Role of glutamate synthase in biofilm formation by *Bacillus subtilis*

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Running Head: The effect of the ΔgltA mutation on biofilm formation

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ABSTRACT

Bacillus subtilis forms robust biofilms in the presence of large amounts of carbon sources, such as glycerol. However, little is known about the importance of the metabolic systems, or the relationship between metabolic systems and regulatory systems, involved in biofilm formation. Glutamate synthase, encoded by gltAB, is an enzyme that converts 2-ketoglutarate (a TCA cycle intermediate) and glutamine into glutamate, which is a general amino group donor in metabolisms. Here, we show that a ΔgltA mutant exhibited early arrest of biofilm formation in complex medium containing glycerol. This phenotype was not due to glutamate auxotrophy. Consistent with its biofilm formation phenotype, the ΔgltA mutant exhibited an early decrease in expression of the epsA and tapA operons, which are responsible for production of biofilm matrix polymers. This resulted from decreased activity of their regulator, Spo0A, as evidenced by reduced expression of other Spo0A-regulated genes in the ΔgltA mutant.

The ΔgltA mutation prevented biofilm formation only in the presence of large amounts of glycerol. Moreover, limited expression of citrate synthase (but not other TCA enzymes) restored biofilm-forming ability to the ΔgltA mutant. These results indicate that the ΔgltA mutant accumulates an inhibitory intermediate (citrate) in the TCA cycle in the presence of large amounts of glycerol. The ΔgltA mutant formed biofilms when excess iron was added to the medium. Taken together, the data suggest that accumulation of citrate ions by the ΔgltA mutant causes iron shortage due to chelation, which prevents activation of Spo0A and causes defective biofilm formation.

IMPORTANCE

Bacillus subtilis, a model organism for bacterial biofilm formation, forms robust biofilms in a medium-dependent manner. Although the regulatory network that controls biofilm formation has been well-studied, the importance of the underlying metabolic systems remains to be elucidated. The present study demonstrates that a metabolic
disorder in a well-conserved metabolic system causes accumulation of an inhibitory metabolic intermediate that prevents activation of the system that regulates biofilm formation. These findings increase our understanding of the coordination between cellular metabolic status and the regulatory networks governing biofilm formation.

KEYWORDS

biofilm, glutamate synthase, TCA cycle, Spo0A, iron
INTRODUCTION

In the natural environment, most bacteria attach to biotic or abiotic surfaces, forming surface-associated multicellular communities called biofilms (1). Bacterial cells within biofilms adhere to each other and to surfaces via a matrix of extracellular polymeric substances called a biofilm matrix, which usually consists of exopolysaccharides, proteins, nucleic acids, and/or lipids (2, 3). The biofilm matrix not only supports biofilms structurally but also provides a physical and chemical barrier to member cells, thereby protecting them against environmental stresses, antibiotics, and attack by host immune systems (4, 5). In addition to producing extracellular polymeric substances, biofilm-forming cells express sets of genes different from those of planktonic cells (6, 7, 8, 9); these include metabolic genes, some of which play unique roles in biofilms (10, 11, 12). However, the importance of the metabolic systems that facilitate biofilm formation remain unclear.

Undomesticated strains of the Gram-positive bacterium Bacillus subtilis form robust biofilms; for example, heavily wrinkled pellicles on the surface of liquid medium under static culture conditions and morphologically complex colonies on solid medium (13). B. subtilis biofilms are maintained by a biofilm matrix that consists mainly of exopolysaccharides, TasA amyloid fibers, and BslA hydrophobins, which are produced by proteins encoded by the 15 gene operon, epsABCDEFGHIJKLMNO (hereafter referred to as the epsA operon), the tapA-sipW-tasA operon (hereafter referred to as the tapA operon), and bslA, respectively (13, 14, 15, 16, 17, 18). These genes are directly or indirectly repressed by the transcriptional repressors AbrB and SinR (19, 20, 21, 22). Phosphorylation of the response regulator Spo0A is required to initiate biofilm formation; that is, phosphorylated Spo0A (Spo0A–P) represses abrB transcription and induces expression of proteins SinI and SlrR, which antagonize SinR (23, 24, 25, 26, 27, 28). Moreover, the two-component system DegS-DegU and the transcriptional activator RemA are required to induce transcription of these biofilm matrix genes (17, 29, 30, 31).
Thus, the regulatory network that controls *B. subtilis* biofilm formation is complex and involves multiple transcriptional regulators.

In addition to biofilm formation, Spo0A governs multiple cellular processes that occur during different growth periods (32). These processes include cannibalism and sporulation (32 and references therein). Differential expression of Spo0A-regulated genes appears to occur because Spo0A-regulated genes have promoters with different affinities for Spo0A–P (33). Specifically, low levels of Spo0A–P repress *abrB* and induce *sinI* (to stimulate biofilm formation), as well as the *skfA* and *sdpA* operons (to stimulate cannibalism toxin production), whereas high levels of Spo0A–P induce expression of genes that drive sporulation, including the *spoIIA* and *spoIIG* operons that encode sporulation-specific sigma factors (33 and references therein). Four sensor histidine kinases (KinA, KinB, KinC, and KinD) phosphorylate Spo0A via a multicomponent phosphotransfer system known as the phosphorelay, in which phosphoryl groups from auto-phosphorylating sensor kinases are transferred successively to relay proteins Spo0F and Spo0B, and finally to Spo0A (34, 35, 36, 37). These sensor kinases appear to play distinct roles. Specifically, KinC and KinD are responsible for production of low levels of Spo0A–P (37, 38), which are enough to induce biofilm formation and cannibalism toxin production (33, 39, 40, 41), whereas KinA and KinB are responsible for production of high levels of Spo0A–P, which are required to induce sporulation (36). However, the functional differences between these kinases are not strict, and they vary according to nutrient conditions (38, 42). In addition, cellular levels of Spo0A–P are controlled by multiple regulators, including phosphatases and the Fe-S cluster-containing RicAFT protein complex (43, 44, 45, 46, 47, 48, 49).

