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Identification of *Bacillus subtilis* CysL, a Regulator of the *cysJI* Operon, Which Encodes Sulfite Reductase

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The way in which the genes involved in cysteine biosynthesis are regulated is poorly characterized in *Bacillus subtilis*. We showed that CysL (formerly YwfK), a LysR-type transcriptional regulator, activates the transcription of the *cysJI* operon, which encodes sulfite reductase. We demonstrated that a *cysL* mutant and a *cysJI* mutant have similar phenotypes. Both are unable to grow using sulfate or sulfite as the sulfur source. The level of expression of the *cysJI* operon is higher in the presence of sulfate, sulfite, or thiosulfate than in the presence of cysteine. Conversely, the transcription of the *cysH* and *cysK* genes is not regulated by these sulfur sources. In the presence of thiosulfate, the expression of the *cysJI* operon was reduced 11-fold, whereas the expression of the *cysH* and *cysK* genes was increased, in a *cysL* mutant. A *cis*-acting DNA sequence located upstream of the transcriptional start site of the *cysJI* operon (positions −76 to −70) was shown to be necessary for sulfur source- and CysL-dependent regulation. CysL also negatively regulates its own transcription, a common characteristic of the LysR-type regulators. Gel mobility shift assays and DNase I footprint experiments showed that the CysL protein specifically binds to *cysJ* and *cysL* promoter regions. This is the first report of a regulator of some of the genes involved in cysteine biosynthesis in *B. subtilis*.

All living organisms require sulfur for the synthesis of proteins and essential cofactors. Sulfur can be assimilated either from inorganic sources, such as sulfate and thiosulfate, or from organic sources, such as sulfate esters, sulfamates, and sulfonates. In *Escherichia coli*, sulfate is transported into the cell via an ATP-binding cassette-type sulfate-thiosulfate transport system (5, 11). Sulfate is subsequently reduced to sulfide by a series of enzymatic steps involving ATP sulfurylase, adenosine 5′-phosphosulfate kinase, 3′-phosphoadenosine 5′-phosphosulfate (PAPS) sulfotransferase, and sulfite reductase (Fig. 1). An *O*-acetyl-L-serine thiol-lyase condenses sulfide and *O*-acetylserine to form cysteine. In *E. coli* and *Salmonella enterica* serovar Typhimurium, at least 22 genes are required for the transport and reduction of sulfate and for its incorporation into cysteine. Most of these genes are coordinately regulated in the cysteine regulon (11). The high-level expression of these genes requires CysB, a LysR-type transcriptional activator, the inducer *N*-acetylserine, and sulfur-limiting conditions (11, 26). The CysB protein binds as a tetramer just upstream of the −35 promoter region of the positively regulated *cys* genes. The interaction of CysB with the inducer causes the activator to undergo a conformational change, allowing it to interact with the activation sites of the *cysJ*, *cysK*, and *cysP* promoters (7, 23). L-Cysteine, sulfide, and thiosulfate downregulate L-cysteine biosynthesis (11, 12).

In *Bacillus subtilis*, the assimilation of sulfate and the biosynthesis of cysteine may occur via similar pathways (Fig. 1). Indeed, the enzymes and the genes involved in the conversion of sulfate into sulfide and the incorporation of sulfide into cysteine are present in *B. subtilis* (14, 28). The *cysH* gene, which encodes PAPS sulfotransferase, is the first gene of an operon encoding a sulfate permease (CysP) and enzymes catalyzing the first steps of the sulfate assimilation pathway (18, 19). The *cysJ* and *cysI* genes, encoding the two subunits of the sulfite reductase, were recently identified (37).

Little is known about the regulation of cysteine biosynthesis in gram-positive bacteria. CmbR, a new LysR-type transcriptional activator, was shown to be important for the expression of the *metC-cysK* operon in *Lactococcus lactis* (2). In gram-positive bacteria, several genes involved in methionine or cysteine biosynthesis form a regulon controlled by a global transcriptional termination system called the S-box regulon (4). The *cysH* operon of *B. subtilis* contains an S-box motif in its leader region. However, the expression of this operon is controlled at the transcription initiation level by an unidentified repressor rather than at the level of premature termination of transcription (18).

The *ssu* operon is required for the assimilation of sulfur from sulfonates by *B. subtilis*. The expression of this operon is repressed by cysteine and sulfate and derepressed by taurine and glutathione (38). This operon is regulated at the level of transcription initiation and transcription termination by an S-box-independent mechanism (36). However, no regulator has been identified yet.

In this report, we demonstrate that the genes involved in the biosynthesis of cysteine in *B. subtilis* are regulated differently depending on the available sulfur source. We identified CysL (formerly YwfK), a LysR-type transcriptional activator of the *cysJI* operon.

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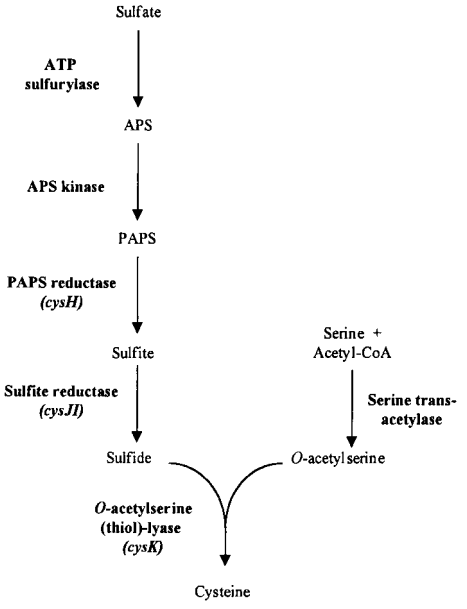

FIG. 1. Biosynthesis of L-cysteine in bacteria. The different enzymes involved in sulfate assimilation and cysteine biosynthesis are indicated. The *B. subtilis* genes used in this study are in parentheses. APS, adenosine 5′-phosphosulfate; CoA, coenzyme A.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *B. subtilis* strains used in this work are listed in Table 1. *E. coli* TGI (K-12 Δ[*lac pro*] *supE thi hsd5*/F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*Δ*M15*) was used for the cloning experiments. *E. coli* cells were grown in Luria-Bertani broth. *B. subtilis* was grown in SP medium (31) or minimal medium (6 mM K_2HPO_4, 4.4 mM KH_2PO_4, 0.3 mM trisodium citrate, 5 mM $MgCl_2$, 0.5% glucose, 50 mg of L-tryptophan liter^{-1}, 22 mg of ferric ammonium citrate liter^{-1}, 0.1% L-glutamine, 200 mM xylose) supplemented with a sulfur source (either 1 mM K_2SO_4, 1 mM L-methionine, 2.5 mM glutathione, 1 mM thiosulfate, 0.5 mM sulfite, 0.5 mM sulfide, or L-cysteine supplied as 1 mM L-cystine, depending on the experiment). Minimal medium plates were prepared by adding 17 g of Noble agar (Difco) liter^{-1} to the liquid medium. Antibiotics were added at the following concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 5 μg ml^{-1}; kanamycin, 5 μg ml^{-1}; spectinomycin, 100 μg ml^{-1}; erythromycin plus lincomycin, 1 and 25 μg ml^{-1}, respectively. Standard procedures were used to transform *E. coli* (30) and *B. subtilis* (15). All experiments were performed in accordance with European regulations concerning the use of genetically modified organisms (level 1 containment; agreement 2735).

