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RESEARCH ARTICLE

# Inactivation of *cysL* Inhibits Biofilm Formation by Activating the Disulfide Stress Regulator Spx in *Bacillus subtilis*

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**ABSTRACT** *Bacillus subtilis* forms biofilms in response to internal and external stimuli. I previously showed that the *cysL* deletion mutant was defective in biofilm formation, but the reason for this remains unidentified. CysL is a transcriptional activator of the *cysJI* operon, which encodes sulfite reductase, an enzyme involved in cysteine biosynthesis. Decreased production of sulfite reductase led to biofilm formation defects in the Δ*cysL* mutant. The Δ*cysL* mutation was suppressed by disrupting *cysH* operon genes, whose products function upstream of sulfite reductase in the cysteine biosynthesis pathway, indicating that defects in cysteine biosynthesis were not a direct cause for the defective biofilm formation observed in the Δ*cysL* mutant. The *cysH* gene encodes phosphoadenosine phosphosulfate reductase, which requires a reduced form of thioredoxin (TrxA) as an electron donor. High expression of *trxA* inhibited biofilm formation in the Δ*cysL* mutant but not in the wild-type strain. Northern blot analysis showed that *trxA* transcription was induced in the Δ*cysL* mutant in a disulfide stress-induced regulator Spx-dependent manner. On the basis of these results, I propose that the Δ*cysL* mutation causes phosphoadenosine phosphosulfate reductase to consume large amounts of reduced thioredoxin, inducing disulfide stress and activating Spx. The *spx* mutation restored biofilm formation to the Δ*cysL* mutant. The Δ*cysL* mutation reduced expression of the *eps* operon, which is required for exopolysaccharide production. Moreover, overexpression of the *eps* operon restored biofilm formation to the Δ*cysL* mutant. Taken together, these results suggest that the Δ*cysL* mutation activates Spx, which then inhibits biofilm formation through repression of the *eps* operon.

**IMPORTANCE** *Bacillus subtilis* has been studied as a model organism for biofilm formation. In this study, I explored why the *cysL* deletion mutant was defective in biofilm formation. I demonstrated that the Δ*cysL* mutation activated the disulfide stress response regulator Spx, which inhibits biofilm formation by repressing biofilm matrix genes. Homologs of Spx are highly conserved among Gram-positive bacteria with low G+C contents. In some pathogens, Spx is also reported to inhibit biofilm formation by repressing biofilm matrix genes, even though these genes and their regulation are quite different from those of *B. subtilis*. Thus, the negative regulation of biofilm formation by Spx is likely to be well conserved across species and may be an appropriate target for control of biofilm formation.

**KEYWORDS** *Bacillus subtilis*, Spx, biofilms, cysteine biosynthesis, disulfide stress

Biofilms are structured, multicellular communities of bacteria in which bacterial cells adhere to each other on a surface via a biofilm matrix. The biofilm matrix consists of polymeric exopolysaccharides, proteins, and/or nucleic acids (1, 2) produced by the bacteria that make up the biofilm. Biofilms are resistant to environmental stresses, including antibiotics, bactericidal chemicals, metals, and host defense mechanisms (3, 4). Most bacteria in nature are capable of forming biofilms and surviving. Some biofilms cause serious problems in man-made environments, such as those associated with

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contamination and infectious diseases, and therefore reagents and treatments to effectively remove, prevent, or inhibit biofilms are in demand.

The Gram-positive bacterium *Bacillus subtilis* forms floating biofilms (pellicles) on the surface of liquid medium under static culture conditions or colony biofilms on solid medium (5). *B. subtilis* biofilms produce a biofilm matrix composed of exopolysaccharides, TasA amyloid fibers, and BslA hydrophobins produced by proteins of the *epsABCDEFGHIJKLMNO* operon, the *tapA-sipW-tasA* operon, and *bslA*, respectively (6–10). These genes are directly or indirectly repressed by the transcriptional repressors AbrB and SinR (11–14). One of the triggers for the initiation of biofilm formation is a self-produced and secreted antibiotic, surfactin, which is considered to stimulate autophosphorylation of histidine kinase KinC, leading to the activation of antagonistic regulatory mechanisms against AbrB and SinR repression (15, 16). Although regulatory mechanisms for *B. subtilis* biofilm formation have been extensively studied, unanswered questions still remain.

I previously tested the biofilm formation ability of 285 transcriptional regulation mutants and identified ninety regulators involved in biofilm formation (17). One of these is *cysL*, which encodes a transcriptional activator of the cysteine biosynthesis genes, *cysJI* (18). However, why inactivation of *cysL* causes biofilm formation defects remains unexplained. In this study, I investigated the phenotype of the Δ*cysL* mutant and identified a previously unknown mechanism for the regulation of *B. subtilis* biofilm formation.

## RESULTS AND DISCUSSION

**The Δ*cysL* mutant blocks biofilm formation.** I previously found that inactivation of *cysL* impaired the ability of *B. subtilis* to form pellicle biofilms under static culture conditions in 2× Schaeffer's sporulation medium supplemented with glucose and glycerol (2×SGG) medium, which is a nutrient broth (Difco)-based complex medium supplemented with sugars and metals (17). Since the previous study was conducted using *B. subtilis* strain ATCC 6051, I reexamined the effects of the Δ*cysL* mutation on biofilm formation using another *B. subtilis* strain, NCIB3610 (5), which has been widely used in studies of *B. subtilis* biofilm formation. As shown in Fig. 1A, the wild-type strain NCIB3610 formed thick pellicles with densely wrinkled morphology in 2×SGG medium, whereas the Δ*cysL* mutant of NCIB3610 formed thin pellicles lacking macroscopic structures, such that the brown color of the medium was seen through them. The addition of cysteine to the medium suppressed the biofilm formation-defective phenotype of the Δ*cysL* mutant (Fig. 1A). Since *cysL* encodes an LysR-type transcriptional regulator that activates the transcription of the *cysJI* operon encoding sulfite reductase (18, 19), decreased expression of *cysJI* was thought to cause the biofilm formation-defective phenotype of the Δ*cysL* mutant. To test this idea, the Δ*cysI* mutant was cultured in 2×SGG medium without agitation. The Δ*cysI* mutant formed very thin pellicles similar to those of the Δ*cysL* mutant (Fig. 1A). The addition of cysteine to the medium restored pellicle formation to the Δ*cysI* mutant (Fig. 1A). Thus, Δ*cysL* and Δ*cysI* mutants displayed the same phenotype, supporting my assumption.

