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Bacillus subtilis Cysteine Synthetase Is a Global Regulator of the Expression of Genes Involved in Sulfur Assimilation

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The synthesis of L-cysteine, the major mechanism by which sulfur is incorporated into organic compounds in microorganisms, occupies a significant fraction of bacterial metabolism. In *Bacillus subtilis* the *cysH* operon, encoding several proteins involved in cysteine biosynthesis, is induced by sulfur starvation and tightly repressed by cysteine. We show that a null mutation in the *cysK* gene encoding an *O*-acetylserine-(thiol)lyase, the enzyme that catalyzes the final step in cysteine biosynthesis, results in constitutive expression of the *cysH* operon. Using DNA microarrays we found that, in addition to *cysH*, almost all of the genes required for sulfate assimilation are constitutively expressed in *cysK* mutants. These results indicate that CysK, besides its enzymatic role in cysteine biosynthesis, is a global negative regulator of genes involved in sulfur metabolism.

Sulfur is a vital element for all living organisms since it is required for the synthesis of proteins and essential cofactors. Sulfur can be assimilated from inorganic sources such as sulfate and thiosulfate or from organic ones such as sulfate esters, sulfamates, and sulfonates. The assimilatory reduction of sulfate and formation of cysteine has been extensively studied in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (18). At least 22 genes required for the transport and reduction of sulfate and its incorporation into cysteine have been identified in these bacteria. Most of these genes form part of the cysteine regulon (18). Full expression of this regulon requires a LysR-type transcriptional activator, encoded by *cysB*, sulfur limitation, and a signal of sulfur limitation provided by *N*-acetyl-L-serine (NAS), which functions as an internal inducer. CysB protein binds just upstream of the −35 region of positively regulated promoters where, in the presence of inducer, it facilitates formation of a transcription initiation complex. Sulfide and thiosulfate provide additional regulation, acting as anti-inducers by inhibiting binding of CysB protein to *cys* promoters (18, 26). CysB also controls the expression of the *ssu* and *tau* genes which are required for the utilization of taurine and other aliphatic sulfonates as the sulfur source (40). Expression of these genes also requires the LysR-type regulator Cbl (15). The expression of *cbl* itself is under the control of CysB, so CysB may be considered the main regulator for sulfur assimilation in *E. coli*. In the case of the *tau* genes both regulators, Cbl and CysB, must bind to the promoter region in order to activate their expression (40). The requirement of CysB binding for *ssu* genes expression is less well understood (39).

The pathway by which *Bacillus subtilis* utilizes inorganic sulfate as sulfur source seems to be very similar to that used by *E. coli*. Indeed, *B. subtilis* possess all of the genes and enzymes required to convert sulfate into sulfide and for the incorporation of sulfide into cysteine (20, 27) (Fig. 1). The *B. subtilis* *cysH* operon encodes a 3′-phospho-adenosine-5′-phosphosulfate (PAPS) reductase, a sulfate permease (CysP) and the enzymes catalyzing the reduction of sulfate to sulfite (22, 23). The *cysI* and *cysJ* genes encoding the two subunits of the sulfite reductase are part of an operon and were recently identified (37). Once sulfide is synthesized it is condensed with *O*-acetyl-L-serine (OAS) in a reaction catalyzed by a *O*-acetylserine-(thiol)-lyase (or cysteine synthetase), coded by the *cysK* gene, to give cysteine. In addition, *B. subtilis* contains two *cysK* paralogs, *yrhA* and *ytkP*. However, CysK seems to be the main enzyme catalyzing this final step. This conclusion is based on the observation that although a *cysK* mutant can still grow with sulfate, betanesulfonate, and sulfite as sole sulfur sources, its growth rates are significantly lower than those of the wild-type strain growing in similar conditions (37).

Many efforts have been made to understand the regulation of sulfur assimilation in gram-positive bacteria. Several genes involved in methionine biosynthesis form a regulon controlled by a global transcriptional termination system called the S-box regulon (12). In this system, *S*-adenosylmethionine (SAM) seems to be the effector directly binding to and inducing changes in the leader RNA. In this way, SAM would control the transcription level of this regulon (8, 24). Although the *cysH* operon contains an S-box like motif, its expression, which is induced by sulfur starvation and repressed by cysteine, is regulated at the transcription initiation level by a mechanism that appears to involve a transcriptional repressor (21). A model was proposed in which an increase in the intracellular levels of OAS would promote the dissociation of a putative transcriptional repressor (CysR) from its operator, allowing high level expression of the *cysH* operon (21). It has been shown that the *cysJI* operon transcription is activated by a LysR-type transcriptional regulator named CysL (14). The *cysJI* operon is transcribed at a higher level when cells are grown in the presence of sulfate or sulfite. CysL also represses its own transcription but independently of the sulfur source