Amylase activity was detected as previously described (33). β-Galactosidase-specific activity was measured according to the method described by Miller (22) with cell extracts obtained by lysozyme treatment. One unit of β-galactosidase is defined as the amount of enzyme that produces 1 nmol of *O*-nitrophenol min^{-1} at 28°C. Protein concentrations were determined by the method of Bradford.

DNA manipulation and plasmid construction. Plasmid DNA from *E. coli* and chromosomal DNA from *B. subtilis* were prepared according to standard procedures. Restriction enzymes, *Taq* DNA polymerase, and phage T4 DNA ligase were used as recommended by the manufacturers. PCR products were purified by use of the Qiaquick kit (Qiagen). DNA sequences were determined by the dideoxy chain termination method with the Thermo Sequenase kit (Amersham Pharmacia Biotech).

Nucleotides are numbered relative to the transcriptional start sites of the different genes.

A 6,187-bp DNA fragment containing the *cysL* gene (formerly *ywfK*) was cloned into plasmid pDIA5304 to give pDIA5373 (29). A spectinomycin resistance cassette (25) was inserted into the unique *Xba*I site of the *cysL* gene within pDIA5373. The resulting plasmid, pDIA5619, was linearized with *Sca*I and used to transform *B. subtilis* strain 168. The *cysL* gene was disrupted with the spectinomycin resistance cassette by a double-crossover event, giving rise to strain BSIP1168.

The *cysL* gene was cloned into pX containing a xylose-inducible promoter (10) to complement the *cysL*::*spc* mutant. The complete coding sequence of *cysL* (nucleotides +30 to +980) was amplified by PCR and inserted into the *Spe*I and *Bam*HI sites of pX, producing pDIA5561. This plasmid was used to transform BSIP1168, leading to the integration of the *cysL* gene at the *amyE* locus (Table 1).

To disrupt the *cysJI* genes, two DNA fragments containing the 5′ end of the *cysJ* gene (nucleotides −104 to +1091) and the 3′ end of the *cysI* gene (nucleotides +3043 to +3623) were amplified by PCR. These two PCR fragments and a kanamycin resistance cassette (35) were inserted into pUC18 (Roche), producing pDIA5568. This plasmid was linearized at its unique *Sca*I site and used to transform *B. subtilis* strain 168. The *cysJI* genes were deleted by a double-crossover event, giving rise to strain BSIP1206.

pAC6 (33) was used to construct transcriptional fusions between a series of *cysJ* promoter regions with various 5′ deletions and the promoterless *lacZ* gene. Regions ΔA (−163, +82), ΔB (−76, +82), ΔC (−70, +82), and ΔD (−52, +82) were amplified by PCR, such that 5′ *Eco*RI and 3′ *Bam*HI sites were incorporated. The PCR products were inserted into pAC6, giving rise to pDIA5578 (ΔA), pDIA5588 (ΔB), pDIA5589 (ΔC), and pDIA5579 (ΔD), respectively. The different fusions were integrated at the *amyE* locus of *B. subtilis* strains 168 and BSIP1168 (Table 1).

Transcriptional fusions between a series of *cysL* promoter regions with 3′ deletions and the *lacZ* gene were also constructed and integrated at the *amyE* loci of strains 168 and BSIP1168. Regions ΔA (−167, +111), ΔB (−167, +19), and ΔC (−167, +31) were amplified by PCR. The fragments were inserted into pAC6, giving rise to pDIA5559 (ΔA), pDIA5596 (ΔB), and pDIA5584 (ΔC), respectively.

The same strategy was used to introduce a *cysH′-lacZ* fusion and a *cysK′-lacZ* fusion at the *amyE* loci of strains 168 and BSIP1168. A 431-bp *Sma*I-*Bam*HI DNA fragment, corresponding to the *cysH* promoter region (positions −105 to +326), was inserted into pAC6, leading to pDIA5618. A 518-bp *Eco*RI-*Bam*HI fragment, corresponding to the *cysK* promoter region (positions −169 to +349), was inserted into pAC6, producing pDIA5566.

RNA isolation and analysis. The Trizol reagent (Gibco-BRL) was used to extract total RNA from *B. subtilis* 168 that had been grown in minimal medium supplemented with 1 mM thiosulfate. Reverse transcription-PCR (RT-PCR) experiments were performed with the Access RT-PCR introductory system kit (Promega). Three independent reactions with different pairs of primers corresponding to either *cysJ*, *cysI*, or *cysJ* and *cysI*, were used to detect specific transcripts. A control experiment without reverse transcriptase was used to ensure that no contaminating DNA was present.

In the primer extension experiments, oligonucleotides IV156 (*cysJ*) and IV159 (*cysL*) (for the positions of the primers see Fig. 2) were labeled with [γ-^{32}P]ATP

TABLE 1. Bacterial strains used in this study[a]