The Δ*cysL* mutant has cysteine auxotrophy and cannot grow in Spizizen minimal medium (SMM) due to decreased expression of *cysJI* (18) (Fig. 2D). To determine whether the Δ*cysL* mutation had severe effects on growth in 2×SGG complex medium, the wild-type strain and the Δ*cysL* mutant were grown in 2×SGG medium with vigorous shaking, and optical density at 600 nm ($OD_{600}$) was measured over time. The Δ*cysL* mutant showed a comparable growth rate to the wild-type strain during the exponential growth phase, but reached a lower $OD_{600}$ than that of the wild-type strain in the stationary phase (Fig. 1B). The addition of cysteine to the medium raised the $OD_{600}$ of the Δ*cysL* mutant culture in the stationary phase to the wild-type strain level (Fig. 1B). The Δ*cysI* mutant also showed the same growth phenotype. These results indicate that the Δ*cysL* mutation does not severely affect growth during the exponential phase but does suppress growth in the stationary phase, likely caused by decreased cysteine in the medium.

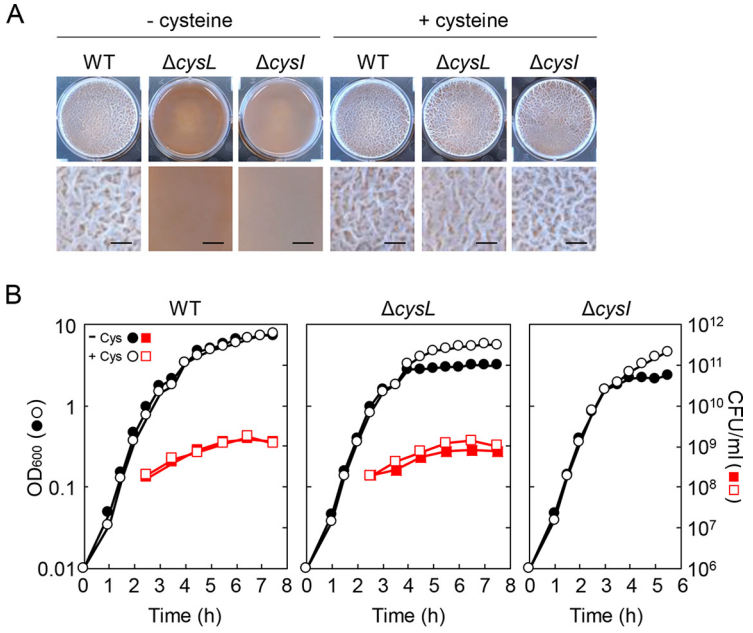


**FIG 1** The Δ*cysL* mutant is defective in biofilm formation. (A) Biofilm formation ability of Δ*cysL* and Δ*cysI* mutants in the presence or absence of 100 $\mu$g/ml cysteine. *B. subtilis* strains were grown at 30°C for 48 h in 2×SGG medium with or without cysteine under static conditions. Top-down photographs of pellicle biofilms are shown. The well diameter is 34 mm. Extended images of each pellicle are also shown. Bars, 2 mm. (B) Growth profiles of the Δ*cysL* and Δ*cysI* mutants. *B. subtilis* strains were grown with vigorous shaking in 2×SGG medium or 2×SGG medium plus 100 $\mu$g/ml cysteine. CFU/ml were determined only for the wild-type strain and the Δ*cysL* mutant.

The Δ*cysL* mutation drastically reduces transcription of sulfite reductase (CysJ/CysI) (18), which is required for the reduction of sulfite to sulfide in cysteine biosynthesis (19) (Fig. 2A). I tested whether disrupting genes involved in earlier steps in the cysteine biosynthesis pathway inhibited biofilm formation. Sulfite is synthesized from sulfate through four steps catalyzed by proteins of the *cysH* operon (Fig. 2B) (20). The first four genes of the *cysH* operon belong to the cysteine biosynthesis pathway; *cysP* encodes sulfate permease (21), while *sat*, *cysC*, and *cysH* encode sulfate adenylyl transferase, adenylyl sulfate kinase, and phosphoadenosine phosphosulfate reductase, respectively, all of which are responsible for synthesizing sulfite from incorporated sulfate in the cysteine biosynthesis pathway (20, 21). The remaining genes, *ylnD*, *ylnE*, and *ylnF*, are probably involved in the synthesis of siroheme, a cofactor of sulfite reductase (22, 23). The *cysH* operon genes were disrupted by the insertion of an integration vector, pMutin (24). The pMutin vector contains a LacI-repressive and isopropyl $\beta$-D-1-thiogalactopyranoside (IPTG)-inducible *spac* promoter, which alleviates the polar effect of the pMutin insertion on downstream genes by expressing them from the *spac* promoter in an IPTG-dependent fashion (Fig. 2B). The pMutin insertion mutants of the *cysH* operon genes were grown statically in 2×SGG medium supplemented with IPTG. Unlike the Δ*cysL* mutant, Δ*cysH*, Δ*cysP*, Δ*sat*, Δ*cysC*, Δ*ylnD*, Δ*ylnE*, and Δ*ylnF* mutants all formed pellicle biofilms comparable to those of the wild-type strain (Fig. 2C). Except for Δ*ylnE* and Δ*ylnF*, these mutants showed cysteine auxotrophy on SMM (Fig. 2D). These results indicate that cysteine biosynthesis deficiency does not always inhibit biofilm formation, and that siroheme synthesis by YlnD, YlnE, and YlnF is not required for sulfite reductase activity in 2×SGG medium.