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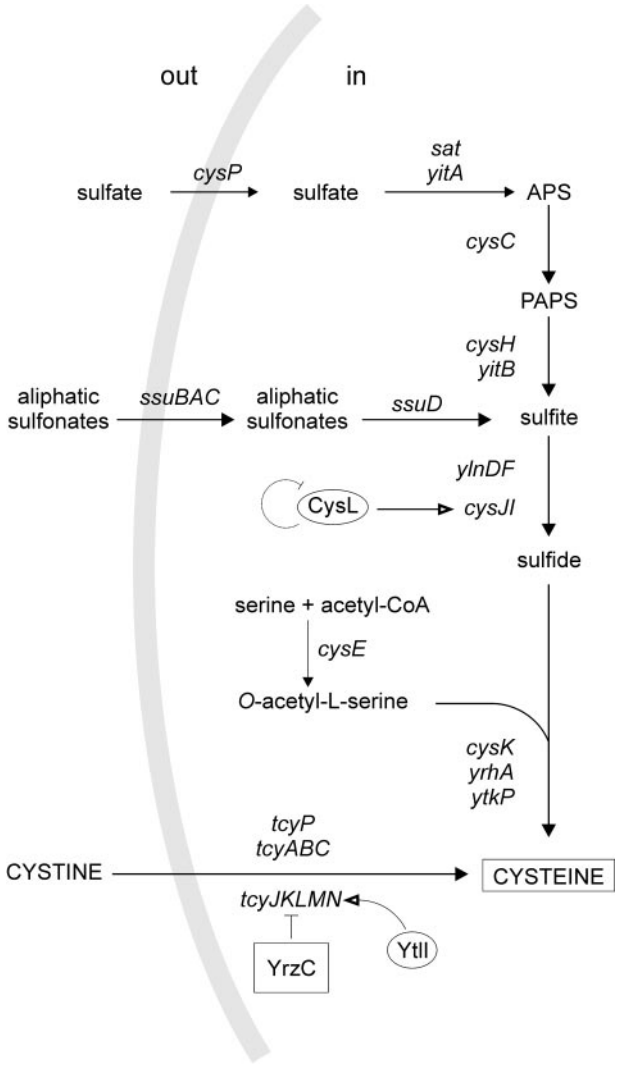

FIG. 1. Pathway for sulfate assimilation and biosynthesis of cysteine in *B. subtilis.* Genes involved in each step are indicated. The effect of the CysL, YtlI, and YrzC transcriptional regulators on their target genes is indicated with open arrowheads (positive regulation) or bars (negative regulation).

added to the medium. Although the expression levels of *cysH* and *cysK* are higher in a *cysL* mutant, CysL is not involved in the regulation of *cysH* and *cysK* genes under sulfur-limiting conditions, suggesting that the cysteine biosynthetic pathway is controlled at different levels in *B. subtilis* (14).

Recently, three systems involved in L-cystine uptake have been characterized. Two of them, TcyABC and TcyJKLMN, are ABC transporters, whereas TcyP is a symporter (4). TcyJKLMN participates also in the uptake of cystathionine, *S*-methylcysteine, djenkolic acid, and other sulfur compounds (4). This high-affinity transporter is part of the *ytmI* operon which also includes genes coding for a riboflavin kinase, a putative acetyltransferase and a putative amidohydrolase (5, 33). The expression of this operon responds to sulfur availability being higher under sulfur limitation (4). YtlI is a positive regulator of the *ytmI* operon and its expression is also regulated in response to the sulfur source being high in the presence of methionine and low in the presence of sulfate (1, 4, 5). Recently, another putative regulator encoded by the *yrzC* gene was involved in the regulation of the *ytmI* operon. YrzC was shown to be a negative regulator of this operon, although no direct binding of YrzC to the *ytmI* promoter was proven until now (33).

The expression of the *ssu* operon, which is required for the assimilation of sulfur from sulfonates, is repressed by cysteine and sulfate and is 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, but no regulator has been identified yet (36). Interestingly, a *B. subtilis* mutant containing a transposon insertion in the *cysK* gene, which does not belong to any of the aforementioned sulfur-related operons, exhibited constitutive expression of the *ssu* operon and the *cysK* gene itself (37). These results suggest that CysK could also have a regulatory function, or that the accumulation of a metabolite as a consequence of CysK inactivation would be responsible for the derepression of the *cysK* and *ssu* transcriptional units (37). Taking into account that sulfonate and sulfate assimilatory pathways are related and that CysK catalyzes the last step of such pathways, we decided to determine whether a *cysK* mutation also affects *cysH* expression.

We show here that *cysH*, as well as almost all of the genes required for sulfate assimilation, is constitutively expressed in *cysK* mutants and that this expression is not due to OAS accumulation. Our results provide evidence for a novel mechanism for global control of sulfur assimilation in which CysK couples the status of sulfur availability with the expression of genes involved in cysteine biosynthesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in the present study are listed in Table 1. *E. coli* and *B. subtilis* strains were routinely grown in Luria-Bertani (LB) broth (30). Spizizen salts (35), supplemented with 0.4% glucose and the required L-amino acids for particular strains, were used as the minimal medium for *B. subtilis*. In nutritional studies, where different sulfur sources were tested, $MgSO_4$ and $(NH_4)_2SO_4$ were substituted by an equimolecular amount of $MgCl_2$ and NH_4Cl, respectively (sulfur-free minimal medium). Glutathione (1 mM) and cysteine supplied as cystine (1 mM), were used as sulfur sources for *B. subtilis*. Since *cysK* mutants grow poorly on sulfate, butanesulfonate and sulfite, we grew them in minimal medium supplemented with 0,05% casein hydrolysate and 0.01% yeast extract. In experiments testing in vivo effects of OAS, the medium was adjusted to pH 6.8 before autoclaving to minimize conversion of OAS to NAS. Antibiotics were added to media at the following concentrations: ampicillin (Amp), 100 μg ml^{-1}; chloramphenicol (Cm), 5 μg ml^{-1}; erythromycin (Em), 1 μg ml^{-1}; lincomycin (Lm), 25 μg ml^{-1}; and spectinomycin (Sp), 100 μg ml^{-1}.