Strain	Genotype	Source
168	*trpC2*	Laboratory stock
BSIP1168	*trpC2 cysL*::*spc*	pDIA5619→168
BSIP1195	*trpC2 cysL*::*spc amyE*::p*xyl-cysL cat*	pDIA5561→BSIP1168
BSIP1196	*trpC2 amyE*::pΔA*cysL′-lacZ cat*	pDIA5559→168
BSIP1197	*trpC2 cysL*::*spc amyE*::pΔA*cysL′-lacZ cat*	pDIA5559→BSIP1168
BSIP1206	*trpC2* Δ*cysJI*::*aphA3*	pDIA5568→168
BSIP1207	*trpC2 amyE*::*cysK′-lacZ cat*	pDIA5566→168
BSIP1210	*trpC2 cysL*::*spc amyE*::*cysK′-lacZ cat*	pDIA5566→BSIP1168
BSIP1219	*trpC2 amyE*::pΔA*cysJ′-lacZ cat*	pDIA5578→168
BSIP1220	*trpC2 cysL*::*spc amyE*::pΔA*cysJ′-lacZ cat*	pDIA5578→BSIP1168
BSIP1221	*trpC2 amyE*::pΔD*cysJ′-lacZ cat*	pDIA5579→168
BSIP1222	*trpC2 cysL*::*spc amyE*::pΔD*cysJ′-lacZ cat*	pDIA5579→BSIP1168
BSIP1234	*trpC2 amyE*::pΔC*cysL′-lacZ cat*	pDIA5584→168
BSIP1235	*trpC2 cysL*::*spc amyE*::pΔC*cysL′-lacZ cat*	pDIA5584→BSIP1168
BSIP1238	*trpC2 amyE*::pΔB*cysJ′-lacZ cat*	pDIA5588→168
BSIP1239	*trpC2 cysL*::*spc amyE*::pΔB*cysJ′-lacZ cat*	pDIA5588→BSIP1168
BSIP1240	*trpC2 amyE*::pΔC*cysJ′-lacZ cat*	pDIA5589→168
BSIP1241	*trpC2 cysL*::*spc amyE*::pΔC*cysJ′-lacZ cat*	pDIA5589→BSIP1168
BSIP1252	*trpC2 amyE*::pΔB*cysL′-lacZ cat*	pDIA5596→168
BSIP1253	*trpC2 cysL*::*spc amyE*::pΔB*cysL′-lacZ cat*	pDIA5596→BSIP1168
BSIP1283	*trpC2 amyE*::*cysH′-lacZ cat*	pDIA5618→168
BSIP1284	*trpC2 cysL*::*spc amyE*::*cysH′-lacZ cat*	pDIA5618→BSIP1168

[a] Arrows indicate construction by transformation. *cat* is the pC194 chloramphenicol acetyltransferase gene, *aphA3* is an *Enterococcus faecalis* kanamycin resistance gene, and *spc* is a spectinomycin resistance gene from *Staphylococcus aureus*.

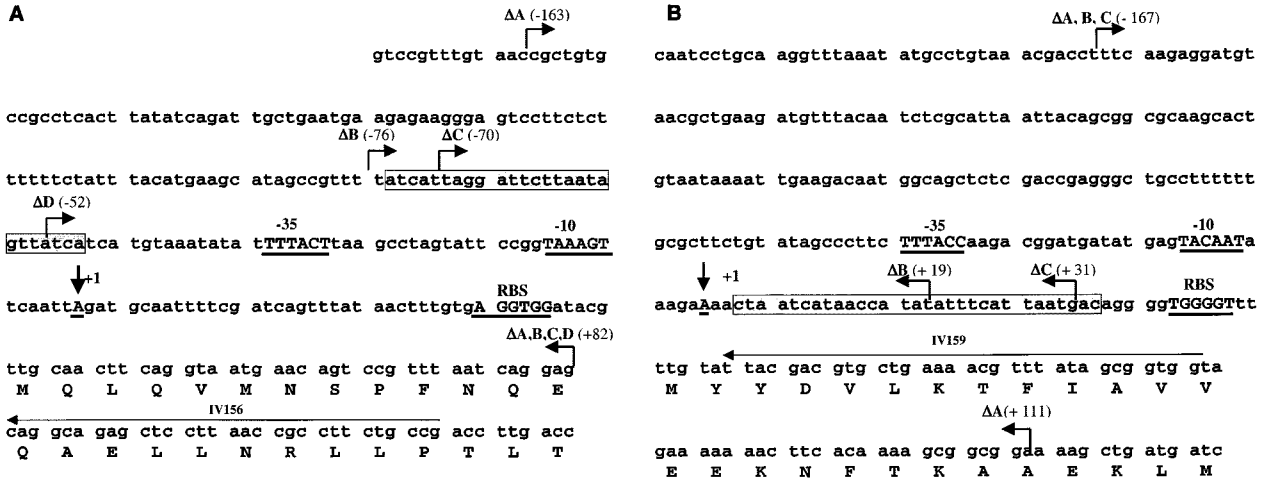

FIG. 2. Promoter regions of the *cysJI* operon (A) and of the *cysL* gene (B). Vertical arrows, transcription start sites (+1) of the *cysJ* and *cysL* promoters. The −35 and −10 regions and the proposed ribosome-binding sites (RBS) are capitalized and underlined. Bent arrows, deletion end points of the different fusions with the *lacZ* gene (numbering is with respect to the transcriptional start site); shaded boxes, DNA regions protected by CysL in the DNase I footprint experiments. Oligonucleotides IV156 and IV159 are indicated.

and hybridized with 10 μg of RNA. For the *cysL* primer extension experiment, RNA was extracted from a strain transformed with pDIA5643, a pHT315 derivative containing the promoter region, and the 5′ part of the *cysL* gene (nucleotides −167 to +448). Elongation of the DNA primer using reverse transcriptase and analysis of the products were performed as previously described (30). The same primers were used for the generation of a sequence ladder.

Overproduction of the CysL protein in *E. coli*. The *cysL* gene (nucleotides +46 to +940) was amplified by PCR such that a 5′ *Nde*I site and a 3′ *Xho*I site were introduced. The amplified gene was cloned into the pET20b$^+$ vector (Novagen), which had been digested with the same enzymes. The resulting plasmid, pET20b$^+$*cysL*, was introduced into *E. coli* strain BL21(DE3) (Novagen), which contains pDIA17 (24), encoding the LacI repressor. Transformants were grown in Luria broth at 30°C to an optical density at 600 nm of 2. The expression of the *cysL* gene was induced by adding 5 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The cells were harvested 2 h later by centrifugation, resuspended in 0.1 M potassium phosphate buffer (pH 7.3), and disrupted in a Fastprep apparatus (Bio 101). The lysate was clarified by centrifugation for 30 min at 8,000 × *g* and used for the gel shift and DNase I footprint experiments. The overproduction of CysL was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Comparison of a crude *E. coli* extract, which contains pET20b$^+$*cysL* or pET20b$^+$ alone, revealed the presence of an additional band at 34 kDa corresponding to CysL (data not shown).