Biofilm formation is a consumptive process, as it is accompanied by production and secretion of large amounts of extracellular polymeric substances. Biofilm formation by *B. subtilis* is a medium-dependent process. Media supporting *B. subtilis* biofilm formation contain 0.5% to 1% carbon sources that can be consumed...
through glycolysis, and these sugars are necessary for robust biofilm formation (13, 50, 51, 52). In addition to sugars, glutamate or glutamine is essential to support biofilm formation in synthetic medium, even though *B. subtilis* strains have no auxotrophy for glutamate and glutamine (53). These requirements suggest that metabolic systems play critical roles in biofilm formation. However, despite our current understanding of the regulatory networks governing *B. subtilis* biofilm formation, little is known about the importance of the metabolic systems, or the relationship between regulatory networks and these metabolic systems. This is, in part, because defects in metabolic systems often cause a growth defect, particularly in synthetic medium in which biofilm formation by *B. subtilis* has been analyzed frequently. During biofilm formation, carbon sources are consumed by glycolysis (Fig. 1). Since genes involved in the tricarboxylic acid (TCA) cycle and nitrogen metabolism are induced during biofilm formation (9), some of its metabolites flow into the TCA cycle and then nitrogen metabolism. Glutamate synthase (also known as glutamine oxoglutarate aminotransferase, GOGAT), a two-subunit enzyme encoded by gltAB, connects the TCA cycle with nitrogen metabolism by converting each molecule of 2-oxoglutarate and glutamine into two molecules of glutamate (54). Glutamine required for this reaction is synthesized from glutamate and ammonium by glutamine synthetase (GS). The reactions of GS and GOGAT form one cycle, which serves as a main route of ammonium assimilation, and its product, glutamate, serves as a general amino group donor during biosynthesis of nitrogen-containing compounds such as amino acids, nucleotides, and polyamines (54, 55, 56). Glutamate, which is normally the cell’s most abundant amino acid, also plays important roles in cellular physiology (56). Since in *B. subtilis*, glutamate dehydrogenases (GDH) are devoted to glutamate degradation and are not involved in its biosynthesis, glutamate synthase (GOGAT) is a main contributor to ammonium assimilation and glutamate biosynthesis (54, 56). In addition to the de novo biosynthetic pathway, glutamate is synthesized by amino acid degradation under conditions of 6
nutrient depletion (57).

A previous report shows that a transposon insertion in gltA impairs biofilm formation in nutrient-rich complex medium (58). The glutamate auxotrophy of the ΔgltA mutant in that study did not appear to be the cause of the biofilm formation defect, as addition of glutamate did not restore biofilm formation to the ΔgltA mutant (58). We were interested in these ΔgltA mutant phenotypes because these observations seem to suggest that an unknown mechanism involved in integrating cellular metabolic status into the regulatory network for biofilm formation might underlie the ΔgltA mutant phenotype. Moreover, since the ΔgltA mutation disturbs both carbon and nitrogen metabolism, its effect on biofilm formation is also intriguing. However, since analysis of the ΔgltA mutant in that study was performed under conditions unsuitable for biofilm formation using a domesticated strain (58), which forms biofilms poorly (59), the ΔgltA mutant phenotype must be verified under appropriate conditions using undomesticated strains that form robust biofilms. Here, we investigated the effect of the ΔgltA mutation on biofilm formation by an undomesticated strain. Based on our findings, we propose how the ΔgltA mutation causes a defect in biofilm formation.
RESULTS

The ΔgltA mutation prevents biofilm formation. To test its effect on biofilm formation on an undomesticated strain background, we introduced the ΔgltA mutation into strain NCIB3610 (hereafter referred to as the wild-type strain) (13), which is used widely for biofilm formation studies. We tested the ability of the ΔgltA mutant to form biofilms in nutrient-rich complex medium 2×SGG (51). When grown statically in liquid 2×SGG, the wild-type strain formed thin pellicles on the surface of the medium 24 h after inoculation, and thick, heavily wrinkled pellicles at 48 h (Fig. 2). The ΔgltA mutant formed thin pellicles at 24 h (like the wild-type strain), but failed to develop thick pellicles; the ΔgltA mutant only formed thin, flat pellicles at 48 h. On solid 2×SGG medium, the wild-type strain formed structurally complex colonies at 48 h, which comprised a central area fully covered with tiny wrinkle structures and a prominent peripheral area surrounding the central area (Fig. 2). The central area of colonies is the footprint of where the founding cells are deposited and therefore contains older than the peripheral expanding zone. Its morphological features resemble that of pellicle biofilms, the heavily wrinkled structures in the central area appeared to represent mature biofilms.

Unlike the wild-type strain, the ΔgltA mutant formed colonies with no wrinkles in the central areas at 48 h (Fig. 2). The thin and flat features within the central area of the ΔgltA mutant colonies were consistent with the thin, flat pellicles of the ΔgltA mutant. The biofilm formation-defective phenotype of the ΔgltA mutant was also observed in MSgg synthetic medium, which contains glutamate as a nitrogen source (13). On solid MSgg medium, the wild-type strain formed whitish colonies covered with wrinkles, whereas the ΔgltA mutant formed brownish colonies with tiny wrinkles only in the central areas (Fig. S1).