These insertion mutations of the *cysH* operon genes were transferred to the Δ*cysL* mutant, and the resultant double mutants were tested for their ability to form biofilms. Δ*cysH* Δ*cysL*, Δ*cysP* Δ*cysL*, Δ*sat* Δ*cysL*, and Δ*cysC* Δ*cysL* double mutants formed normal pellicles, while Δ*ylnD* Δ*cysL*, Δ*ylnE* Δ*cysL*, and Δ*ylnF* Δ*cysL* double mutants did not (Fig. 2C). These double mutants showed cysteine auxotrophy on SMM (Fig. 2D). Growth of

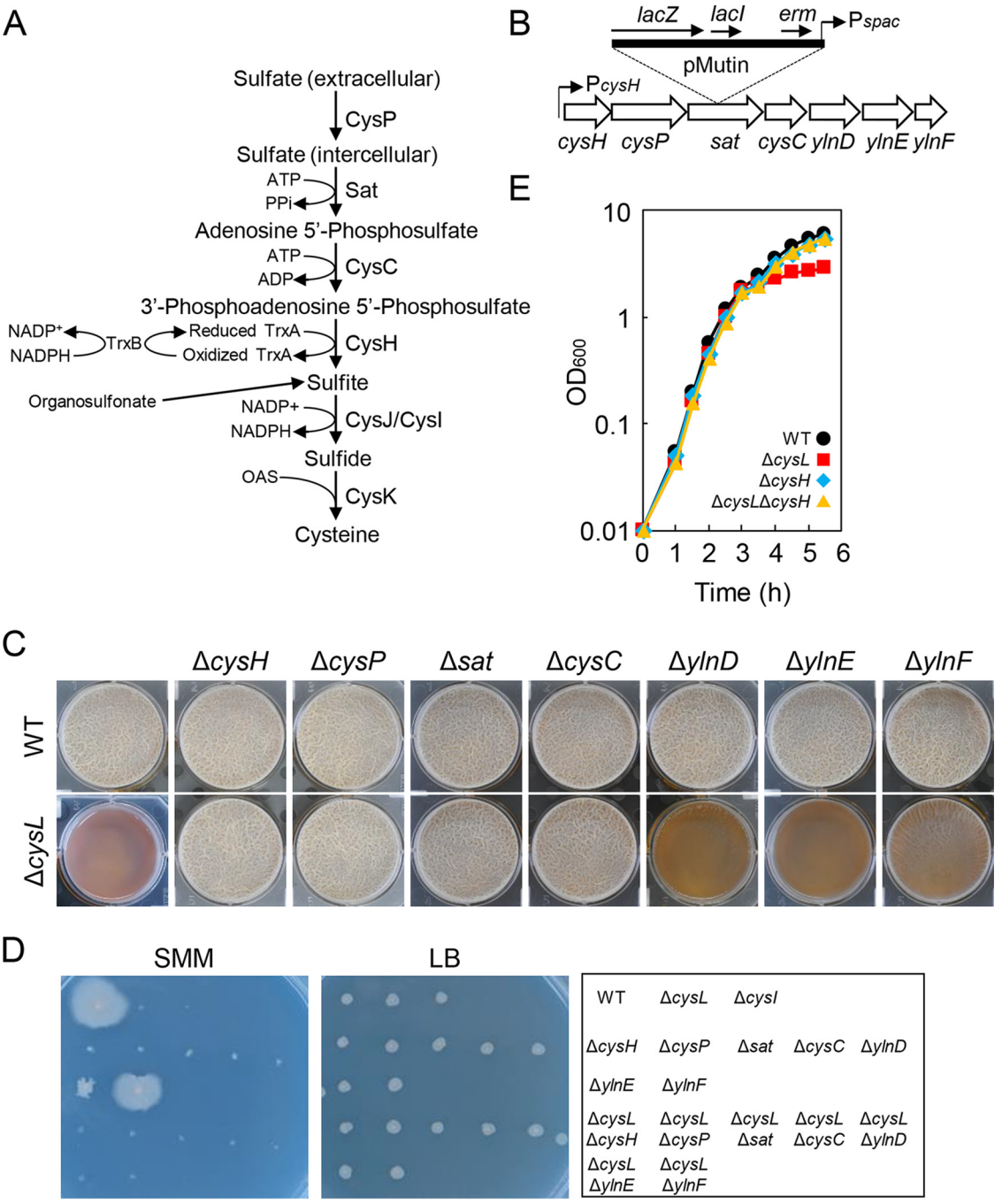


**FIG 2** Null mutations of the *cysH* operon genes suppress the Δ*cysL* mutation. (A) Diagram of the cysteine biosynthesis pathway of *B. subtilis*. The proteins believed to function in the process are shown. (B) Gene organization of the *cysH* operon. A pMutin insertion into *sat* is shown above the gene map as an example. (C) Biofilm formation of *cysH* operon mutants. The wild-type and mutant strains were statically grown for 48 h in 2×SGG medium with 1 mM IPTG. (D) Viability of *cysH* operon mutants on Spizizen minimal medium (SMM) and LB. Strains were grown at 37°C for 48 h on indicated medium supplemented with 1 mM IPTG. Strain positions on the media are shown in the right panel. (E) Growth profiles of Δ*cysH* and Δ*cysH* Δ*cysL* mutants. Strains were grown at 37°C in 2×SGG medium with vigorous shaking.

Δ*cysH* and Δ*cysH* Δ*cysL* mutants was tested in 2×SGG medium with vigorous shaking. Δ*cysH* and Δ*cysH* Δ*cysL* mutants showed growth comparable to that of the wild-type strain from the exponential phase to the stationary phase (Fig. 2E). Thus, disrupting genes involved in steps earlier than sulfite reductase in the cysteine biosynthesis pathway restored biofilm formation and stationary-phase growth to the Δ*cysL* mutant.