Gel mobility shift assay. DNA fragments corresponding to the promoter regions of *cysJ* (nucleotides −163 to +82), *cysL* (nucleotides −167 to +111), *cysH* (nucleotides −105 to +326), and *cysK* (positions −169 to +349) were amplified by PCR using specific primers 5′ end labeled with [γ-^{32}P]ATP. Unincorporated oligonucleotides were removed with the QIAquick-spin PCR purification kit (Qiagen). Protein-DNA complexes were formed in 10-μl volumes by incubating the ^{32}P-end-labeled DNA fragments with different amounts of crude *E. coli* extract containing the CysL protein in binding buffer (20 mM HEPES [pH 7.6], 4 mM $MgCl_2$, 100 mM KCl, 0.5 mM dithiothreitol, 0.2 mM EDTA, 6% glycerol) in the presence of 5 ng of poly (dI-dC) μl^{-1} and 0.1 μg of bovine serum albumin μl^{-1}. After 30 min at 25°C, 5 μl of loading buffer (0.125% [wt/vol] bromophenol blue, 0.125% [wt/vol] xylene cyanol, 10 mM Tris-HCl, 1 mM EDTA, 5% glycerol) was added to the reaction mixture. Samples were separated on a 6% polyacrylamide native gel in Tris-borate-EDTA buffer at 4°C at 10 V cm^{-1} for 1 h.

DNase I footprinting. The labeled DNA fragments corresponding to the *cysJ* and the *cysL* promoter regions were used to analyze the CysL protected regions in DNase I footprinting reactions. Briefly, constant amounts of the 5′-end-labeled 245-bp top strand DNA fragment of the *cysJ* promoter region (nucleotides −163 to +82) or the 278-bp bottom strand DNA fragment of the *cysL* promoter region (nucleotides −167 to +111) were incubated without protein or with various concentrations of the crude *E. coli* extract containing the CysL protein. The binding conditions were similar to those used for gel mobility shift assays. DNase I (0.05 U μl^{-1}; Roche) was added to the reaction mixtures. After 30 s at 25°C, the DNase I was inactivated by adding 40 μl of phenol and 190 μl of stop solution (0.4 M sodium acetate, 2.5 mM EDTA, 50 μg of poly[dI-dC] ml^{-1}, 10 μg of glycogen ml^{-1}). For each reaction, DNA was then subjected to ethanol precipitation followed by electrophoresis in a 7 M urea-polyacrylamide gel containing 6% acrylamide in Tris-borate-EDTA buffer. The sequence ladder (G+A) was produced as previously described (20).

RESULTS

CysL (YwfK) is a regulator of sulfur metabolism in *B. subtilis*. Three LysR-type activators are involved in the regulation of sulfur metabolism in *E. coli*: CysB, Cbl, and MetR (8, 11, 21). Nine of the 14 LysR-type regulators of unknown function in *B. subtilis* (14) were tested for their possible role in the regulation of sulfur metabolism. The genes encoding eight of these regulators (*yofA*, *ytlI*, *yusT*, *yvbU*, *ywqM*, *yclA*, *yxjO*, and *yybE)* were inactivated as part of European and Japanese projects for the functional characterization of the *B. subtilis* genome (http://locus.jouy.inra.fr/cgibin/genmic/madbase/progs/madbase.operl and http://bacillus.genome.ad.jp). We disrupted the *ywfK* gene by inserting a spectinomycin resistance cassette. The resulting mutants were screened on minimal medium plates in the presence of sulfate, cystine, or methionine as the sulfur source. Interestingly, the *B. subtilis ywfK* mutant (BSIP1168) could not grow in the presence of sulfate or sulfite, but grew similarly to the wild-type strain in the presence of sulfide, thiosulfate, cystine, or methionine (Table 2). A single copy of the *ywfK* gene, expressed under the control of the *xylA* promoter, was introduced into the *amyE* locus of the BSIP1168 strain. The presence of *ywfK* in *trans* restored the growth of the *ywfK*::*spc* mutant on all of the sulfur sources tested (Table 2).

The phenotypes of a *B. subtilis ywfK* mutant and of an *E. coli cysB* mutant are very similar, except that the *cysB* mutant is unable to grow in the presence of thiosulfate (11), whereas the *B. subtilis ywfK* mutant can grow on this sulfur source. Each of the LysR-type transcriptional regulators contains an N-terminal DNA-binding domain with a helix-turn-helix (HTH) motif and two regions probably involved in coinducer recognition and/or response (32). A sequence comparison revealed that

TABLE 2. Phenotypes of the *B. subtilis cysL* and *cysJI* mutants grown in the presence of various sulfur sources[a]

Sulfur source	Generation time (min) for strain[b]			
	168	BSIP1168	BSIP1195	BSIP1206
Sulfate	55	NG[c]	55	NG
Sulfite	70	NG	70	NG
Sulfide	55	55	ND[d]	70
Cystine	55	55	55	60
Methionine	50	55	55	ND
Thiosulfate	55	60	55	ND

[a] All strains were grown in minimal medium. This medium was supplemented with a sulfur source at 1 mM except for sulfite and sulfide (0.5 mM). For strain BSIP1195, 5 mM xylose was added to the medium to induce transcription from the *xylA* promoter.
[b] For strain genotypes, see Table 1.
[c] NG, no growth.
[d] ND, not determined.

the YwfK polypeptide has only 28% identity to CysB of *E. coli*. Only 3 of the 18 residues known to be important for the activity of CysB are conserved in YwfK (16). Accordingly, the YwfK polypeptide was not able to restore the growth of a *cysB* mutant (CB64, *cysB93*) in the presence of sulfate (data not shown), indicating that YwfK cannot substitute for CysB. The fact that the *ywfK* gene could not complement the *cysB* mutant led us to rename the YwfK regulator CysL.

Regulation of the expression of the *cysJI* operon. The *cysJ* and *cysI* genes of *B. subtilis* were recently shown to encode the two subunits of the sulfite reductase (37), which reduces sulfite to sulfide (Fig. 1). The *cysJ* and *cysI* genes are adjacent in the *B. subtilis* chromosome, suggesting that these two genes are organized in an operon. To confirm this hypothesis, RT-PCR experiments were performed with total RNA isolated from *B. subtilis* 168 grown in the presence of thiosulfate. Three DNA fragments were amplified by using primer pairs specific to either *cysJ*, *cysI*, or *cysJ* and *cysI* (data not shown). A single DNA fragment corresponding to a *cysJI* common transcript was detected, confirming that *cysJ* and *cysI* form an operon. The translation initiation codon of *cysJ* is a TTG preceded by a consensus ribosome-binding site. The 5′ end of the *cysJI* transcript was identified by primer extension analysis using total RNA extracted from the wild-type strain. Transcription was initiated at a single A located 44-bp upstream of the translational start point (Fig. 3A). This initiation start site is slightly different from the one identified previously, which is located 46 bp upstream of the translation initiation codon of *cysJ* (37). The deduced −35 (TTTACT) and −10 (TAAAGT) boxes of the promoter are quite similar to the consensus sequences for σ^A-dependent promoters.