Next, we confirmed that the biofilm formation-defective phenotypes of the ΔgltA mutant were not due to a growth defect caused by glutamate auxotrophy, although 2×SGG medium contains plenty of glutamate. According to the Difco & BBL manual...
(https://www.bd.com/resource.aspx?IDX=9572), the medium contains about 1.2% glutamate. Wild-type and ΔgltA mutant strains were grown with vigorous shaking in liquid 2×SGG, and the optical density (OD$_{600}$) and pH of the cultures were compared over time. As shown in Figure 3, there was no difference in growth between the wild-type and ΔgltA mutant strains, at least from exponential to early stationary phase. However, the pH in these cultures was different. Specifically, the pH of the wild-type strain culture fell as it grew, reaching 5.8 at the end of the exponential phase (Fig. 3A). This decrease in pH is likely to be due to production of acidic glycolytic products such as acetate, lactate, and pyruvate (Fig. 1). The pH then increased slightly and transiently, but decreased gradually again (to pH 5.5); subsequently, it increased to 7.0. This increase is probably caused by production of ammonium due to consumption of amino acids in the medium. The ΔgltA mutant exhibited a decrease in pH similar to that of the wild-type strain during exponential phase; however, the pH began to increase immediately after the end of the exponential phase. This earlier increase in pH suggests that the ΔgltA mutation hastens the onset of amino acid consumption to compensate for glutamate shortage. Similar pH changes were observed in standing cultures, but the changes were slower and more moderate than those in shaking cultures (Fig. S2A). In standing culture, pH in wild type and ΔgltA mutant cultures fell to 6.1 and then gradually increased. During the increase, ΔgltA mutant culture exhibited higher pH than wild-type strain culture. However, the increase in pH of the ΔgltA mutant culture almost stopped at 38 h probably because the ΔgltA mutant stopped biofilm formation. Thus, the ΔgltA mutation alters the activity of nitrogen metabolism pathways but does not affect growth in 2×SGG complex medium. These results indicate that the ΔgltA mutation causes early arrest of biofilm formation in 2×SGG medium, although this is unlikely to be due to a defect in growth.

**Decreased expression of the eps and tapA operons is responsible for the biofilm formation defect of the ΔgltA mutant.** B. subtilis biofilms are supported by
exopolysaccharides and TasA amyloid fibers, which are synthesized by the products of the epsA and tapA operons, respectively (13, 14, 15, 16). Therefore, we hypothesized that the ΔgltA mutation might affect transcription of these operons. To analyze the promoter activity of these operons, we used the green fluorescent protein (GFP) as a transcriptional reporter and introduced the P_{epsA-gfp} or P_{tapA-gfp} reporter into the chromosomes of the wild-type and ΔgltA mutant strains. The wild-type and ΔgltA mutant strains harboring the P_{epsA-gfp} or P_{tapA-gfp} reporters were grown statically in liquid medium, and expression of the reporters in individual cells was analyzed by flow cytometry. The wild-type strain lacking gfp reporters was used as a negative control. The wild-type and ΔgltA mutant strain harboring P_{epsA-gfp} exhibited very similar, homogeneous expression of GFP at 24 h; however, this then became different (Fig. 4, upper row). Specifically, wild-type cells continued to express GFP even at 72 h, whereas ΔgltA mutant cells showed reduced GFP expression at 48 h. The P_{tapA-gfp} reporter was expressed in a manner similar to the P_{epsA-gfp} reporter; ΔgltA mutant cells exhibited lower expression of GFP from P_{tapA-gfp} than wild-type cells at 48 and 72 h (Fig. 4, lower row). Thus, the ΔgltA mutation causes an early decrease in transcription of the eps and tapA operons during biofilm formation, which is consistent with its biofilm formation phenotype.

Next, we examined whether increased transcription of the eps and tapA operons restored biofilm formation to the ΔgltA mutant. Transcription of these operons is negatively and independently regulated by two repressors, AbrB and SinR (19, 20, 21). Introducing the ΔabrB or ΔsinR mutation restored biofilm formation to the ΔgltA mutant (Fig. 5A). Moreover, we introduced the isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible, LacI-repressible promoter spac-hy upstream of epsA or tapA; then, the P_{spac-hy-eps} operon and P_{spac-hy-tapA} operon constructs were transferred to the ΔgltA mutant. The P_{spac-hy-eps} operon and P_{spac-hy-tapA} operon strains formed biofilms only in the presence of IPTG, although the pellicles of the P_{spac-hy-tapA} operon strain
were somewhat rough (Fig. 5B). Induction of either the *eps* operon or the *tapA* operon from the *spac-hy* promoter with IPTG restored biofilm formation to the Δ*gltA* mutant. These results indicate that decreased transcription of the *eps* and *tapA* operons is responsible for the biofilm formation defect of the Δ*gltA* mutant.

The Δ*gltA* mutation reduces transcription of Spo0A-regulated genes. Transcription of the *eps* and *tapA* operons requires phosphorylated Spo0A, which represses *abrB* transcription and induces antagonistic proteins specific for SinR (23, 24, 25, 26, 27, 28). Therefore, we asked whether the Δ*gltA* mutation affects transcription of Spo0A-regulated genes *skfA* and *spoIIA* (33). Flow cytometry showed that transcription of P_{skfA-*gfp} and P_{spoIIA-*gfp} in the wild-type and Δ*gltA* mutant strains was induced at 48 h, but expression of both was clearly lower in the Δ*gltA* mutant (Fig. 6A). Since the *spoIIA* operon encodes the sporulation-specific sigma factor σ^F^, decreased transcription of the *spoIIA* operon should lead to a decrease in sporulation. To test this, we measured sporulation frequency in pellicles at 72 h. As shown in Figure 6B, 21.7% of wild-type cells formed spores in pellicles, whereas only 3.4% of the Δ*gltA* mutant cells did. Thus, consistent with the small decrease in *spoIIA* transcription, the Δ*gltA* mutant produced 6.3-fold less spores than the wild-type strain. These results indicate that the Δ*gltA* mutation reduces Spo0A~P levels, probably leading to decreased transcription of the *epsA* and *tapA* operons.