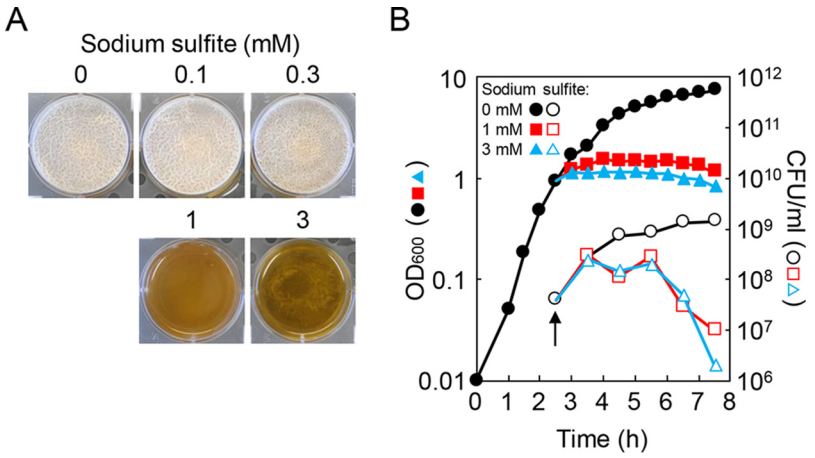


**FIG 3** Sulfite inhibits biofilm formation by killing cells. (A) Impacts of sulfite on biofilm formation. The wild-type strain was statically grown at 30°C for 18 h in 2×SGG medium, and then sulfite was added to the cultures. The cultures were further incubated at 30°C for 30 h. (B) Impact of sulfite on growth. The wild-type strain was grown to an $OD_{600}$ of 1.0, and then the culture was divided into three parts. Each of the cultures was treated with 0, 1, or 3 mM sulfite. An arrow indicates the time point at which sulfite was added to the cultures.

These results indicate that the biofilm formation-defective phenotype of Δ*cysL* mutant is caused by the action of the *cysH* operon proteins, CysH, CysP, Sat, and CysC. As described above, the Δ*cysL* mutation was suppressed by the addition of cysteine. Since expression of cysteine biosynthetic genes, including that of *cysH*, is repressed in the presence of cysteine (20), this suppression is likely to be caused by decreased expression of the *cysH* operon.

**Sulfite accumulation is not responsible for the biofilm formation-defective phenotype of the Δ*cysL* mutant.** CysH, CysP, Sat, and CysC are required for the biosynthesis of sulfite, which is expected to accumulate in the Δ*cysL* mutant due to decreased expression of sulfite reductase (Fig. 2A). Sulfite is often used as a food preservative and an antioxidant and is toxic to bacteria. I therefore hypothesized that accumulated sulfite might inhibit biofilm formation in the Δ*cysL* mutant. To address this hypothesis, the effects of various concentrations of sulfite on biofilm formation were tested. The wild-type strain was statically grown to just before formation of pellicle biofilms, and then the cultures were treated with sulfite. Although the low concentrations of sulfite did not affect biofilm formation, sulfite completely inhibited biofilm formation at 1 mM and 3 mM (Fig. 3A). I examined the effects of those concentrations of sulfite on growth. The wild-type strain was grown to the end of the exponential phase with vigorously shaking. The culture was then divided into three parts, and each of the cultures was treated with 0, 1, or 3 mM sulfite (Fig. 3A). The addition of 1 mM or 3 mM sulfite immediately inhibited growth and caused a decrease in the number of viable cells (Fig. 3B). These results indicate that sulfite inhibits biofilm formation by killing cells. This contradicted the observation that the Δ*cysL* mutation had moderate effects on the number of viable cells (Fig. 1B). Thus, sulfite accumulation is unlikely to be the primary cause of the biofilm formation-defective phenotype of the Δ*cysL* mutant.

**Excessive activity of phosphoadenosine phosphosulfate reductase activates Spx in the Δ*cysL* mutant.** I hypothesized that the biofilm formation-defective phenotype of the Δ*cysL* mutant might be caused by the action of enzymes for sulfite synthesis rather than by their reaction products. I focused on phosphoadenosine phosphosulfate reductase (CysH), which catalyzes the most downstream reaction among the enzymes involved in sulfite synthesis (Fig. 2A). Phosphoadenosine phosphosulfate reductase, which catalyzes the reduction of 3′-phosphoadenosine 5-phosphosulfate to sulfite, requires a small protein, called thioredoxin, encoded by *trxA* (25). The reduced form of thioredoxin (thioredoxin disulfide) serves as an electron donor to phosphoadenosine

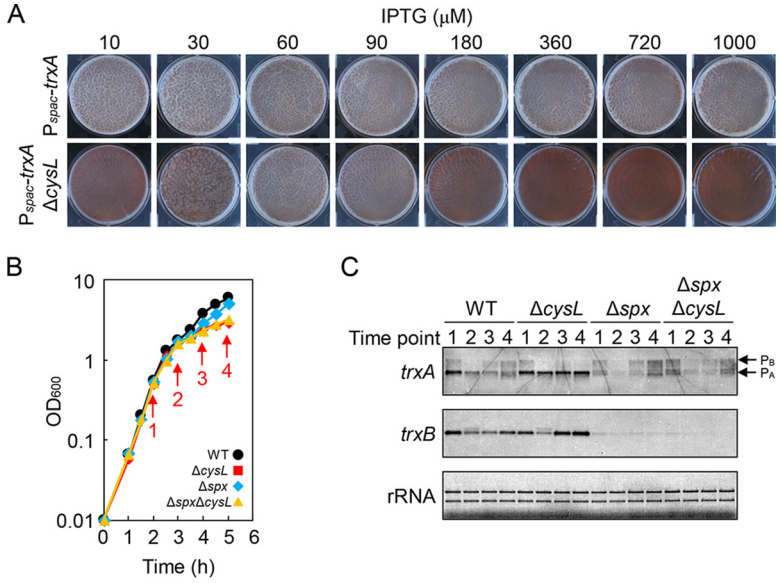


**FIG 4** The Δ*cysL* mutation activates Spx. (A) Low and high levels of *trxA* inhibit biofilm formation in the Δ*cysL* mutant. $P_{spac}$-*trxA* and $P_{spac}$-*trxA* Δ*cysL* mutants were grown at 30°C for 48 h in 2×SGG with the indicated concentrations of IPTG. (B) Growth profiles of Δ*cysL* and Δ*spx* mutants. Strains were grown in 2×SGG medium with vigorous shaking. Arrows indicate the time points at which samples were taken for RNA isolation. (C) Northern blot analysis of *trxA* and *trxB*. Transcripts were detected with gene-specific digoxigenin (DIG)-labeled RNA probes. Time point numbers correspond to the time points shown in panel B. *trxA* transcripts from $P_B$ and $P_A$ promoters are indicated by arrows. rRNA stained with methylene blue is shown as a loading control.