Plasmids and strain construction. The *cysK* gene was amplified by PCR using oligonucleotides CYSK_UP (5′-TACCAAagCttATAGTCGGAAATTGAGGTG-3′) and CYSK_DW (5′-GcaGgAtccAATATAAAAAAACTCCCG-3′) as primers and JH642 chromosomal DNA as a template. Small letters indicate variations respect to wild-type *B. subtilis* sequence. Restrictions sites are underlined. The correct sequence of the amplification product was confirmed by DNA sequence analysis (DNA Sequencing Facility, Maine University).

To construct plasmid pDK1, PCR-amplified *cysK* gene was cloned under the control of the xylose-inducible promoter P*xyl* (17) into pGES45 (G. E. Schujman, unpublished data), which derives from vector pDG795 (13).

In order to obtain *cysK* mutants, the parental strains were transformed with chromosomal DNA of the *cysK* strain SB11 (Table 1) and selected on LB plates supplemented with the proper antibiotics.

Genetic techniques. Plasmid preparations, restriction enzyme digestions, and agarose gel electrophoresis were carried out as described previously (30). *E. coli*-competent cells were transformed with supercoiled plasmid DNA by

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
Bacillus subtilis		
JH642	*trpC2 pheA1*	Laboratory stock.
JH642K	JH642 *cysK*::mini-Tn*10*; Sp^r Cm^r	This study
DA642X	JH642 *amyE*::pJM116X; Cm^r	This study
DA642X-K	DA642X *cysK*::mini-Tn*10*; Sp^r Cm^r	This study
MC530	JH642 *amyE*::P*cysH-lacZ*	21
1A3	*cysE trpC2 purA26*	BGSC[b]
1A3-530	1A3 *amyE*::P*cysH-lacZ*	21
SB11	Sp100 BS168 *cysK*::mini-Tn*10*; Sp^r	37
MCK530	MC530 *cysK*::mini-Tn*10*; Sp^r Cm^r	This study
DAOK-530	1A3-530 *cysK*::mini-Tn*10*; Sp^r Cm^r	This study
MCK530-DK1	MCK530 *thrC*::P*xyl-cysK*; Sp^r Cm^r Em^r Lm^r	This study
MCK530-C795	MCK530 *thrC*::pDG795; Sp^r Cm^r Em^r Lm^r	This study
Escherichia coli DH5α	*supE44 thi-1* Δ*lacU169*(ϕ80*lacZ*ΔM15) *endA1 recA1 hsdR17 gyrA96 relA1 trp6 cysT329*::*lac inm*$^{\lambda p1(209)}$	Laboratory stock
Plasmids		
pJM116	Integrative vector to construct transcriptional fusions to *lacZ*; integrates at the *amyE* locus of *B. subtilis*; Cm^r	6
pJM116X	Contains the xylose promoter of plasmid pRDC9 cloned in the EcoRI-HindIII sites of pJM116	21
pDG795	Integrates at the *thrC* locus of *B. subtilis*; Em^r Lm^r	13
pGES45	Xylose promoter and repressor cloned into pDG795	G. Schujman, personal communication
pDK1	*cysK* cloned under P*xyl* in pGES45	This study

[a] Cm^r, Em^r, Lm^r, and Sp^r denote resistance to chloramphenicol, erythromycin, lincomycin, and spectinomycin, respectively.
[b] Strain obtained from the *Bacillus* Genetic Stock Center.

using the calcium chloride procedure (30). Transformation of *B. subtilis* was carried out by the method of Dubnau and Davidoff-Abelson (7). The *amy* phenotype was assayed as previously described (32).

β-Galactosidase assays. *B. subtilis* strains harboring *cysH-lacZ* fusions were grown overnight in LB medium. The cells were collected by centrifugation and diluted in sulfur-free minimal medium containing cysteine as the sulfur source. When cultures reached exponential phase of growth, cells were collected by centrifugation, washed several times with sulfur-free minimal medium, and finally resuspended in sulfur-free minimal medium supplemented with the appropriate compounds, as described in each experiment. Samples were taken at 1-h intervals or at the indicated times after resuspension and assayed for β-galactosidase activity as previously described (22). Specific activity was expressed in Miller units (MU) (25). In cases where xylose was used as inducer, glycerol was used as carbon source to prevent catabolic repression.

DNA microarrays analysis. RNA was extracted, labeled, and analyzed with *B. subtilis* DNA microarrays as described previously (3). Briefly, *B. subtilis* JH642 strain was grown overnight in sulfur-free minimal medium containing glutathione or cysteine as the sulfur source to compare the expression profile in both sulfur sources. In order to compare wild-type and *cysK* mutant expression profile, the strains were grown overnight in sulfur-free minimal medium with cystine as a sulfur source supplemented with casein hydrolysate and yeast extract. Cells were collected by centrifugation and diluted in the same medium to an optical density at 600 nm (OD_{590}) of 0.2. The cultures were grown at 37°C for 4 h. Appropriate volumes of culture were used in each case according to the optical density at the collection time. Culture samples were fixed with −20°C methanol and RNA extracted with RNeasy minikit (QIAGEN). Labeled cDNA was generated from RNA samples by direct incorporation of Cy3- or Cy5-labeled dUTP into cDNA. Differentially labeled samples from two different conditions were mixed and hybridized to the DNA microarrays, and each experiment was done three times. Images were processed and analyzed with GenePix 3.0 software (Axon Instruments, Inc.). Background signal was not subtracted from the signal intensity of the spots. Once spots with significant signals were identified (to be considered a valid signal, 40% of the pixels in a spot had to be at least 1 standard deviation above the local background in at least one of the channels), the two channels were normalized by making the total signal in each of the channels equal. The ratios from the independent samples were $\log_2$ transformed, and then the data for each individual spot were averaged between the three replicate experiments (independently grown and prepared samples). The geometric mean and standard deviation of the entire population were then calculated. Any spot that had a ratio that was more than 2.5 standard deviations away from the mean was considered an outlier. All of the microarrays data are available upon request.