The phenotype of the *cysL* mutant corresponds to the phenotype expected for a mutant inactivated in the *cysJI* genes. The Δ*cysJI*::*aphA3* mutant (BSIP1206) was unable to grow when sulfate or sulfite was the sole sulfur source (Table 2). To study the regulation of the expression of the *cysJI* operon in response to sulfur availability and the role of CysL in this regulation, a *cysJ′-lacZ* transcriptional fusion containing the promoter region from positions −163 to +82 was integrated at the *amyE* locus of *B. subtilis* 168 (strain BSIP1219) and of the *cysL* mutant (strain BSIP1220). In strain BSIP1219, the level of β-galactosidase activity was high in the presence of sulfate or sulfite but was 10-fold lower when cystine was the sulfur source (Table 3). An intermediate level of expression was observed in the presence of thiosulfate, and a low level of expression was detected in the presence of glutathione, methionine, or cystine (Table 3). These results confirm that the transcription of the *cysJI* operon is sulfur source dependent. To investigate the role of CysL in the regulation of the *cysJI* operon, we then compared the level of expression of this fusion in the wild-type and *cysL* backgrounds. The expression of a *cysJ′-lacZ* fusion was 11-, 4-, and 2.5-fold lower in the *cysL* mutant than in the wild-type strain for cells grown in the presence of thiosulfate,

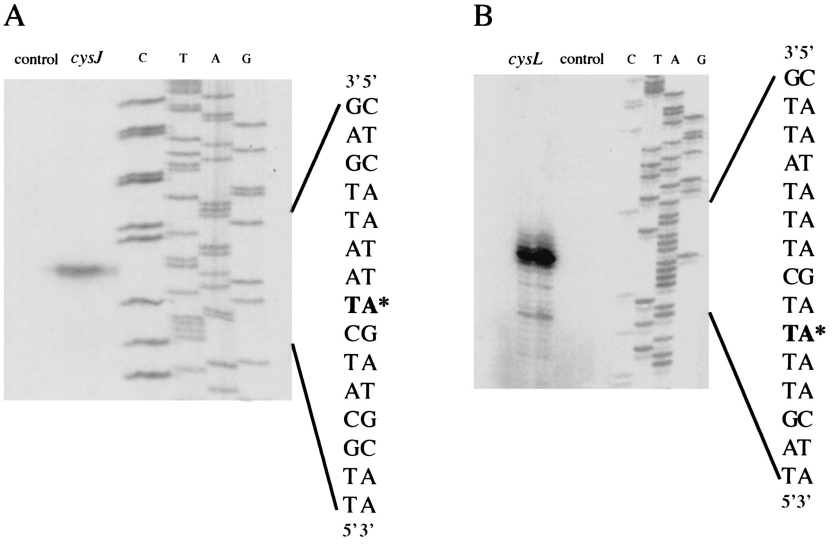

FIG. 3. Mapping of the transcription start sites of the *cysJI* operon (A) and *cysL* gene (B) by primer extension. Primer extension experiments were performed using oligonucleotides IV156 (A) and IV159 (B), which hybridized with the *cysJ* and *cysL* genes, respectively. The labeled oligonucleotides were loaded as a control. Sequencing reactions (lanes C, T, A, and G) were performed with the same oligonucleotides and pDIA5578 (*cysJ*) or pDIA5582 (*cysL*) as the template. ∗, 5′ end of mRNA.

TABLE 3. Expression of *cysJ′-lacZ*, *cysH′-lacZ*, and *cysK′-lacZ* fusions in the presence of different sulfur sources

Sulfur source	β-Galactosidase activity (U mg of protein^{-1})[a] in the indicated strain for:					
	cysJ′-lacZ		*cysH′-lacZ*		*cysK′-lacZ*	
	BSIP1219 (*cysL*$^+$)	BSIP1220 (*cysL::spc*)	BSIP1283 (*cysL*$^+$)	BSIP1284 (*cysL::spc*)	BSIP1207 (*cysL*$^+$)	BSIP1210 (*cysL::spc*)
Sulfate	190	NG	600	NG	120	NG
Sulfite	260	NG	470	NG	90	NG
Thiosulfate	75	7	760	1,590	120	910
Cystine	20	5	400	360	110	130
Glutathione	40	50	1,035	1,360	680	640
Methionine	40	15	625	660	ND	ND

[a] Cells were grown in minimal medium in the presence of the indicated sulfur source (1 mM) except for sulfite (0.5 mM) and glutathione (2.5 mM). The β-galactosidase activities were obtained from cultures in mid-exponential growth. The values represent means of at least three independent experiments. Standard deviations were less than 20% of the means. NG, no growth; ND, not determined.

cystine, or methionine as the sole sulfur source (Table 3). The CysL regulator is therefore involved in the transcriptional activation of the *cysJI* operon.

Role of the CysL regulator in the expression of the *cysH* operon and of the *cysK* gene. To determine whether CysL is necessary for the regulation of *cysH* and *cysK* (Fig. 1), *cysH′-lacZ* and *cysK′-lacZ* transcriptional fusions were integrated at the *amyE* loci of a wild-type strain and of a *cysL* mutant (Table 1). β-Galactosidase activity was measured after growth of the resulting strains in minimal medium in the presence of different sulfur sources (Table 3). The levels of expression of each fusion in the presence of cystine, sulfate, sulfite, or thiosulfate were similar. In contrast, the levels of expression of the *cysH′-lacZ* and *cysK′-lacZ* fusions were 2.5- to 6-fold higher in the presence of glutathione than in the presence of cystine. In a *cysL* mutant, the expression of the *cysH′-lacZ* and the *cysK′-lacZ* fusions, after growth in the presence of thiosulfate, were 2- and 7.5-fold higher than that for the wild-type strain, respectively (Table 3). For these fusions, there was no difference in expression between the wild-type and *cysL* strains after growth in the presence of cystine or glutathione. Therefore, the transcription of the *cysH* operon and that of the *cysK* gene are increased in a *cysL* background in the presence of thiosulfate.