phosphosulfate reductase. Thioredoxin reductase TrxB reduces oxidized thioredoxin using NADPH and maintains cellular levels of thioredoxin disulfide (25). Because the thioredoxin system contributes to a wide variety of physiological functions as a major thiol-disulfide oxidoreductase, *trxA* and *trxB* are essential for growth in *B. subtilis* (26). I examined the effects of modulating thioredoxin levels on biofilm formation. For this purpose, the *trxA* gene was placed under the control of the *spac* promoter, and the resultant conditional mutant $P_{spac}$-*trxA* was grown statically in 2×SGG medium supplemented with various concentrations of IPTG. The $P_{spac}$-*trxA* mutant was able to grow and form biofilms at 10 μM or greater IPTG (Fig. 4A). In contrast, the $P_{spac}$-*trxA* Δ*csyL* mutant was able to grow at 10 μM IPTG or more but formed normal biofilms only at 60 to 90 μM IPTG (Fig. 4A). In particular, its pellicles completely lost macroscopic structures, as seen in the pellicles of the Δ*cysL* mutant, at high concentrations of IPTG (360, 720, and 1,000 μM) (Fig. 4A). Thus, unlike in the wild-type strain, low and high expression of *trxA* inhibits biofilm formation in the Δ*cysL* mutant.

I next explored the possibility that transcription of *trxA* and *trxB* is altered in the Δ*cysL* mutant. The wild-type strain and the Δ*cysL* mutant were grown in 2×SGG medium with vigorous shaking. Cells were taken at 1 h intervals from the mid-exponential phase to the stationary phase, and total RNA was isolated (Fig. 4B). Transcription of *trxA* and *trxB* was analyzed by Northern blotting (Fig. 4C). *trxA* is transcribed from two promoters, $P_B$ and $P_A$ (26). The upstream promoter $P_B$ is induced by the general stress sigma factor $\sigma^B$, and the downstream promoter $P_A$ is recognized by the major sigma factor $\sigma^A$. Under my culture conditions, *trxA* was mainly transcribed from $P_A$, and its transcription levels were almost the same between the wild-type strain and the Δ*cysL* mutant during the exponential phase (time point 1 in Fig. 4C). However, *trxA* transcription from $P_A$ decreased during the stationary phase in the wild-type strain, whereas it was observed consistently throughout the growth period in the Δ*cysL* mutant (time points 3 and 4 in Fig. 4C). Thus, *trxA* transcription levels were higher in the Δ*cysL* mutant than in the wild-type strain in the stationary phase. Likewise, *trxB* transcription levels were higher in the Δ*cysL* mutant than in the wild-type strain in the

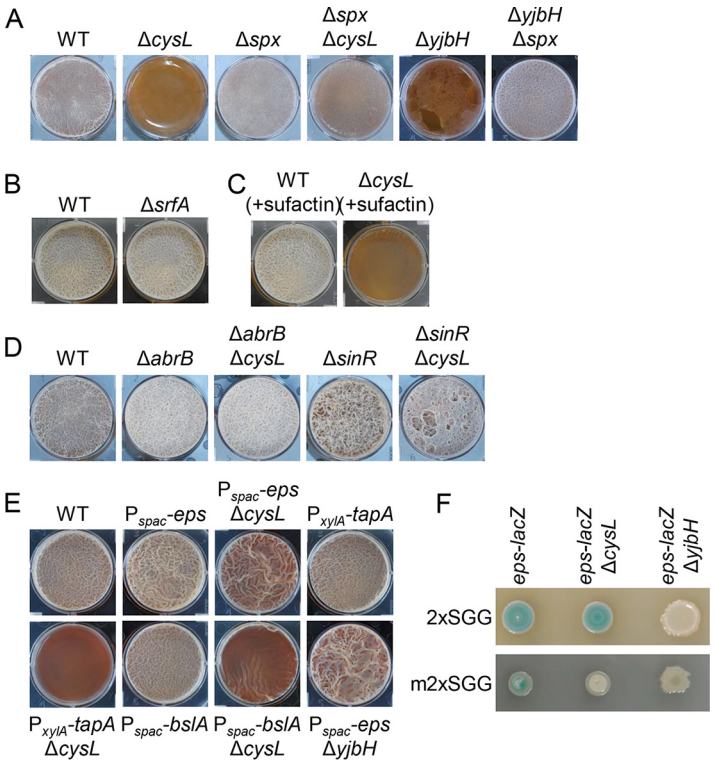


**FIG 5** Δ*cysL* mutation inhibits expression of biofilm matrix genes. (A) Biofilm formation of Δ*spx* and Δ*yjbH* mutants. (B) The Δ*srfA* mutation has little or no effect on biofilm formation in in 2×SGG. (C) Surfactin does not restore biofilm formation to the Δ*cysL* mutant. The wild-type strain and Δ*cysL* mutants were grown in 2×SGG medium with 0.1 μg/ml surfactin. (D) Δ*abrB* and Δ*sinR* mutations restore biofilm formation to the Δ*cysL* mutant. (E) Effects of the artificial expression of biofilm matrix genes on biofilm formation of the Δ*cysL* mutant. $P_{spac}$-*eps* operon and $P_{spac}$-*bslA* strains were grown in 2×SGG medium with 1 mM IPTG. (F) Expression of the *eps* operon. *epsH*::pMutin strains harboring the *eps-lacZ* reporter were grown for 24 h on 2×SGG medium or modified 2×SGG (m2×SGG) medium with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). m2×SGG medium contains four times less nutrient broth than does 2×SGG medium. All photographs of biofilms were taken 48 h after inoculation.

stationary phase (Fig. 4C). Transcription of *trxA* from $P_A$ and *trxB* is induced by the transcription factor Spx (27). The effect of the Δ*spx* mutation on *trxA* and *trxB* transcription was then examined under the same culture conditions (Fig. 4B). The Δ*spx* mutation drastically reduced transcription of *trxA* from $P_A$ and *trxB* in both the wild-type strain and the Δ*cysL* mutant throughout the growth period (Fig. 4C). In the Northern blot of *trxA*, some faint bands were observed around the position of *trxA* transcripts from $P_A$ in Δ*spx* and Δ*spx* Δ*cysL* mutants. However, those transcripts seemed to be degradative products of *trxA* transcripts from $P_B$ because (i) the size of those transcripts was slightly larger or smaller than that of *trxA* transcripts from $P_A$, and (ii) the intensity of those transcripts was proportional to that of *trxA* transcripts from $P_B$. These results indicate that Spx regulates transcription of *trxA* from $P_A$ and *trxB* in both the wild-type strain and in the Δ*cysL* mutant. Unlike in the wild-type strain, Spx activity does not decrease during the stationary phase and continues to induce transcription of *trxA* and *trxB* in the Δ*cysL* mutant.