RESULTS

***B. subtilis O*-acetylserine-(thiol)-lyase, encoded by *cysK*, is involved in the transcriptional regulation of the *cysH* operon.** Recently, it has been shown that a *B. subtilis* mutant containing a transposon insertion in the *cysK* gene exhibited constitutive expression of genes involved in the utilization of sulfur from aliphatic sulfonates (37). Since sulfonates and sulfate assimilatory pathways are related (22, 37), we attempted to determine whether the *cysH* operon was regulated by CysK. To this end, we constructed a *cysK* mutant derived from strain MC530, a JH642 derivative which contains a *cysH-lacZ* fusion in the nonessential *amyE* locus of *B. subtilis* genome (Table 1). This mutant strain was named MCK530. Strains MC530 and MCK530 were assayed for β-galactosidase activity, according to the protocol described in Materials and Methods, in the absence or presence of cystine. As shown in Fig. 2A, MCK530 exhibited high β-galactosidase activity levels regardless of the presence of this amino acid in the growth medium. Four hours after resuspension, the β-galactosidase activity of MCK530 in the presence of cystine was 10-fold higher than the activity of MC530 under the same culture conditions, indicating that repression by this amino acid was lost in the *cysK* strain. Moreover, this effect was specific since a mutation in *cysK* did not affect the transcription from the non-sulfur-dependent promoter P*xyl* (strains DA642X and DA642X-K, Fig. 2B).

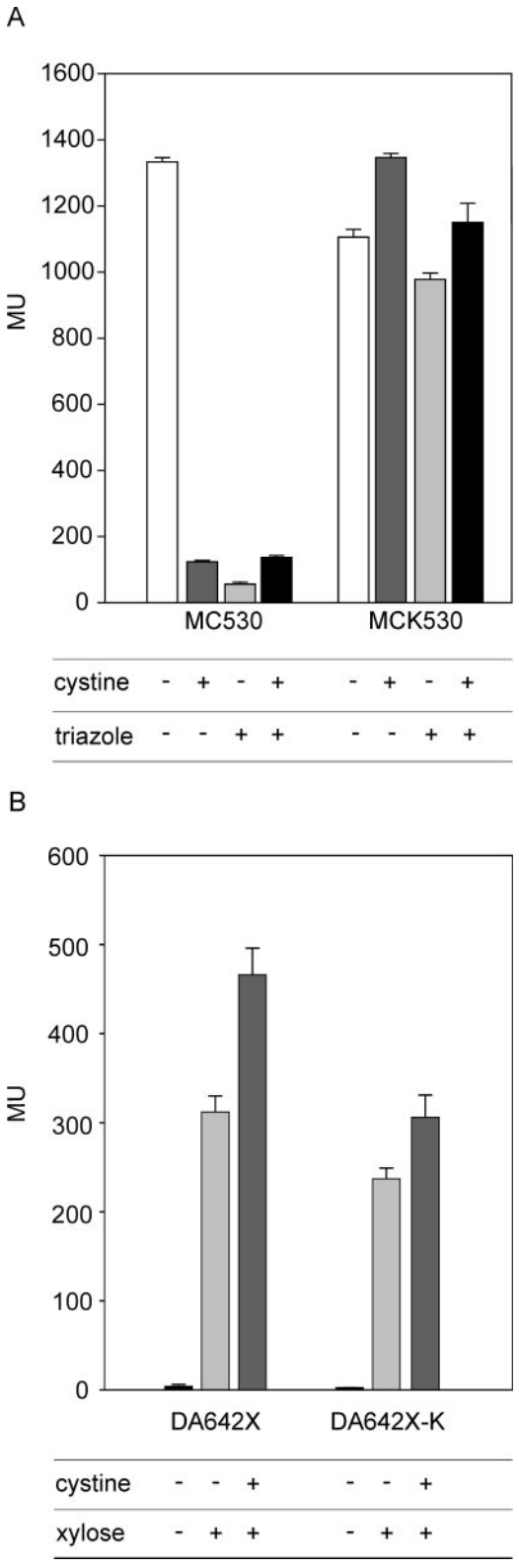

FIG. 2. Constitutive expression of the *cysH* operon in a *cysK* background. Strains MC530 (P*cysH-lacZ*) and MCK530 (P*cysH-lacZ*, *cysK*) (A) and DA642X (P*xyl*-lacZ) and DA642X-K (P*xyl-lacZ*, *cysK*) (B) were grown in sulfur-free minimal media supplemented with cystine, casein hydrolysate, and yeast extract. Cells were recovered by centrifugation and inoculated in sulfur-free minimal medium in the absence or presence of cystine. The addition of 5 mM 1,2,4-triazole or 0.8% xylose is indicated. Samples were taken 4 h after resuspension. β-Galactosidase activities are expressed in MU.