The Δ*gltA* mutation inhibits the phosphorelay. Cellular Spo0A~P levels are positively controlled by four sensor kinases in the phosphorelay (34, 35, 36, 37, 38). Therefore, we examined the possibility that the Δ*gltA* mutation might inhibit the activity of a certain sensor kinase in the phosphorelay. As previously observed (39), the Δ*kinC* and Δ*kinD* mutations prevented biofilm formation in 2×SGG (Fig. S3). Likewise, the Δ*kinC* and Δ*kinD* mutations exacerbated the biofilm formation-defective phenotype of the Δ*gltA* mutant. These results indicate that, like the wild-type strain, formation of thin biofilms by the Δ*gltA* mutant depends on KinC and KinD. To examine KinC and KinD
activity separately, we placed *kinC* or *kinD* under the control of the *spac-hy* promoter and deleted the other sensor kinase genes for the phosphorelay. The resultant strains, P<sub>spac-hy-kinC ΔkinABD</sub> and P<sub>spac-hy-kinD ΔkinABC</sub>, did not form pellicles in the absence of IPTG, but they did form pellicles (like the wild-type strain) in the presence of 30 µM IPTG, and thicker pellicles in the presence of ≥100 µM IPTG (Fig. 7A). Thus, the P<sub>spac-hy-kinC ΔkinABD</sub> and P<sub>spac-hy-kinD ΔkinABC</sub> strains formed biofilms in a KinC- or KinD-dependent manner, respectively. To examine the effect of the Δ*gltA* mutation on KinC or KinD-dependent biofilm formation, we introduced the Δ*gltA* mutation into these strains. If the Δ*gltA* mutation specifically inhibits either KinC or KinD, the Δ*gltA* mutation might prevent biofilm formation in one of the strains. However, the P<sub>spac-hy-kinC ΔkinABD ΔgltA</sub> and P<sub>spac-hy-kinD ΔkinABC ΔgltA</sub> strains both formed only thin pellicles (like the Δ*gltA* mutant) in the presence of 30 µM IPTG. Thus, the Δ*gltA* mutation did not inhibit KinC or KinD specifically. However, in the presence of ≥100 µM IPTG, the P<sub>spac-hy-kinC ΔkinABD ΔgltA</sub> and P<sub>spac-hy-kinD ΔkinABC ΔgltA</sub> strains formed thick pellicles, as did P<sub>spac-hy-kinC ΔkinABD</sub> and P<sub>spac-hy-kinD ΔkinABC</sub>. Thus, overexpression of KinC or KinD fully restores biofilm formation to the Δ*gltA* mutation. These results indicate that the Δ*gltA* mutation prevents biofilm formation by reducing Spo0A~P levels, but that inhibition does not occur via activity of KinC or KinD.

Cellular Spo0A~P levels are also negatively controlled by multiple phosphatases (43, 44, 45). Among these, *sda* and *spo0E* are regulated by LexA and the stress-responsive alternative sigma factor σ<sup>B</sup>, which respond to DNA damage and environmental and metabolic stresses, respectively (45, 61, 62). We hypothesized that the Δ*gltA* mutation might induce some stress responses, leading to decreased expression of Spo0A~P through the activity of Sda or Spo0E. To test this, we measured transcription of *sda* and *spo0E*. However, P<sub>*sda-gfp*</sub> and P<sub>*spo0E-gfp*</sub> were expressed similarly in the wild-type and Δ*gltA* mutant strains (Fig. 7B). Thus, the Δ*gltA* mutation did not induce transcription of these phosphatase genes. Taken together, the results...
indicate that the ΔgltA mutation negatively affects a certain step in the phosphorelay, but that this step does not relate to sensor kinases or phosphatases.

The defect in biofilm formation due to the ΔgltA mutation is dependent on large amounts of glycerol. We sought to determine whether the metabolic change caused by the ΔgltA mutation led to decreased Spo0A-P and, thereby, to a defect in biofilm formation. *B. subtilis* forms robust biofilms in 2×SGG medium in a manner dependent on large amounts of glucose and glycerol. These carbon sources are assimilated through glycolysis, and some of the metabolites flow into the TCA cycle and nitrogen metabolism (Fig. 1). Since glutamate synthase, encoded by *gltAB*, serves as a conduit from the TCA cycle to nitrogen metabolism, disruption of this conduit by the ΔgltA mutation could cause excess flow or accumulation of metabolic intermediates of carbon metabolism upstream of glutamate synthase as well as an inflow shortage to nitrogen metabolism downstream of glutamate synthase. The observation that 2×SGG medium contains plenty of glutamate and can support growth of the ΔgltA mutant suggested that an abnormal flow in carbon metabolism, rather than an inflow shortage in nitrogen metabolism, leads to the biofilm formation defect of the ΔgltA mutant.

To test this hypothesis, we examined the effect of the concentration of carbon sources on biofilm formation by the wild-type and ΔgltA mutant strains. 2×SGG medium contains 0.1% glucose and 1% glycerol as primary carbon sources. To simplify the analysis, we removed these carbon sources and added various concentrations of glycerol instead. When grown statically in liquid medium, the wild-type strain formed only very thin pellicles in the absence of glycerol; however, it formed thicker wrinkled pellicles as the concentration of glycerol increased (Fig. 8). On solid medium in the absence of glycerol, the wild-type strain formed colonies with only a few wrinkles in the central areas, but formed colonies with more and larger wrinkles as the concentration of glycerol increased. The ΔgltA mutant formed pellicles and colonies similar to those of the wild-type strain in the absence or presence of 0.1% glycerol. However, addition of
higher concentrations of glycerol inhibited rather than promoted biofilm formation by the ΔgltA mutant. The negative effect of high concentrations of glycerol was more pronounced with respect to colony biofilm formation; in the presence of 0.5% and 1% glycerol, the ΔgltA mutant formed colonies with no wrinkles in the central areas. Thus, the ΔgltA mutant exhibited a defect in biofilm formation that was dependent on high concentrations of glycerol. These results support our hypothesis that the ΔgltA mutation causes abnormal carbon metabolism, leading to a biofilm formation defect. We also tested the effect of glucose on biofilm formation. However, the high concentration of glucose did not stimulate biofilm formation even in the wild-type strain (Fig. 8), probably because biofilm formation is subject to catabolite repression (63).