Spx activates or represses transcription of a large number of genes in response to thiol-specific oxidative (disulfide) stress (27, 28). To determine whether Spx inhibited biofilm formation in the Δ*cysL* mutant, I tested the ability of the Δ*spx* and Δ*spx* Δ*cysL* mutants to form biofilms. The Δ*spx* mutant formed biofilms, but its pellicles were slightly thinner than pellicles formed by the wild-type strain (Fig. 5A). The Δ*spx* Δ*cysL* double mutant also formed slightly thin pellicles, as observed in the Δ*spx* mutant. Thus, the Δ*spx* mutation largely restored biofilm formation to the Δ*cysL* mutant. I tested the biofilm formation ability of the Δ*yjbH* mutant, which has high levels of Spx (47). The

Δ*yjbH* mutant formed only very thin pellicles, and this phenotype was suppressed by the Δ*spx* mutation (Fig. 5A). These results indicate that activated or increased Spx inhibits biofilm formation. Based on these results, I concluded that the Δ*cysL* mutation leads to activation of Spx, causing the biofilm formation-defective phenotype. I propose that excessive activity of phosphoadenosine phosphosulfate reductase (CysH) induces disulfide stress and then activates Spx in the Δ*cysL* mutant as in the following description. Cysteine biosynthesis genes, including the *cysH* operon, are expected to be induced in the Δ*cysL* mutant in 2×SGG medium, as cysteine levels in the medium decrease with growth (29). However, the induction of cysteine biosynthesis genes does not bring about cysteine synthesis in the Δ*cysL* mutant. Consequently, phosphoadenosine phosphosulfate reductase continues to work and consume thioredoxin disulfide. Since thioredoxin disulfide maintains intracellular thiol-disulfide redox homeostasis and reduces abnormal disulfide bonds, the consumption of significant amounts of thioredoxin disulfide or a shortage of thioredoxin disulfide could have great impacts on intracellular thiol-disulfide redox balance and induce disulfide stress, which activates Spx.

**Spx negatively regulates biofilm matrix genes.** I investigated which critical genes for biofilm formation were inhibited by the Δ*cysL* mutation or activated Spx. Spx is a global regulator, but little is known about its function in *B. subtilis* biofilm formation (27, 30). One of the known targets of Spx is the *srfA* operon; Spx inhibits its transcription (27). The *srfA* operon is required for the production of the lipopeptide antibiotic surfactin, which serves as a self-produced trigger for biofilm formation and induces biofilm matrix genes, the *eps* operon and the *tapA* operon (15). However, the deletion of the *srfA* operon had little or no effect on biofilm formation in 2×SGG medium (Fig. 5B), and the addition of surfactin to medium did not restore biofilm formation to the Δ*cysL* mutant (Fig. 5C), indicating that Spx may inhibit other important genes for biofilm formation.

Two transcriptional repressors, AbrB and SinR, negatively regulate biofilm formation in *B. subtilis* (11–14). Double mutants Δ*abrB* Δ*cysL* and Δ*sinR* Δ*cysL* formed thick pellicles in standing culture, as observed for Δ*abrB* and Δ*sinR* mutants (Fig. 5D), showing that both Δ*abrB* and Δ*sinR* mutations can suppress the Δ*cysL* mutation in biofilm formation. Since these repressors directly and indirectly repress biofilm matrix genes, the *eps* operon, the *tapA* operon, and *bslA* (11–14), I examined whether induction of one of these biofilm matrix genes restored biofilm formation to the Δ*cysL* mutant. For this purpose, the IPTG-inducible *spac* promoter was inserted upstream of the *eps* operon and *bslA*, and a constitutive *xylA* promoter from *Bacillus megaterium* (31) was inserted upstream of the *tapA* operon. These constructs, the $P_{spac}$-*eps* operon, the $P_{xylA}$-*tapA* operon, and $P_{spac}$-*bslA*, were transferred to the Δ*cysL* mutant. Induction of the *eps* operon largely restored biofilm formation to the Δ*cysL* mutant, while induction of the *tapA* operon or *bslA* did not (Fig. 5E). Moreover, induction of the *eps* operon restored biofilm formation to the Δ*yjbH* mutant (Fig. 5E). To determine whether Δ*cysL* and Δ*yjbH* mutations reduced expression of the *eps* operon, expression of the *eps* operon was analyzed using the Δ*epsH*::pMutin mutant harboring the *lacZ* reporter within *epsH*. The Δ*epsH*::pMutin mutant formed colonies with clear blue pigmentation on 2×SGG solid medium supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), whereas the Δ*epsH*::pMutin Δ*yjbH* mutant formed white colonies (Fig. 5F). However, in contrast to the above results, the Δ*epsH*::pMutin Δ*cysL* mutant formed blue colonies similar to those of the Δ*epsH*::pMutin mutant. Since the Δ*cysL* mutant phenotype appeared after cysteine levels in medium decreased with growth, I suspected that the Δ*cysL* mutant might form blue colonies before this decrease. Accordingly, *eps-lacZ* activity was examined on a modified 2×SGG (m2×SGG) medium, which contained four times less nutrient broth as a nitrogen source than 2×SGG medium. On the modified 2×SGG medium, the Δ*epsH*::pMutin mutant formed pale blue colonies, whereas the Δ*epsH*::pMutin Δ*cysH* mutant and the Δ*epsH*::pMutin Δ*yjbH* mutant formed white colonies (Fig. 5F). These results indicate that the *eps* operon is the primary target of Spx