To test whether *cysK* provided in *trans* was able to reestablish cysteine-dependent regulation of *cysH*, we constructed strain MCK530-DK1, which is a derivative of MCK530 containing the *cysK* gene under the control of the xylose-inducible P*xyl* promoter ectopically integrated at the *thrC* locus (Table 1). Strains MCK530-DK1 and the control strain MCK530-C795 (Table 1) were grown in minimal medium supplemented with threonine, cystine, casein hydrolysate, yeast extract, and xylose. When cultures reached the exponential phase of growth, cells were recovered by centrifugation and washed several times with sulfur-free minimal medium. Then, cells were inoculated in minimal medium supplemented with threonine and xylose in the presence or in the absence of cystine. As shown in Fig. 3A, in strain MCK530-DK1 cystine repressed the expression of *cysH-lacZ*, as observed in a *cysK*$^+$ wild-type strain. However, in the control strain MCK530-C795 the β-galactosidase activity coded by *cysH-lacZ* was not repressed by cystine. It is important to note that when the same experiment was carried out in the absence of xylose, both strains exhibited high levels of β-galactosidase activities regardless the presence of cystine (Fig. 3B).

These results show that in the absence of CysK the *cysH* operon is constitutively expressed, indicating that CysK plays a key role in the sulfur-dependent transcriptional control of the *cysH* operon.

Constitutive expression of *cysH* in a *cysK* background is not due to OAS accumulation. In *Salmonella enterica* serovar Typhimurium, CysK catalyzes a reaction (triazolylase) between OAS and 1,2,4-triazole, giving 1,2,4-triazole-1-alanine as a product (19), which reduces the intracellular concentration of OAS. We have previously reported that OAS is an inducer of *cysH* expression and that this effect on *cysH* transcription is independent of sulfur starvation and insensitive to cystine repression (21). Accordingly, when cultures of strains MC530 grown in sulfur-free minimal medium were supplemented with 5 mM 1,2,4-triazole, transcriptional activation of *cysH* did not take place either in the absence or in the presence of cystine (Fig. 2A). However, addition of 1,2,4-triazole to the *cysK* mutant MCK530 (Fig. 2A) did not result in repression of *cysH* transcription, presumably because this strain is unable to reduce the OAS pools in the presence of the inhibitor. Since CysK catalyzes the condensation of OAS with sulfide to form cysteine, these data suggested that derepression of *cysH* in a *cysK* mutant could be due to accumulation of OAS. To test this possibility, we constructed strain DAOK-530, a *cysK* mutant derived from strain 1A3-530 which is an OAS auxotroph. Strains 1A3-530 and DAOK-530 were treated as indicated in Materials and Methods for β-galactosidase activity determination. In the last step, the cells were diluted in sulfur-free minimal medium supplemented either with cystine or cystine plus OAS. Four hours after resuspension, *cysH* expression was low in strain 1A3-530 unless the inducer OAS was present (Fig. 4). In contrast, in the *cysK* strain DAOK-530 *cysH* expression was derepressed in the absence of external OAS, although this