The ΔgltA mutant causes a defect in biofilm formation due to the accumulation of citrate. In the presence of large amounts of glycerol, large amounts of glycolytic metabolites are expected to flow into the TCA cycle; some of these metabolites are pumped from the TCA cycle to nitrogen metabolism by glutamate synthase (Fig. 1). Therefore, we hypothesized that the ΔgltA mutation might generate excessive accumulation of TCA cycle intermediates, leading to a biofilm formation defect. If this was the case, disrupting TCA cycle genes could restore biofilm formation to the ΔgltA mutant. The first and second enzymes in the TCA cycle are citrate synthase (CitZ and CitA) and aconitase (CitB) (64). B. subtilis possesses two citrate synthases, CitZ and CitA, but CitZ is responsible for the bulk of citrate synthase activity (63). To test our hypothesis, we deleted citZ and citB, and tested the ability of the mutants to form biofilms. We found that the ΔcitZ and ΔcitB mutants formed only very thin pellicles in standing culture (Fig. S4), which made it difficult to test whether these mutations could improve biofilm formation by the ΔgltA mutant. To overcome this problem, we replaced the native promoters of TCA enzyme genes with the spac-hy promoter and examined whether the limited induction of TCA enzyme genes from the spac-hy promoter in the presence of low concentrations of IPTG could restore biofilm formation.
formation to the ΔgltA mutant. The Pspac-hy-citZ strain formed very thin pellicles in the absence of IPTG, but formed thicker wrinkled pellicles as IPTG concentrations increased (Fig. 9). In the presence of 16 µM IPTG, the Pspac-hy-citZ strain formed pellicles comparable with those of the wild-type strain. The Pspac-citZ ΔgltA strain formed pellicles similar to the Pspac-citZ strain in the presence of 0 to 4 µM IPTG, and formed wrinkled pellicles in the presence of 2 and 4 µM IPTG. However, its pellicle formation was clearly inhibited in the presence of ≥8 µM IPTG; the Pspac-citZ ΔgltA strain formed very thin pellicles (like the ΔgltA mutant) in the presence of 16 µM IPTG. Thus, limited expression of citZ restored biofilm formation to the ΔgltA mutant. We also tested the effect of limited expression of other TCA cycle genes, citB, citC, mdh, and citG (see Fig. 1), on biofilm formation by the ΔgltA mutant. However, limited induction of these genes from the spac-hy promoter did not restore biofilm formation to the ΔgltA mutant; specifically, on the ΔgltA mutant background, the Pspac-hy-citB, Pspac-hy-citC, Pspac-hy-mdh, and Pspac-hy-citG strains did not form wrinkled pellicles at any of the IPTG concentrations tested. These results indicate that limited induction of citZ suppresses the ΔgltA mutation not by reducing the overall activity of the TCA cycle but by reducing CitZ activity, i.e., by reducing citrate production.

**Addition of excess iron suppresses the ΔgltA mutant phenotype.** The results obtained thus far suggest that ΔgltA mutant cells probably accumulate citrate, which prevents biofilm formation. Since accumulated citrate can chelate cellular divalent metal ions (65), we examined the possibility that the biofilm formation defect of the ΔgltA mutant was due to chelation of metal ions important for biofilm formation by citrate ions. Since iron and manganese stimulate biofilm formation (52, 67, 68, 69), we examined whether the addition of excess iron or manganese might improve biofilm formation by the ΔgltA mutant. Wild-type and ΔgltA mutant strains were grown in medium containing a 5-fold, 25-fold, or 125-fold excess of FeSO₄ or MnCl₂ relative to that in 2×SGG medium. In the presence of a 25-fold or 125-fold excess of FeSO₄, the
ΔgltA mutant formed wrinkled pellicles (Fig. 10A). As described above, an increase in pH of the ΔgltA mutant culture stopped when the ΔgltA mutant arrested biofilm formation. In the presence of 125-fold excess of FeSO₄, the pH of the ΔgltA mutant culture continued to increase as observed for that of the wild-type strain culture (Fig. S2B). Although we could not test the effect of a 25-fold or 125-fold excess of MnCl₂ due to inhibitory effects on biofilm formation, a 5-fold excess of MnCl₂ did not improve biofilm formation by the ΔgltA mutant. These results are supportive of the conclusion that in the ΔgltA mutant, accumulated citrate causes an iron shortage, leading to a biofilm formation defect.

To test the requirement of iron for biofilm formation, the wild-type strain was grown in 2×SGG medium without added FeSO₄. Note, because the nutrient broth in 2×SGG contains a trace of iron, no added FeSO₄ does not equate to zero iron. Not adding FeSO₄ had no significant effect on pellicle formation (Fig. S6). However, the wild-type strain formed colonies with no wrinkles in the central areas on solid medium containing 1/10 or no additional FeSO₄, as observed for ΔgltA mutant colonies on normal 2×SGG (Fig. 10B). This result suggests that shortage of iron can prevent biofilm formation.
DISCUSSION

Glutamate synthase is a key enzyme that connects carbon and nitrogen metabolism. Here, we demonstrate that the ΔgltA mutation caused early arrest of biofilm formation in nutrient-rich 2×SGG medium. Unexpectedly, this defect was not caused by disruption of some important metabolic systems that underlie biofilm formation; indeed, artificial expression of either the epsA or tapA operon restored biofilm formation to the ΔgltA mutant. Instead, the ΔgltA mutation interferes with the regulatory system for biofilm formation. Our results indicate that the ΔgltA mutant probably accumulates an inhibitory metabolic intermediate, citrate. The accumulated citrate reduces cellular iron levels, which prevent phosphorylation of Spo0A~P, leading to reduced expression of the epsA and tapA operons.