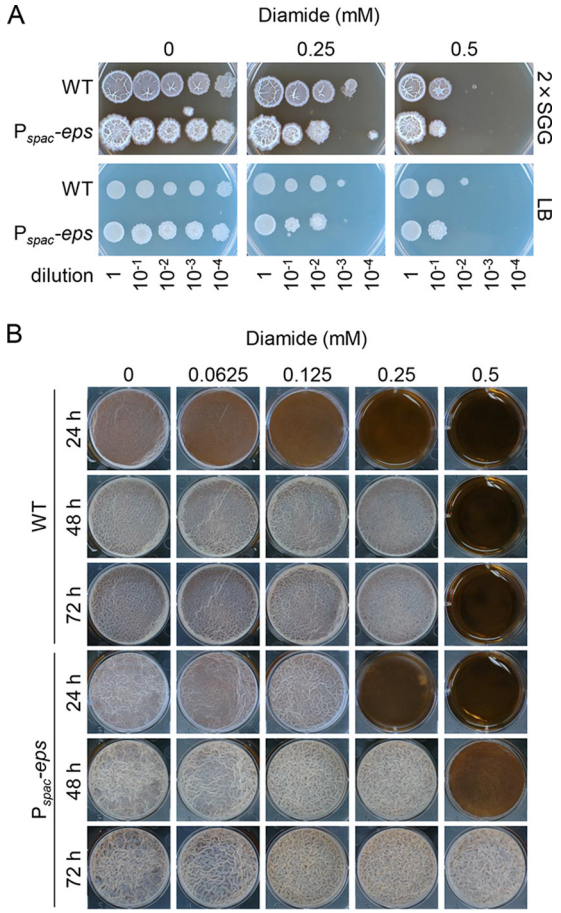


**FIG 6** The effects of diamide on biofilm formation. (A) Diamide resistance. Stationary-phase cultures of the wild-type strain and the P$_{spac}$-*eps* operon mutant in LB were serially diluted 10-fold, and 2-$\mu$l aliquots of the indicated dilutions were spotted on 2×SGG or LB solid medium supplemented with 1 mM IPTG. (B) Biofilm formation in the presence of diamide. Stationary-phase cultures of the wild-type strain and the P$_{spac}$-*eps* operon mutant were diluted 10-fold, and 10 $\mu$l of the dilutions was added to each well containing 10 ml of 2×SGG medium with 1 mM IPTG and the indicated concentrations of diamide.

in biofilm formation, although my results did not show whether Spx directly represses the *eps* operon.

**The effects of disulfide stress on biofilm formation.** My finding that the disulfide stress response regulator Spx inhibited biofilm formation suggested the possibility that inducing biofilm formation is detrimental to cells during disulfide stress. If this were the case, biofilm-inducing conditions or overexpression of the *eps* operon should adversely affect resistance to disulfide stress. To examine this possibility, stationary-phase cultures of the wild-type strain and the P$_{spac}$-*eps* operon mutant in LB medium were 10-fold serially diluted, and the dilutions were spotted onto biofilm formation medium (2×SGG) or non-biofilm formation medium (LB) supplemented with IPTG and diamide, the latter of which is a thiol-specific oxidant used to induce disulfide stress (Fig. 6A). I did not observe a significant difference between the two media or between the two strains in diamide resistance. Thus, inducing biofilm formation appears not to have a negative effect on diamide resistance. At present, I cannot answer the question of why biofilm formation is inhibited by Spx in response to disulfide stress.

I further examined whether overexpression of the *eps* operon led to disulfide stress-resistant biofilm formation. The wild-type strain and the P$_{spac}$-*eps* operon mutant were statically grown in 2×SGG medium supplemented with IPTG and diamide (Fig. 6B). The addition of diamide to medium delayed or inhibited biofilm formation of the wild-type strain as its concentration increased. Specifically, a slight delay was