Regulation of the expression of the *cysL* gene. The translation initiation codon of *cysL* is a TTG preceded by a consensus ribosome binding site (Fig. 2B). The 5′ end of the *cysL* transcript was identified by primer extension using total RNA extracted from a strain containing a plasmid carrying the promoter region and the 5′ part of the *cysL* gene (nucleotides −167 to +448). Transcription was initiated at a single A located 46 bp upstream of the translational start point (Fig. 3B). The deduced −35 (TTTACC) and −10 (TACAAT) boxes (Fig. 2B) of the promoter are quite similar to the consensus sequences for σ^A-dependent promoters.

To study the regulation of expression of the *cysL* gene, the *cysL* promoter region (positions −167 to +111) was fused to the *lacZ* gene to create a transcriptional *cysL′-lacZ* fusion. A single copy of this fusion was integrated at the *amyE* loci of the wild-type strain and of the *cysL* mutant, resulting in strains BSIP1196 and BSIP1197, respectively. The β-galactosidase activity was measured for these strains after growth in minimal medium in the presence of various sulfur sources. The expression of the *cysL* gene was low and was not modulated by the sulfur source (data not shown). However, the expression of a *cysL′-lacZ* fusion was twofold higher in a *cysL* mutant (8 U mg of protein^{-1}) than in a wild-type strain (4 U mg of protein^{-1}). This result suggests that the CysL protein negatively regulates its own transcription, as observed for other LysR-type transcriptional regulators (9, 32).

Binding of the CysL regulator to the promoter regions of genes involved in cysteine biosynthesis. We used electrophoretic mobility shift assays to investigate the capacity of CysL for binding to the DNA of the promoter regions of the *cysJ*, *cysL*, *cysH*, and *cysK* genes. The *B. subtilis* CysL protein was overproduced in *E. coli*, and the cell-free crude extract was used in the DNA-binding experiments.

A 245-bp radiolabeled DNA fragment corresponding to the *cysJ* promoter region (positions −163 to +82) was incubated with increasing amounts of the CysL protein. Native polyacrylamide gel electrophoresis revealed that the band corresponding to the 245-bp labeled fragment was shifted when the CysL protein was present (Fig. 4A). Indeed, the DNA fragment containing the *cysJ* promoter region was fully retarded by 50 ng of the *E. coli* extract containing CysL (Fig. 4A, lane 9). This shift was not observed with 200 ng of the *E. coli* extract without CysL (Fig. 4A, lane 2). The addition of an excess of an unlabeled DNA fragment corresponding to the *cysJ* promoter region prevented CysL binding to the same labeled DNA fragment. In contrast, an excess of a cold noncompetitor DNA fragment (the *serA* promoter region) did not prevent CysL binding to the *cysJ* promoter region (data not shown). These results demonstrate that CysL binds specifically to the *cysJ* promoter region.

As CysL regulates its own transcription, a 278-bp DNA fragment containing the *cysL* promoter region (positions −167 to +111) was used in similar binding studies (Fig. 4B). A gel shift was observed only with the *E. coli* extract overproducing CysL, and 50 ng of the CysL crude extract caused complete retardation of the DNA fragment containing the *cysL* promoter region (Fig. 4B, lane 9). The specificity of this interaction was verified under the same experimental conditions as those described for the binding of CysL to the *cysJ* promoter region. The CysL regulator bound specifically to its own promoter in a dose-dependent manner.

Gel mobility shift assays were also performed to determine if CysL can directly interact with the *cysH* and *cysK* promoter regions. The labeled DNA fragments containing the *cysH* and *cysK* promoter regions (nucleotides −105 to +326 and −169 to +349, respectively) were tested with increasing amounts of the *E. coli* crude extract containing CysL (10 ng to 1 μg). A limited amount of the DNA fragment corresponding to the *cysK* promoter region was retarded in the presence of 1 μg of an *E. coli* crude extract containing CysL (data not shown). The affinity of CysL for the *cysK* promoter region was about 20-fold lower than its affinity for the *cysJ* or *cysL* promoter regions. No detectable binding of CysL to the *cysH* promoter region was observed in the conditions tested (data not shown).

Characterization of a *cis*-acting target in the promoter re-

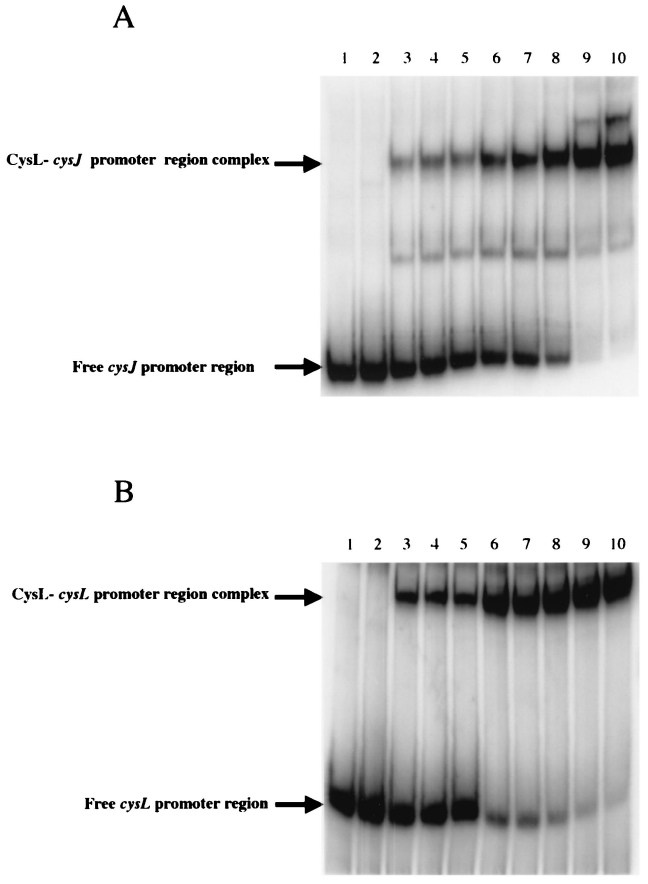

FIG. 4. Binding of the CysL regulator to a *cysJ* or *cysL* promoter fragment in a gel mobility shift assay. A 245-bp 5′-labeled fragment containing the *cysJ* promoter region (positions −163 to +82) (A) or a 278-bp fragment containing the *cysL* promoter (nucleotides −167 to +111) (B) was incubated with various amounts of a cell-free crude *E. coli* extract either containing overproduced CysL or not. Lane 1, free DNA probe; lane 2, negative control with a crude *E. coli* extract without CysL (200 ng); lanes 3 to 10, increasing amounts of the *E. coli* extract overproducing CysL (10, 12, 15, 18, 20, 30, 50, and 100 ng, respectively).