*B. subtilis* biofilm formation depends on large amounts of carbon sources such as glycerol. We propose the following scenario for carbon metabolism and the function of glutamate synthase during biofilm formation. Since acetoin synthesis is required for biofilm formation under our culture conditions (51), *B. subtilis* cells grow with overflow metabolism during an early phase of biofilm formation (see Fig. 1). This is probably the reason why the ΔgltA mutant did not exhibit a biofilm formation defect for the first 24 h. Subsequently, glycolytic metabolites pyruvate and acetyl CoA flow into the TCA cycle, and citrate synthase converts these compounds into citrate. Citrate inhibits the transcriptional repressor CcpC, thereby derepressing its target genes (the *citZ* operon and *citB*) (70). The gene products (citrate synthase, aconitase, and isocitrate dehydrogenase) together increase production of 2-ketoglutarate, an inducer of the transcriptional regulator GltC, which activates *gltAB* transcription (71). Previous studies demonstrate that GltC activity is inhibited directly by glutamate, even in the presence of 2-ketoglutarate (71). Moreover, in the presence of glutamate, GltC is sequestered by moonlighting proteins glutamate dehydrogenases RocG and GudB (72, 73). These mechanisms are predicted to keep GltC inactive and to keep *gltAB* transcription at low.
levels in 2×SGG medium, which contains plenty of glutamate and other amino acids. However, given that the ΔgltA mutant probably accumulates citrate, a substantial amount of 2-ketoglutarate in the wild-type strain is converted to glutamate by glutamate synthase. Thus, these existing regulatory mechanisms for controlling gltAB transcription do not seem to fully explain the phenomena observed herein. These regulatory models are constructed based on experiments in which biofilm formation-deficient, domesticated strains were grown in synthetic medium under non-biofilm formation conditions (71, 72, 73). Recently, Pisithkul et al. (9) reported comprehensive analysis of the transcription of metabolic genes and the dynamics of metabolic systems during biofilm formation by an undomesticated strain. Although their analysis was carried out using synthetic medium supplemented with glutamate, transcription of gltAB was induced during biofilm formation. These observations suggest that gltAB is specifically induced during biofilm formation through unknown mechanisms and plays important roles in biofilms. One possible function for active glutamate synthase is to provide materials that allow biosynthesis of nitrogen-containing compounds to be well-coordinated with robust growth of biofilm-forming cells. However, since the ΔgltA mutant expressing the epsA or tapA operon was able to form robust biofilms comparable with those of the wild-type strain, glutamate synthase activity seems to be excessive. We therefore propose that B. subtilis biofilm-forming cells employ a mechanism that sends excessive metabolites from the TCA cycle to nitrogen metabolism to generate storage compounds. This mechanism may prevent metabolites from accumulating in carbon metabolic pathways and stimulate carbon assimilation. B. subtilis produces poly-γ-glutamate in biofilms (50). The cellular levels of some amino acids and nucleotides, which are produced from glutamate, increase during biofilm formation (9). These can be considered as storage compounds. Often, bacteria in biofilms compete with other species for space and nutrients. The ability to assimilate favorable nutrients more ravenously than other bacteria and then produce some storage compounds may
confer *B. subtilis* with a competitive advantage within biofilms. Future studies should explore the possibility that three enzymes of the TCA cycle branch (citrate synthase, aconitase, and isocitrate dehydrogenase) and glutamate synthase play important roles in cell competition and survival within biofilms.

A previous study reports that the phosphorelay is inhibited by accumulated citrate (65). A null mutant of *citB* accumulated citrate and exhibited a severe defect in sporulation. However, the Δ*gltA* mutation had only a minor effect on sporulation compared with the Δ*citB* mutation. This suggests that citrate levels in the Δ*gltA* mutant may be lower than those in the Δ*citB* mutant, and that biofilm formation may be much more sensitive to cellular citrate than sporulation. Aconitase encoded by *citB* is a bifunctional protein that also acts as an RNA-binding protein (74). Under low iron conditions, aconitase binds to the 5’ leader region of *citZ* mRNA and restricts the synthesis of citrate synthase. This mechanism likely suppresses accumulation of citrate to some extent in the Δ*gltA* mutant. Differences in sensitivity to citrate between sporulation and biofilm formation are probably due to the fact that these two processes have different requirements for Spo0A–P. Low levels of Spo0A are enough to induce biofilm formation, whereas high levels of Spo0A–P are required to induce sporulation (33). Therefore, the activity of the phosphorelay producing Spo0A–P is much lower during biofilm formation, which can make biofilm formation more sensitive to inhibitors of the phosphorelay. Citrate ions can chelate divalent cations. Since addition of excess iron restored biofilm formation to the Δ*gltA* mutant, accumulated citrate probably causes an iron shortage in the Δ*gltA* mutant, which inhibits the phosphorelay. Since the Δ*gltA* mutant expressing the *epsA* or *tapA* operon was able to form robust biofilms, this iron shortage is not severe enough to inhibit growth. These observations suggest that some components of the phosphorelay are very sensitive to low levels of cellular iron. Possible targets for iron shortage are iron-containing proteins. The RicAFT protein complex carrying two [4Fe-4S]2+ clusters regulates biofilm formation and
sporulation by accelerating the phosphorylation of Spo0A (46, 47, 48, 49). The respiratory chain is required for biofilm formation (12). Iron shortage may disrupt these functions during biofilm formation.
MATERIALS AND METHODS

Bacterial strains and media. The *B. subtilis* strain NCIB3610 and its derivatives used in this study are listed in Table 1. Construction of *B. subtilis* mutants is described in supplemental material. Primers used for the strain construction are listed in Table S1. Strains NRS2242 and NRS2394 were kind gifts from Prof. Nicola Stanley-Wall. *B. subtilis* strains were maintained in LB (LB Lennox; BD Difco, Franklin Lakes, NJ, USA). Biofilm-forming ability was tested in 2×SGG medium, which comprises 1.6% (w/v) nutrient broth (BD Difco), 0.2% (w/v) KCl, 0.1% (w/v) glucose, 1% (w/v) glycerol, 2 mM MgSO$_4$, 1 mM Ca(NO$_3$)$_2$, 0.1 mM MnCl$_2$, and 1 µM FeSO$_4$ (51).

*Escherichia coli* strains JM105 and HB101 were used for construction and maintenance of plasmids.

Biofilm formation. For pellicle biofilms, overnight cultures at 28°C in LB were diluted 100-fold, and 10 µl of each dilution were inoculated into 10 ml 2×SGG in the wells of 6-well plates. For colony biofilms, 2 µl of the same dilutions were spotted onto 2×SGG 1.5% (w/v) agar or MSgg 1.5% (w/v) agar plates. These plates were incubated at 30°C. Biofilm formation tests were carried out at least three times, and representative examples are shown in the figures.