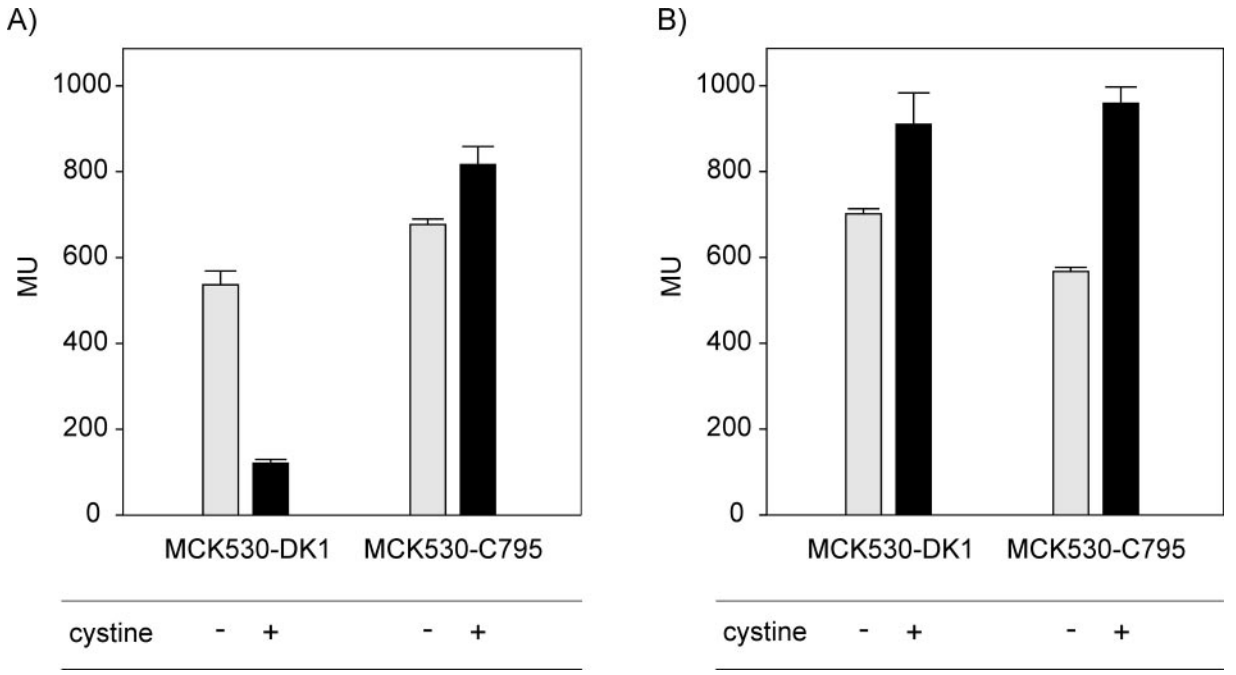

FIG. 3. *cysK* is a *trans*-acting regulator of *cysH* expression. (A) Strains MCK530-DK1 (P*cysH-lacZ*, *cysK thrC*::P*xyl-cysK*) and MCK530-C795 (P*cysH-lacZ*, *cysK thrC*::pDG795) were grown in sulfur-free-minimal medium supplemented with threonine, cystine, casein hydrolysate, yeast extract, and xylose. When cultures reached the exponential phase of growth, cells were recovered by centrifugation and washed several times with sulfur-free minimal media. The cells were then resuspended in sulfur-free minimal media supplemented with threonine and xylose in the absence or in the presence of cystine. Samples were taken 4 h after resuspension. β-Galactosidase activities are expressed in MU. (B) Cells were treated as described in panel A legend, but the experiment was carried out in the absence of xylose.

strain is unable to synthesize the inducer, and it was not affected by the addition of OAS (Fig. 4).

These results indicate that *cysH* derepression in *cysK* mutants was not due to an accumulation of OAS, thus suggesting that CysK could play a more direct role in the transcriptional regulation of this operon.

***cysK* is a global negative regulator of genes involved in sulfur metabolism.** To see whether other genes involved in sulfur metabolism, in addition to the *cysH* and *ssu* operons, are upregulated in a *cysK* mutant, we compared the whole transcriptome of strain JH642 grown with either glutathione or cystine as the sole sulfur source with that of a *cysK* isogenic strain, JH642K, grown with cystine as a sulfur source. To this end, the cultures were grown for 4 h in these media, and the genomic expression profiles of the samples were analyzed by using DNA microarrays containing 4074 of the 4106 protein coding genes of *B. subtilis* (see Materials and Methods).

As expected from our previous results (21) and those from Van der Ploeg et al. (38), the amount of RNA from the *cysH* and *ssu* operons was greatly increased in cells grown in the presence of glutathione, a poor sulfur source. As shown in Table 2, in addition to the genes mentioned above, almost all of the genes coding for proteins with similarity to enzymes involved in cysteine biosynthesis were significantly induced in cells grown in the presence of glutathione. Exceptions were *ytkP*, an *O*-acetylserine-(thiol)-lyase paralog, two open reading frames, *yvdB* and *ybaR*, that would code for proteins similar to sulfate permeases belonging to the SulP family, and the serine transacetylase paralog *yvfD* (Table 2). The fact that *ytkP*, one

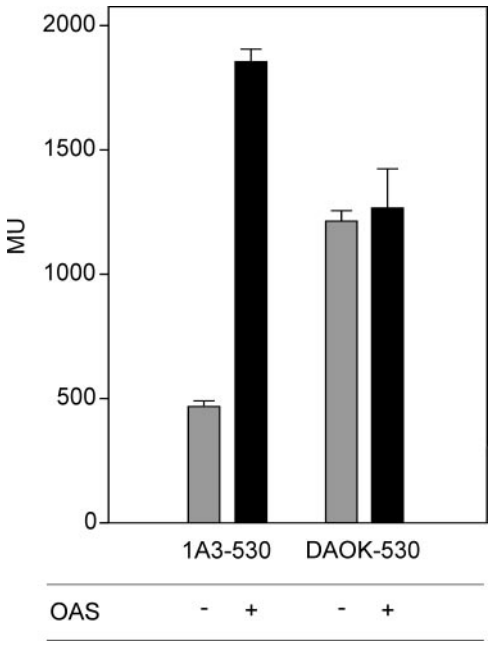

FIG. 4. Derepression of the *cysH* operon in a *cysK* background is not due to OAS accumulation. Strains 1A3-530 (*cysE*, P*cysH-lacZ*) and DAOK-530 (*cysE*, P*cysH-lacZ*, and *cysK*) were grown and recovered by centrifugation as described in Fig. 2. Washed cells were then inoculated in sulfur-free minimal media supplemented with cystine or cystine plus OAS. Samples were taken 4 h after resuspension. β-Galactosidase activities are expressed in MU.

TABLE 2. Expression profile of sulfur metabolism-related genes in strains JH642 and JH642K