gions of the *cysJ* and the *cysL* genes. To identify the DNA sequences involved in the sulfur-dependent regulation of the *cysJI* operon, *cysJ* promoter regions containing various 5′ deletions were fused to the promoterless *lacZ* gene (Fig. 2A). A single copy of these fusions was introduced at the *amyE* locus of *B. subtilis* 168. β-Galactosidase activities in the different strains grown in minimal medium in the presence of sulfate, thiosulfate, or cystine as a sulfur source were measured (Table 4). The ΔA (−163, +82) and ΔB (−76, +82) *cysJ′-lacZ* fusions were expressed 10- and 4-fold more highly in the presence of sulfate or thiosulfate than in the presence of cystine. The ΔC (−70, +82) and ΔD (−52, +82) *cysJ′-lacZ* fusions, which were expressed at lower levels, were weakly or not regulated in these conditions. This indicates that the DNA fragment located between positions −76 and −70 of the *cysJ* promoter is important for the sulfur source-dependent regulation of the *cysJI* operon. To identify *cis*-acting DNA sequences required for activation of the *cysJI* operon by the CysL regulator, the same fusions were introduced into a *cysL* background. β-Galactosidase activities of the wild-type and *cysL*::*spc* strains after growth in the presence of thiosulfate were compared (Table 4). The ΔA (−163, +82) and ΔB (−76, +82) *cysJ′-lacZ* fusions were expressed 10-fold more highly in the wild-type strain than in the *cysL* mutant. No activation by CysL was observed with the ΔC (−70, +82) and the ΔD (−52, +82) *cysJ′-lacZ* fusions. Thus, the same DNA fragment (positions −76 to −70) is important for both the CysL-dependent and the sulfur source-dependent regulation of the *cysJI* operon.

TABLE 4. Effect of the sulfur source and of the CysL regulator on the expression of different *cysJ′-lacZ* transcriptional fusions[a]

Transcriptional fusion of the *cysJ* promoter at the *amyE* locus	β-Galactosidase activity (U mg of protein^{-1}) in the presence of:			
	Sulfate[b] (*cysL*$^+$)	Cystine[b] (*cysL*$^+$)	Thiosulfate for strain with genotype:	
			cysL$^+$	*cysL*::*spc*
ΔA (−163, +82) *cysJ′-lacZ*	190	20	75	7
ΔB (−76, +82) *cysJ′-lacZ*	200	20	90	9
ΔC (−70, +82) *cysJ′-lacZ*	20	4	7	6
ΔD (−52, +82) *cysJ′-lacZ*	3	2	3	6

[a] β-Galactosidase activities were determined in extracts prepared from exponentially growing cells (optical density at 600 nm, 0.8 to 1). The values represent means of at least three independent experiments. Standard deviations were less than 15% of the means. The *cysL*$^+$ and *cysL*::*spc* strains used in this study are described in Table 1.
[b] Relevant strain genotype is in parentheses.

To identify the *cis*-acting DNA target required for the autoregulation of *cysL* expression, the *cysL* promoter region with different 3′ deletions was fused to the *lacZ* gene. The fusions were introduced into strains 168 and BSIP1168, and the β-galactosidase activity was measured after growth of the strains with thiosulfate as a sulfur source. The ΔA (−167, +111) and ΔC (−167, +31) *cysL′-lacZ* fusions were twofold more highly expressed in the *cysL* mutant (8 and 22 U mg of protein^{-1}, respectively) than in the wild-type strain (4 and 9 U mg of protein^{-1}, respectively). No regulation by CysL was observed with the ΔB (−167, +19) *cysL′-lacZ* fusion. These results indicate that the DNA fragment located between nucleotides +19 and +31 of the *cysL* promoter is important for negative autoregulation by CysL.

DNase I protection assays of the *cysJ* and *cysL* promoter regions by CysL. DNase I footprint experiments were carried out to determine the precise location of the CysL binding site(s) within the *cysJ* and *cysL* promoter regions. Comparison of the sequence patterns produced in the absence of CysL and in the presence of saturating concentrations of CysL located protected regions consisting of 25 nucleotides on the top strand of the *cysJ* promoter region (Fig. 5) and 29 nucleotides on the bottom strand of the *cysL* promoter region (data not shown). The binding sites extended from positions −75 to −50 and from positions +4 to +33 relative to the transcriptional start sites of *cysJ* and *cysL,* respectively.

These results are consistent with the roles of the CysL protein as activator of the expression of the *cysJI* operon and repressor of the expression of the *cysL* gene. These experiments also confirm the location of the *cis*-acting target sequences identified by using deletions of the *cysJ* and of the *cysL* promoter regions.

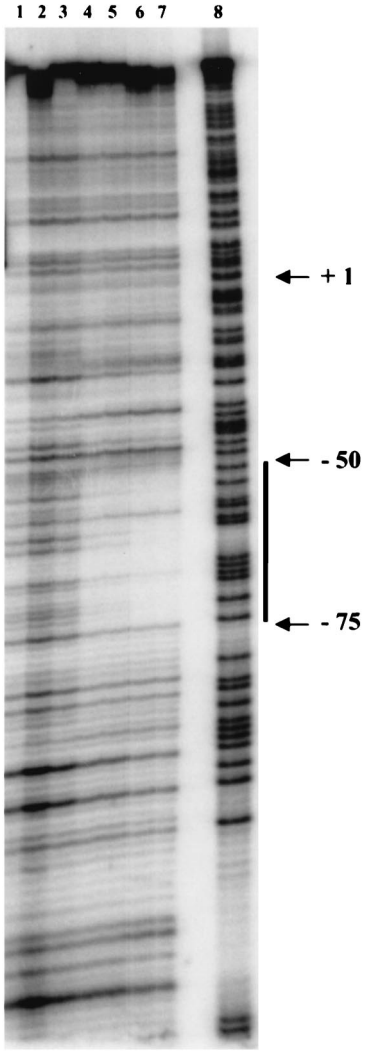

FIG. 5. DNase I footprint of the CysL regulator on the *cysJ* promoter region. The 245-bp PCR fragment representing the coding strand of the *cysJ* promoter region (positions −163 to +82) was 5′ end labeled, and incubated in separate reaction mixtures without protein (lane 1), in the presence of 200 ng of a crude protein extract without CysL (lane 2), or in the presence of 10 (lane 3), 50 (lane 4), 100 (lane 5), 200 (lane 6), or 300 ng (lane 7) of the crude *E. coli* extract overproducing CysL and subjected to DNase I digestion. Lane 8, G+A sequencing ladder (20). Vertical bar, position of the protected region. Numbers indicate the distances from the transcription initiation site of the *cysJI* operon.

