these genes are not only conserved and clustered but also grouped with the \textit{gal} genes (Fig. 6B). A similar genetic organization of these genes was also found in a gut-colonizing bacterium, \textit{Lactobacillus acidophilus}. This gene cluster was indeed recently proposed to be involved in the utilization of host-released polymers of galactose by \textit{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, \textit{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). \textit{E. coli} strains were grown in LB medium at 37°C, and strain DH5α 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 \textit{B. subtilis} strains: 10 \(\mu\)g ml\(^{-1}\) tetracycline, 100 \(\mu\)g ml\(^{-1}\) spectinomycin, 10 \(\mu\)g ml\(^{-1}\) kanamycin, 5 \(\mu\)g ml\(^{-1}\) chloramphenicol, and 1 \(\mu\)g ml\(^{-1}\) erythromycin. For \textit{E. coli} strains, ampicillin was added at 100 \(\mu\)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 mutations (36).

The insertional mutations in \textit{galK-galT}, \textit{epsA-O}, and \textit{cycB-ganPQAB} gene clusters were generated by long flanking PCR mutagenesis by using primers \textit{galTK-P1}, \textit{P2}, \textit{P3}, \textit{P4}, \textit{epsP1}, \textit{P2}, \textit{P3}, \textit{P4}, and \textit{ganP1}, \textit{P2}, \textit{P3}, \textit{P4} (see Table S3), respectively. To construct the inducible \textit{galK-galT} fusion (\textit{p hypersac-galTK}), the coding region of \textit{galK-galT} was amplified by PCR using 3610 genomic DNA as the template and the primers \textit{galTK-F1} and \textit{galTK-R1}. The PCR products were cloned into Nhel and HindIII sites of the vector \textit{pDR111} (gift of D. Rudner). The resulting recombinant plasmid (\textit{pYC245}) was first introduced into PY79 by transformation. The
reporter fusion at the amyE locus was then introduced into other 3610 derivatives by SPP1 phage-mediated transduction.

To construct the inducible galK fusion (P_{hspsK}-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 DH5α. The resulting recombinant plasmid contains functional galK only under the control of the hspsK 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 epsP_{hspsK}-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 × 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 Spc^r and resistance to macrolides, lincosamides, and streptogramins (MLS^r) 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.

**β-Galactosidase assays.** A detailed protocol for assays of β-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-OMutant 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 acid methanolation. Methyl glycosides were first prepared from 500 µg of the dry sample by methanolysis in 1 M HCl in methanol at 80°C for 18 to 22 h, followed by 2-deoxy-galactose. Gene

**SUPPLEMENTAL MATERIAL**

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

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