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

| Strain | Relevant feature(s) | Reference or construction[a] |
|---|---|---|
| NCIB3610 | Prototroph | 5 |
| NTF234 | Δ*cysL*::*cat* | WTF237 (17)→ NCIB3610 |
| N502 | Δ*cysI*::pMutin (*erm*) | YVGRd (48)→NCIB3610 |
| N503 | Δ*cysH*::pMutin (*erm*) | CYSHd (48)→NCIB3610 |
| N504 | Δ*cysP*::pMutin (*erm*) | CYSPd (48)→NCIB3610 |
| N505 | Δ*sat*::pMutin (*erm*) | SATd (48)→NCIB3610 |
| N506 | Δ*cysC*::pMutin (*erm*) | CYSCd (48)→NCIB3610 |
| N507 | Δ*ylnD*::pMutin (*erm*) | YLNDd (48)→NCIB3610 |
| N508 | Δ*ylnE*::pMutin (*erm*) | YLNEd (48)→NCIB3610 |
| N509 | Δ*ylnF*::pMutin (*erm*) | YLNFd (48)→NCIB3610 |
| N510 | Δ*cysH*::pMutin (*erm*) Δ*cysL*::*cat* | N503→NTF234 |
| N511 | Δ*cysP*::pMutin (*erm*) Δ*cysL*::*cat* | N504→NTF234 |
| N512 | Δ*sat*::pMutin (*erm*) Δ*cysL*::*cat* | N505→NTF234 |
| N513 | Δ*cysC*::pMutin (*erm*) Δ*cysL*::*cat* | N506→NTF234 |
| N514 | Δ*ylnD*::pMutin (*erm*) Δ*cysL*::*cat* | N507→NTF234 |
| N515 | Δ*ylnE*::pMutin (*erm*) Δ*cysL*::*cat* | N508→NTF234 |
| N515 | Δ*ylnF*::pMutin (*erm*) ΔcysL::cat | N509→NTF234 |
| N519 | *trxA*:pMutinNC ($P_{spac}$-*trxA erm*) | TRXAp (48)→NCIB361 |
| N520 | *trxA*:pMutinNC ($P_{spac}$-*trxA erm*) Δ*cysL*::*cat* | N519→NTF234 |
| N523 | Δ*spx*::*kan* | This study |
| N524 | Δ*spx*::*kan* Δ*cysL*::*cat* | N520→NTF234 |
| N744 | Δ*yjbH*::pMutinNC (*erm*) | This study |
| N751 | Δ*yjbH*::pMutinNC (*erm*) Δ*spx*::*kan* | N744→N523 |
| N743 | Δ*yjbH*::*kan* | This study |
| N440 | Δ*srfA operon* | This study |
| NTF2 | Δ*abrB*::*kan* | WTF2 (17)→NCIB3610 |
| N552 | Δ*abrB*::*kan* Δ*cysL*::*cat* | NTF2→NTF234 |
| NTF92 | Δ*sinR*::*cat* | WTF92 (17)→NCIB3610 |
| N553 | Δ*sinR*::*kan* Δ*cysL*::*cat* | NTF92→NTF234 |
| N901 | *epsA*::pMutinT3-hy ($P_{spac\text{-}hy}$-*epsABCDEFGHIJKLMNO erm*) | This study |
| N902 | *epsA*::pMutinT3-hy ($P_{spac\text{-}hy}$-*epsABCDEFGHIJKLMNO erm*) Δ*cysL*::*cat* | NTF234→N901 |
| N903 | *epsA*::pMutinT3-hy ($P_{spac\text{-}hy}$-*epsABCDEFGHIJKLMNO erm*) Δ*yjbH*::*kan* | N743→N901 |
| N963 | *tapA*::pCA*xylAtapA* ($P_{xylA}$-*tapA-sipW-tasA spc*) | This study |
| N964 | *tapA*::pCA*xylAtapA* ($P_{xylA}$-*tapA-sipW-tasA spc*) Δ*cysL*::*cat* | NTF234→N963 |
| N904 | *bslA*::pMutinT3-hy ($P_{spac\text{-}hy}$-*bslA tet*) | This study |
| N905 | *bslA*::pMutinT3-hy ($P_{spac\text{-}hy}$-*bslA tet*) Δ*cysL*::*cat* | NTF234→N904 |
| N978 | Δ*epsH*::pMutin *(epsA-H-lacZ erm)* | YVERd (48) →NCIB3610 |
| N979 | Δ*epsH*::pMutin *(epsA-H-lacZ erm)* Δ*cysL*::*cat* | NTF234→N978 |
| N980 | Δ*epsH*::pMutin *(epsA-H-lacZ erm)* Δ*yjbH*::*kan* | N743→N978 |

[a]Arrows indicate *B. subtilis* transformation, donor strain → recipient strain.

observed even in the presence of a low concentration (0.0625 mM) of diamide, and its biofilm formation was completely inhibited for 72 h by 0.5 mM diamide. Low concentrations of diamide did not affect biofilm formation of the $P_{spac}$-*eps* operon mutant. The $P_{spac}$-*eps* operon mutant formed biofilms even in the presence of 0.5 mM diamide. These observations supported my findings that Spx inhibits biofilm formation through repressing the *eps* operon.

**Concluding remarks.** In this study, I investigated why the Δ*cysL* mutant was defective in biofilm formation. I showed that the Δ*cysL* mutation resulted in the overworking of phosphoadenosine phosphosulfate reductase and thereby induced disulfide stress, leading to the activation of Spx. Spx was previously shown to negatively regulate cysteine biosynthesis genes in *B. subtilis* (32, 33). Moreover, since *B. subtilis* has a large cysteine pool (34), cysteine biosynthesis is expected to be highly active in *B. subtilis*. Taken together, my findings suggest that the negative regulation of cysteine biosynthesis by Spx plays a vital role in coordinating cysteine biosynthesis, a potential producer of disulfide stress, with the cellular redox state. I demonstrated that Spx negatively regulates biofilm formation by repressing the *eps* operon in *B. subtilis*. Spx is highly conserved among low-G+C-content Gram-positive bacteria or members of the *Firmicutes* (35). The negative regulation of biofilm formation by Spx has also been reported in several pathogens of *Firmicutes*, including *Staphylococcus aureus* (36), *Staphylococcus epidermidis* (37), and *Streptococcus mutans* (38). In these pathogens, Spx

also inhibits transcription of biofilm matrix genes, despite the fact that these bacteria have different biofilm matrix genes with different regulatory systems from those of *B. subtilis* (36, 37). Bacterial pathogens have evolved to adapt to their host environment and robustly respond to reactive oxygen species (ROS) generated by host immune cells to kill invading pathogens (38). In many pathogens of *Firmicutes*, Spx has also evolved to play a critical role in virulence and survival within the host (39–44). However, my findings indicate that the negative regulation of biofilm formation by Spx is not specific to those pathogens but widespread among many *Firmicutes* species. Drugs that induce Spx may offer a promising new method for controlling biofilm formation in *Firmicutes*.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *B. subtilis* strain NCIB3610 and its derivatives used in this study are listed in Table 1. Construction of *B. subtilis* mutants is described in the supplemental material. Primers used for strain construction are listed in Table S1 in the supplemental material. *B. subtilis* strains were grown in LB (LB Lennox; BD Difco, Franklin Lakes, NJ), 2×SGG (17), or SMM (45). Cysteine was added to medium at a final concentration of 100 $\mu$g/ml where indicated. For the biofilm formation test, a fresh single colony of tested strains was inoculated into 10 ml of 2×SGG medium in a well of a 6-well plate and cultured at 30°C for 48 h without agitation. *Escherichia coli* strains HB101 and JM105 were used for construction and maintenance of plasmids. Sodium sulfite and surfactin were obtained from Fuji Wako Pure Chemicals (Osaka, Japan), and diamide was obtained from Sigma-Aldrich (St. Louis, MO).

**Northern blot analysis.** RNA samples were prepared from cells grown at 37°C in 2×SGG medium with vigorous shaking, as described previously (46). Northern blot analysis was carried out as described previously (46). Primers *trxA*-N-F, *trxA*-N-T7R, *trxB*-N-F, and *trxB*-N-T7R were used for RNA probe synthesis. Primer sequences are shown in Table S1 in the supplemental material.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00712-18.

**SUPPLEMENTAL FILE 1**, PDF file, 0.6 MB.

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