DISCUSSION

The regulation of methionine and cysteine biosynthesis genes in *E. coli* involves two LysR-type transcriptional activators (CysB and MetR) and a repressor (MetJ) (11). In this study, we identified a new member of the LysR regulator family, CysL (YwfK), which is involved in the regulation of expression of the *cysJI* operon, encoding both subunits of the sulfite reductase of *B. subtilis*. The CysL protein is the first regulator of at least one step of the sulfur-containing amino acid biosynthesis pathway to be identified in *B. subtilis*. Proteins with a high level of similarity to CysL have been found in *Bacillus stearothermophilus* (60% identity), *Bacillus anthracis* (45% identity), *Clostridium difficile* (44% identity), *Clostridium acetobutylicum* (40% identity), and *Listeria monocytogenes* (45% identity). No CysL-like protein could be detected in *L. lactis*, *Bacillus halodurans*, or *Staphylococcus aureus*. CmbR, a newly characterized LysR-type transcriptional activator of *L. lactis*, controls the expression of the *metC-cysK* operon (2). The CysL and CmbR proteins show only 25% identity, suggesting that these two regulators are not functionally equivalent. Moreover, the genes responsible for sulfate uptake and reduction are absent from the *L. lactis* genome. Sulfur metabolism and its regulation are probably different in *B. subtilis* and *L. lactis*.

Most of the genes encoding members of the LysR family are adjacent to and transcribed divergently from the genes they regulate (32). This is not the case for the *B. subtilis cysL* gene. A few other LysR-type transcriptional regulator genes, such as *E. coli cysB* and *oxyR*, are also located some distance from their target genes on the chromosome. Interestingly, a *B. stearothermophilus* gene encoding a protein with high similarity to CysL was found to be located adjacent to a *cysJI*-like operon (http://www.genome.ou.edu). The CysL-like activator of *B. stearothermophilus* probably controls the expression of this operon.

In enteric bacteria, the *cysJ* and *cysI* genes are cotranscribed with the *cysH* gene (17, 27). In contrast, in *B. subtilis* and *B. stearothermophilus*, *cysJ* and *cysI* form an operon, whereas *cysH* belongs to another operon encoding the first steps of the sulfate assimilation pathway (18). The location of *cysH* and *cysJI* in two independent transcriptional units suggests that the synthesis of PAPS sulfotransferase could be regulated differently from the synthesis of sulfite reductase in *B. subtilis*. Indeed, the *cysJI* operon was transcribed at a high level when the cells were grown in the presence of sulfate or sulfite. Transcription was 10-fold lower when cells were grown in the presence of cystine. The levels of expression of the *cysH* and *cysK* (encoding the cysteine synthase) genes were not regulated by these sulfur sources (this work; 1, 18, 37). The transcription of the *cysJI* operon is positively controlled by the CysL activator, whereas the transcription of the *cysH* and *cysK* genes is higher in a *cysL* mutant. These results indicate that the transcription of the *cysJI* operon in response to sulfur availability is regulated differently from that of the *cysH* and *cysK* genes. The expression of the *cysJI* operon can either be repressed by cysteine or induced by an intermediate compound from the sulfate assimilation pathway, via the CysL regulator. As in *E. coli* and *S. enterica* serovar Typhimurium (11, 13, 17), the *cysK* and *cysH* genes of *B. subtilis* were expressed at the highest levels in sulfur-limiting conditions (in the presence of glutathione or during cystine limitation) (this work; 1, 18). A yet-unidentified repressor, CysR, was recently proposed to prevent the transcription of *cysH* in these conditions (18). The activity of this CysR regulator is probably controlled by the intracellular concentration of *O*-acetylserine, which may induce the expression of the *cys* genes. The CysL regulator is not this CysR repressor. Indeed, CysL is not involved in the regulation of the *cysK* and *cysH* genes in sulfur-limiting conditions (Table 3) (I. Guillouard, unpublished results). This suggests that the cysteine biosynthetic pathway is controlled at several levels in *B. subtilis* and that this control involves at least two regulators, CysR and CysL. The precise roles of these regulators deserve further investigation.

Like most members of the LysR family, the CysL protein can also inhibit the transcription of its structural gene. The expression of the *cysL* gene is independent of the sulfur source added to the medium. A genetic approach showed that the expression of four different genes or operons (*cysH*, *cysJ*, *cysK*, and *cysL*) is modified in a *cysL* background. The CysL protein can specifically bind in vitro to the *cysJ* and *cysL* promoter regions in the absence of any added cofactor, as observed for several LysR-type regulators (32). CysL may also interact with the *cysK* promoter region, but with a much lower affinity. The specificity and the physiological significance of this low binding deserve further experiments. The mobility shift assays did not show that CysL can bind to the *cysH* promoter region. The derepression of *cysH* expression in a *cysL* mutant in the presence of thiosulfate could be due to the depletion of sulfite reductase synthesis, which may modify the concentrations of several metabolites of the cysteine biosynthesis pathway.

The DNA sequence of the *cysJ* promoter region found to be protected in the DNase I footprint experiment is located between positions −76 and −50. This is consistent with the genetic data indicating that the −76 to −70 DNA region is important for sulfur- and CysL-dependent regulation. Similar experiments with the *cysL* promoter region showed that the DNA sequence extending from nucleotides +19 to +31 is necessary for *cysL* negative autoregulation. These locations correspond to the positions of the recognition sites of other LysR-type regulators (32). Many LysR-type-regulated promoters contain characteristic sequence $TN_{11}A$ at the core of a larger motif with dyad symmetry (3, 32). Sequence ATTA-N_7-TAAT, consistent with this consensus motif, was found upstream of the *B. subtilis* and *B. stearothermophilus cysJI* operons. However, this motif was not found in the *cysK* promoter region and in the protected DNA sequence of the *cysL* promoter region. The target sequences of the OxyR and CysB regulators, which control a large set of genes, are not highly conserved (6, 34). This could be also the case for CysL. Additional experiments are necessary to determine the precise DNA sequences required for recognition by CysL.

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