Gene	Localization (°)	Function and/or similarity	Ratio	
			JH642[a] Glut/Cys	JH642K/JH642[b]
cysS	9	Cysteinyl-tRNA synthetase	(1.3)[c]	(1)
cysE	9	Serine acetyltransferase	(1.3)	(1)
yvfD	300	Similar to serine acetyltransferase	(−)	(0.9)
cysH	139	PAPS reductase	4.7	7
cysP	139	Sulfate permease	5	4
sat	139	Similar to ATP sulfurilase	9	13
cysC	139	Similar to APS kinase	9	15
ylnD	139	UROIII methyltransferase	9	15
ylnF	139	Ferrokelatase	9	5
cysJI	293	Similar to sulfite reductase	3	6
cysL	330	LysR transcriptional regulator	(0.7)	(0.5)
cysK	7	OAS-thiol liase	5.5	3
yrhA	238	Similar to OAS-thiol liase	14	14
ytkP	262	Similar to OAS-thiol liase	(−)	(1)
yisZ	100	Similar to APS kinase	2.7	(0.7)
yitA	100	Similar to ATP sulfurilase	(1.7)	(0.6)
yitB	100	Similar to PAPS reductase	(1.7)	(0.6)
ykuP	127	Similar to sulfite reductase	(0.6)	(1.2)
yvdB	304	Similar to SulP permease	(−)	(0.8)
ybaR	15	Similar to SulP permease	(−)	(0.9)
ssuBACD	82	Sulfonates utilization	7.7	12
ytlI	257	LysR transcriptional regulator	4	4
ytmI	257	Unknown	13	13
tcyJ	257	L-Cystine and sulfur compounds ABC transporter	12	10
tcyK	257	L-Cystine and sulfur compounds ABC transporter	15	10
tcyL	257	L-Cystine and sulfur compounds; ABC transporter	7.8	16
tcyM	257	L-Cystine and sulfur compounds; ABC transporter	5	8
hisP	257	Histidine transporter	16	15
ytmO	257	Similar to monooxygenase	16	22
ytnI	257	Unknown	16	14
ytnJ	256	Similar to nitrilotriacetate monooxigenase	16	20
ribR	256	Riboflavin kinase	16	11.5
hipO	256	Hippurate hydrolase	10	6
ytnM	256	Unknown	10	7
tcyP	84	L-Cystine symporter	2.5	6
tcyA	35	L-Cystine ABC transporter	(1.0)	3
yrzC	240	Negative regulator of *ytmI* operon	(0.8)	(0.9)

[a] Ratio of expression of genes in wild-type cells grown in the presence glutathione (Glut) as a sulfur source compared to wild-type cells grown in the presence of cystine (Cys) as a sulfur source. The average ratios of three independent experiments are shown.

[b] Ratio of expression of genes in *cysK* mutant cells grown in the presence of cystine as sulfur source supplemented with casein hydrolysate and yeast extract compared to wild-type cells grown in the same medium. The average ratios of three independent experiments are shown.

[c] Values in parentheses indicate genes not significantly induced or repressed.

of the two *cysK* paralogs, is not induced would agree with the results obtained by Van der Ploeg et al. (38), which showed that a *cysK yrhA* double mutant is unable to grow with sulfate or thiosulfate as sole sulfur sources. Other induced open reading frames were the recently characterized L-cystine ABC-type transporter TcyJKLMN and the symporter TcyP (4), remarking their role in the adaptation of *B. subtilis* to an environment with a poor sulfur source. We also detected a pronounced induction of the *ytlI* gene and the *ytmI* operon (which includes the *tcyJKLMN* genes) involved in sulfur assimilation (4, 5). These results agree with those obtained by Coppée et al., who compared the protein expression pattern of *B. subtilis* grown in the presence of either sulfate or glutathione (5). Thus, the transcriptional induction observed for *cysK*, *ytmI*, *ssuA*, *ssuD*, and *ytmI* is also reflected in an increase in the synthesis of the corresponding proteins. However, these authors were unable to detect an increase in the synthesis of proteins coded by the *cysH* operon or several other proteins probably involved in cysteine metabolism (5). Our results also agree with those of Burguiere et al., who characterized the L-cystine transporters (4). Surprisingly, *cysE* and *cysS*, which code for the enzymes serine transacetylase and cysteinyl-tRNA synthetase that catalyze the synthesis of OAS and the attachment of cysteine to its cognate tRNA, respectively (11), showed low levels of induction under sulfur limitation. It has been suggested that *cysE* and *cysS*, which are part of an operon, are transcriptionally regulated by the "T box" antitermination mechanism (11), which is widely used by gram-positive bacteria for control of amino acid biosynthesis and aminoacyl-tRNA synthetase gene expression. Although we expected that transcription of the *cysE* and *cysS* genes should respond to variations in the levels of cysteine we cannot discard a different sensitivity of the tRNA charging response.

Notably, the transcriptome analysis of the *cysK* strain JH642K grown in the presence of cystine was practically the same than that of the $cysK^+$ strain JH642, grown in the presence of glutathione (Table 2). This finding shows that in a *cysK* strain all of the cysteine-repressed genes are upregulated, mim-

icking the growth in a medium containing a poor sulfur source. The upregulation induction of the *cysH* operon, the *ssuBACD* operon and *cysK* itself observed in the transcriptome analysis of a *cysK* mutant fully agrees with the β-galactosidase assays of strains bearing fusions to the promoter regions of the *cysH* operon (Fig. 2A), *ssuA* and *cysK* genes (37). These data strongly imply that CysK, in addition to catalyzing the last step in cysteine biosynthesis, may have a global role controlling the expression of genes involved in sulfate assimilation.

DISCUSSION

Expression of *B. subtilis ssu* and *cysH* operons is repressed when sulfate and cysteine serve as sulfur sources, but the mechanism by which repression by these two sulfur compounds is achieved is not well understood. Although expression of the *ssu* genes seems to be regulated at the level of both transcription initiation and transcription attenuation (36), the *cysH* operon is regulated primarily at the level of transcription initiation (21). It has been proposed that a hypothetical repressor, named CysR, prevents transcription of the *cysH* operon unless OAS is present, which would promote dissociation of the repressor from its operator (21). It has also been suggested that a putative repressor could be involved in the regulation of transcription initiation of the *ssu* operon (36). Irrespective of whether or not transcription control of the *ssu* and *cysH* operons involves the same or different proteins, OAS appears to play an important role as an effector in the regulation of these two operons.