Growth curve analysis. Wild-type and ΔgltA mutant strains grown at 30°C on LB plates overnight were inoculated into 5 ml of 2×SGG and grown at 37°C to mid-exponential phase with vigorous shaking. These cultures were then added to 50 ml of pre-warmed 2×SGG in a 500 ml baffled flask to yield an OD$_{600}$ of 0.005. These cultures were shaken at 37°C, and the OD$_{600}$ and pH were measured over time. The experiments were performed three times, and representative results are shown in the figures.

Flow cytometry analysis. Pellicle biofilms grown in 5 ml of 2×SGG in 12-well plates were disrupted by pipetting, and 1 ml of the suspension was transferred to a 1.5 ml tube. Cells were pelleted by centrifugation at 17,400 × g for 2 min and then
suspended in 1 ml PBS buffer. Cells were further dispersed by repetitive pipetting and then fixed for 7 min in 4% (w/v) paraformaldehyde (75). Prior to flow cytometry analysis, the cells were subjected to mild sonication to obtain single cells (75). Single cell fluorescence was measured and analyzed using an Accuri C6 Plus flow cytometer, and data were analyzed using BD Accuri C6 Plus software (BD Biosciences, Franklin Lakes, NJ, USA). The number of recorded events was 50,000. Experiments were performed at least twice. Representative examples are shown in the figures.

**Sporulation in pellicle biofilms.** Pellicles grown for 72 h in 5 ml of 2×SGG in 12-well plates were disrupted by repetitive pipetting. Next, 1 ml of the suspension was transferred to a 1.5 ml tube. Cells were then subjected to mild sonication to obtain single cells (75). The cell suspensions were used to count the number of colony-forming units before and after heat treatment (80°C, 10 min). Sporulation frequency (%) (number of heat-resistant spores/total number of cells × 100) was calculated and expressed as the mean value from three independent experiments.

**ACKNOWLEDGMENTS**

We thank Prof. Nicola Stanley-Wall for kindly providing strains NRS2242 and NRS2394, and Prof. Hisaji Maki and Associate Prof. Masahiro Akiyama for helpful advice and support. K. K. was supported by JSPS KAKENHI [Grant Number: JP17K07721].
REFERENCES


22. Verhamme DT, Murray EJ, Stanley-Wall NR. 2009. DegU and Spo0A jointly control transcription of two loci required for complex colony development by...


the gene for a protein kinase which phosphorylates the sporulation-regulatory proteins Spo0A and Spo0F of Bacillus subtilis. J. Bacteriol. 171:6187-6196.


Table 1. *B. subtilis* strains used in this study

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<td>This study</td>
</tr>
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<td>ΔgltA::neo</td>
<td>This study</td>
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<td>N694 → NRS2242</td>
</tr>
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<td>N694 → NRS2394</td>
</tr>
<tr>
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<td>ΔabrB::kan</td>
<td>W1050 (19) → NCIB3610</td>
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<td>ΔabrB::kan ΔgltA::cat</td>
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This study

N557 \( \Delta \text{kinD} \) This study

N1830 \( \Delta \text{kinD} \Delta \text{gltA::cat} \) N694 \( \rightarrow \) N557

N541 \( \Delta \text{kinA} \Delta \text{kinB} \) This study

N1827 \( \Delta \text{kinA} \Delta \text{kinB} \Delta \text{gltA::cat} \) N694 \( \rightarrow \) N541

N733 \( \text{kinC::pMutinT3-hy (P}_{spac-hy}\text{-kinC operon, erm)} \) This study

\( \Delta \text{kinA} \Delta \text{kinB} \Delta \text{kinD} \)

N1743 \( \text{kinC::pMutinT3-hy (P}_{spac-hy}\text{-kinC operon, erm)} \) N1802 \( \rightarrow \) N733

N635 \( \text{kinD::pMutinT3-hy (P}_{spac-hy}\text{-kinD operon, erm)} \) This study

\( \Delta \text{kinA} \Delta \text{kinB} \Delta \text{kinC} \)

N1744 \( \text{kinD::pMutinT3-hy (P}_{spac-hy}\text{-kinD operon, erm)} \) N1802 \( \rightarrow \) N635

N1878 \( \Delta \text{amyE::P}_{sda}\text{-gfp (tet)} \) This study

N1879 \( \Delta \text{amyE::P}_{sda}\text{-gfp (tet)} \Delta \text{gltA::cat} \) N1878 \( \rightarrow \) N694

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N795 \( \Delta \text{citZ::cat} \) This study

N794 \( \Delta \text{citB::cat} \) This study

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N1848 \( \text{citZ::pMutinT3-hy (P}_{spac-hy}\text{-citZ operon, erm)} \) N1840 \( \rightarrow \) N694

\( \Delta \text{gltA::cat} \)

N1837 \( \text{citB::pMutinT3-hy (P}_{spac-hy}\text{-citB,erm)} \) This study

N1845 \( \text{citB::pMutinT3-hy (P}_{spac-hy}\text{-citB,erm)} \Delta \text{gltA::cat} \) N1837 \( \rightarrow \) N694

N1838 \( \text{citC::pMutinT3-hy (P}_{spac-hy}\text{-icd-mdh,erm)} \) This study

N1846 \( \text{citC::pMutinT3-hy (P}_{spac-hy}\text{-icd-mdh operon,erm)} \Delta \text{gltA::cat} \) N1838 \( \rightarrow \) N694

N1839 \( \text{citG::pMutinT3-hy (P}_{spac-hy}\text{-citG,erm)} \) This study
N1847  
\[ \text{citG::pMutinT3-hy (Pspac-hy\-citG, erm) } \Delta \text{gltA::cat N1839→ N694} \]

N1842  
\[ \text{mdh::pMutinT3-hy (Pspac-hy\-mdh, erm) } \]
This study

N1850  
\[ \text{mdh::pMutinT3-hy (Pspac-hy\-mdh, erm) } \Delta \text{gltA::cat N1842→ N694} \]

*Arrows indicate *B. subtilis* transformation: donor strain → recipient strain.