Here we show that, as previously reported for the *ssu* operon (37), disruption of the *cysK* gene, which encodes the enzyme that catalyzes the final step in the biosynthesis of cysteine, results in constitutive expression of the *cysH* operon. Notably, this effect is not due to the accumulation of OAS. Moreover, the expression of almost all genes with a putative function in cysteine biosynthesis, as well as of many genes probably involved in sulfur assimilation, is derepressed in the absence of CysK. Since all of these genes are repressed in cells growing on good sulfur sources, the simplest model for the function of CysK in global regulation of sulfur assimilation is that this protein is a sensor of the status of sulfur availability. It is worth mentioning that in a *cysK* background, the *cysJI* operon, which is under the control of CysL activator (14), is also derepressed. This could indicate that CysK is controlling the expression of *cysJI* at another level, possibly in response to a different signal, or that the effector sensed by CysL is accumulating in a *cysK* background.

Another global transcriptional regulator of sulfur amino acid metabolism was recently described in *Lactococcus lactis.* In this organism FhuR, a LysR-type activator, controls the expression of genes involved in the novo synthesis of cysteine, methionine as well as of genes involved in the interconversion of both amino acids (34). In this case it was also suggested that OAS acts as a cofactor of FhuR inducing the expression of the regulon (34). This regulatory mechanism, however, differs significantly from the one used by *E. coli* and *B. subtilis*, where the diversity of regulators and signals allows the independent control of the genes required for the synthesis and interconversion of cysteine and methionine (34).

It is interesting that, similar to the *B. subtilis* sulfur assimilation system, other regulatory circuits contain proteins that have a dual function with enzymatic and regulatory activities. In *E. coli* the Mal regulon is positively regulated by the transcriptional activator MalT. In addition to the positive effectors maltotriose and ATP, three proteins are able to interact with MalT modifying its activity. These are MalK, the ATP-hydrolyzing subunit of the high-affinity maltose transport system (2), Aes, an enzyme with acetyl esterase activity (16, 28) and MalY, an enzyme with cystathionase activity. It has been suggested that these three proteins would inactivate MalT by inhibiting its oligomerization (31). Another example is the E1 subunit of the pyruvate dehydrogenase complex from *Azotobacter vinelandii*, a central enzyme in aerobic respiration, which binds specifically to the *fpr* promoter modulating its transcription. However, the exact mechanism of action has not been determined (29). In *B. subtilis* the glutamine synthetase (GS) is involved in the regulation of the genes involved in nitrogen metabolism (10). GS, which is encoded by the *glnA* gene, synthesizes glutamine from ammonium and glutamate and is the only enzyme capable of assimilating ammonium into cellular metabolites (9). It is important to note the resemblance between the regulation of sulfate and ammonium assimilation in this organism. Both are assimilative reductive pathways and the final product is an amino acid, cysteine or glutamine, respectively. In the sulfate assimilatory pathway CysK, which synthesizes cysteine from sulfide and OAS, seems to be the main enzyme by which sulfide is incorporated into cysteine. In each case, the last enzyme of the synthesis of cysteine and glutamine, CysK and GlnA, respectively, seems to be involved in the regulation of the expression of the genes coding for these proteins. That is, null mutations in *cysK* or *glnA* lead to constitutive activity of their respective promoters (10; this study). However, in contrast to the preliminary description of the role of CysK in the regulation of sulfur metabolism reported here, the control of nitrogen assimilation by GS in *B. subtilis* is well characterized. In this organism, GS directly interacts with the nitrogen regulatory factor TnrA and regulates its DNA-binding activity. In conditions of nitrogen excess, the feedback-inhibited form of GS interacts with TnrA blocking the binding of this transcription factor to its target promoters (41). Our results seems to indicate that similar to the role of GS as a nitrogen sensor, CysK transmits the signal for sulfate availability to as-yet-unidentified transcription factors. Since CysK does not contain a consensus DNA-binding motif and attempts to demonstrate binding of CysK to the *cysK* promoter have failed (J. R. van der Ploeg, unpublished data), we propose that CysK would control gene expression of *cysH* through protein-protein interaction with the CysR putative repressor. Moreover, *cysK* is an autoregulated gene since its expression is increased in the absence of its gene product. Thus, it is tempting to speculate that the regulatory activity of CysK is controlled by a ligand that is an integral component of the sulfur assimilation pathway. A reasonable candidate for the regulatory ligand is OAS because (i) the *cysE* gene responsible for OAS synthesis is not under CysK control (Table 2), (ii) OAS is required for transcriptional activation of the *cysH* and *ssu* operons (21, 36) and cannot be replaced by *N*-acetyl-L-serine (data not shown), and (iii) *cysH* is actively transcribed regardless of the absence of OAS in a *cysK* background

(Fig. 4). In addition, OAS binds to the active site of CysK since this enzyme catalyzes the synthesis of cysteine from sulfide and OAS. In the light of these arguments we propose that the intracellular levels of OAS modulate the ability of CysK to interact with the putative repressor CysR. When intracellular levels of sulfide are low, OAS accumulates. The binding of OAS to CysK presumably alters the protein conformation and decreases the affinity of CysK for CysR, promoting the dissociation of the complex from its operator. In a similar way CysK could also interact with other regulators of sulfate assimilation. Further analysis of this hypothesis, as well as the identification of the putative transcription factors proposed in this work, will be of great interest.

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