**FIGURE LEGENDS**

**FIG 1.** Simplified metabolic pathways for carbon and nitrogen metabolism. The enzymes described in the text are shown along with their corresponding gene names in blue. Glutamate synthase is shown in red. Abbreviations: CS, citrate synthase; ACN, aconitase, IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; FUM, fumarase; GOGAT, glutamate synthase; GS, glutamine synthase; and GDH, glutamate dehydrogenase.

**FIG 2.** The \( \Delta \text{gltA} \) mutation causes early arrest of biofilm formation. Wild-type and \( \Delta \text{gltA} \) mutant strains were grown statically in liquid 2×SGG medium in 6-well plates ("pellicle" in the Figure) and on solid 2×SGG medium ("colony" in the Figure). Top-down views of entire biofilms are shown. Magnified images of biofilms taken at 48 h with a stereo microscope (see right side of each series of photos). A well without bacteria inoculated (medium) is shown as a reference. Scale bar, 5 mm.

**FIG 3.** The glutamate auxotrophy of the \( \Delta \text{gltA} \) mutant is not the cause of its biofilm formation defect. Growth and pH profiles of the wild-type and \( \Delta \text{gltA} \) mutant strains. The wild-type strain (black circles) and the \( \Delta \text{gltA} \) mutant (red circles) were grown in 2×SGG at 37°C with vigorous shaking, and the OD\(_{600}\) (close circles) and pH (open circles) of the cultures were compared over time.

**FIG 4.** The \( \Delta \text{gltA} \) mutation causes an early decrease in transcription of \( \text{epsA} \) and \( \text{tapA} \)
operons. Strains harboring the \(P_{\text{epsA}}\)-gfp or \(P_{\text{tapA}}\)-gfp reporter were grown statically in 2×SGG, and expression of GFP in individual cells was analyzed by flow cytometry. The wild-type strain lacking the gfp reporter was used as a negative control.

**FIG 5.** Reduced expression of \(\text{epsA}\) and \(\text{tapA}\) operons is responsible for the biofilm formation defect in the \(\Delta\text{gltA}\) mutant. (A) \(\Delta\text{abrB}\) and \(\Delta\text{sinR}\) mutations suppress the \(\Delta\text{gltA}\) mutation during biofilm formation. Pellicle biofilms grown for 48 h are shown. (B) Induction of the \(\text{epsA}\) or \(\text{tapA}\) operons restores biofilm formation to the \(\Delta\text{gltA}\) mutant. The \(\text{P}_{\text{spac-by-epsA}}\) operon and \(\text{P}_{\text{spac-by-tapA}}\) operon strains were grown for 48 h with or without 1 mM IPTG. Scale bar, 5 mm.

**FIG 6.** The \(\Delta\text{gltA}\) mutation reduces transcription of Spo0A-regulated genes. (A) Expression of the \(\text{P}_{\text{ska-}}\)-gfp and \(\text{P}_{\text{spoIIA-}}\)-gfp reporters was analyzed by flow cytometry. The wild-type strain lacking the gfp reporter was used as a negative control. (B) Sporulation frequency of wild-type and \(\Delta\text{gltA}\) mutant strains in pellicle biofilms. These strains were grown statically for 72 h, and the cultures were used to determine sporulation frequency. Data are expressed as the mean and standard deviation of three independent experiments.

**FIG 7.** The \(\Delta\text{gltA}\) mutation prevents phosphorelay. (A) The \(\Delta\text{gltA}\) mutation does not inhibit \(\text{KinC}\) or \(\text{KinD}\) specifically. The \(\text{P}_{\text{spac-by-kinC}}\ \Delta\text{kinABD}\) and \(\text{P}_{\text{spac-by-kinD}}\ \Delta\text{kinABC}\) strains with or without the \(\Delta\text{gltA}\) mutation were grown for 48 h in liquid 2×SGG supplemented with the indicated concentrations of IPTG. (B) Expression of Sda and Spo0E phosphatases by the \(\Delta\text{gltA}\) mutant. Wild-type and \(\Delta\text{gltA}\) mutant strains harboring the \(\text{P}_{\text{sdar-}}\)-gfp or \(\text{P}_{\text{spo0E-}}\)-gfp reporter were grown statically for 48 h in 2×SGG, and expression of these reporters was analyzed by flow cytometry. The wild-type strain
lacking the gfp reporter was used as a negative control. Scale bar, 5 mm.

**FIG 8.** ΔgltA mutation-mediated prevention of biofilm formation is dependent on large amounts of glycerol. Wild-type and ΔgltA mutant strains were grown for 48 h in modified 2×SGG, which contained the indicated concentrations (w/v %) of glycerol or glucose instead of 0.1% glucose and 1% glycerol. Magnified images of colony biofilms taken with a stereo microscope are also shown. Scale bar, 5 mm.

**FIG 9.** Limited expression of citZ restores biofilm formation to the ΔgltA mutant. The indicated genes for the TCA cycle in the wild-type and ΔgltA mutant strains were placed under the control of the spac-hy promoter. These strains were grown statically for 48 h in 2×SGG supplemented with the indicated concentrations of IPTG. To make the wrinkle structures of pellicles easier to see, magnified images are shown. Full images of the pellicles are shown in Figure S5. Scale bar, 5 mm.

**FIG 10.** Excess iron suppresses the ΔgltA mutation during biofilm formation. (A) Wild-type and ΔgltA mutant strains were grown statically for 48 h in modified 2×SGG supplemented with the indicated concentrations of FeSO₄ or MnCl₂ instead of 1 µM FeSO₄ or 0.1 mM MnCl₂. The relative ratios of FeSO₄ or MnCl₂ concentrations to that in original 2×SGG medium are also shown. (B) Effect of iron limitation on biofilm formation. The wild-type strain was grown on modified 2×SGG medium supplemented with 1/10 FeSO₄ or without FeSO₄. Scale bar, 5 mm.
### A

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### B

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