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Functional analysis of the *Bacillus subtilis cysK* and *cysJI* genes

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Abstract

The function of the *Bacillus subtilis cysK* and *cysJI* (previously designated *yvgQR*) genes, expected to be involved in the assimilatory sulfate reduction pathway, was investigated. A *B. subtilis* mutant with a deletion in the *cysJI* genes was unable to use sulfate or sulfite as sulfur source, which confirmed that these genes encode sulfite reductase. A mutant with a transposon insertion in the *cysK* gene, whose deduced protein sequence showed similarity to cysteine synthases, grew poorly on sulfate and butanesulfonate. A strain in which *cysK* and *yrhA*, a *cysK* paralog, were inactivated was unable to grow with sulfate. Whereas expression of the *cysJI* genes was induced by sulfate, expression of *cysK* was repressed both by sulfate and by cysteine.

Keywords: Sulfate; Cysteine biosynthesis; Sulfite reductase; Cysteine synthase; *Bacillus subtilis*

1. Introduction

The pathway by which *Bacillus subtilis* utilizes inorganic sulfate as source of sulfur appears to be very similar to the one in *Escherichia coli* (Fig. 1). Sulfate is transported by a sulfate permease [1], rather than by an ABC type transport system like in *E. coli* [2]. The gene encoding sulfate permease, *cysP*, resides in an operon together with the *cysH* gene, which encodes 3′-phospho-adenosine-5′-phosphosulfate (PAPS) reductase [3]. Two other genes in this operon, *ylnB* and *ylnC*, are expected to encode enzymes responsible for activation of sulfate to PAPS [1]. In addition, the 6.0-kb *cysH* operon also contains the genes *ylnD*, *ylnE* and *ylnF*. *ylnD* and *ylnF* are required for biosynthesis of the siroheme cofactor of sulfite reductase [4], and the function of *ylnE* is not known. Until now, little is known about the genes and enzymes involved in the last two steps of cysteine biosynthesis in *B. subtilis*, the reduction of sulfite to sulfide and incorporation of sulfide into *O*-acetylserine (OAS).

The expression of the *cysH* operon is thought to be regulated at the level of transcription initiation [5]. OAS appears to be an important signalling molecule for sulfur limitation, since addition of this compound to the medium resulted in elevated expression of a *cysH′–lacZ* fusion [5]. OAS is synthesized from serine and acetyl-CoA by the product of the *cysE* gene, which is located in one operon together with *gltX*, encoding glutamyl-tRNA synthetase, and *cysS*, which encodes cysteinyl-tRNA synthetase [6]. The expression of *cysE* and *cysS* is regulated by cysteinyl-tRNA-mediated anti-termination of transcription [6].

In the absence of sulfate, *B. subtilis* can utilize aliphatic sulfonates as a source of sulfur. This is enabled by the genes of the *ssuBACDygaN* operon [7], and we have carried out studies to identify effectors and regulators involved in expression of the *ssu* genes [8]. Within this framework, we have investigated the function of the *cysK* gene, encoding cysteine synthase, and that of the *cysJI* genes, which encode sulfite reductase.

2. Materials and methods

2.1. Chemicals

All chemicals used as sulfur source were of the highest quality available and were obtained from Fluka, Acros, Aldrich or Sigma. OAS was from Sigma.

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Table 1
Strains, plasmids and used in this study

Strain	Genotype or relevant properties	Ref. or source
E. coli		
DH5α	*supE44 ΔlacU169*(ϕ80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Life Technologies
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (*Tet*^{r})]	Stratagene
NK3	*ΔtrpE5 leu6 thi cysK cysM rbs*$^+$ *r*$^+$ m_k^+	M. Hryniewicz
B. subtilis		
1A1	*trpC2*	BGSC
BFA2063	*trpC2 yrhA-lacZ ΔyrhA*	W. Schumann
MS-11	*hisA1 trpC2 thr-5 ssuD::lacZ* (Tn*917-lac*)	[7]
MS11-6	*hisA1 trpC2 thr-5 ssuD::lacZ* (Tn*917-lac*) *cysK*::Tn*10*	this study
SB11	*trpC2 cysK*::Tn*10*	this study
SB12	*trpC2 ΔyvgQr*::Nm^r	this study
SB36	*trpC2 amyE*::pME4834	this study
SB27	*trpC2 amyE*::pME4881	this study
SB28	*trpC2 cysK*::Tn*10 amyE*::pME4881	this study
SB29	*trpC2 amyE*::pME4886	this study
SB30	*trpC2 cysK*::Tn*10 amyE*::pME4886	this study
SB49	*trpC2 ΔytkP*::Nm^r	this study
SB50	*trpC2 yrhA-lacZ ΔyrhA cysK*::Tn*10*	this study
SB51	*trpC2 yrhA-lacZ ΔyrhA ΔytkP*::Nm^r	this study
SB52	*trpC2 ΔytkP*::Nm^r, *cysK*::Tn*10*	this study
SB53	*trpC2 yrhA-lacZ ΔyrhA cysK*::Tn*10 ytkP*::Nm^r	this study
Plasmid		
pBluescript II KS	*E. coli* cloning vector, Ap^r	Stratagene
pUC19	*E. coli* cloning vector, Ap^r	[20]
pDH32M	Ap^r Cm^r *amyE* front *amyE* back *lacZ*	[16]
pMLK83	Ap^r Nm^r *amyE* front *amyE* back *gus*	[17]
pRB374	*E. coli*/*B. subtilis* shuttle vector, Nm^r	[21]
pRSM40	pT7T3, *cysK* from *S. typhimurium*	N. Kredich
pBEST501	Ap^r, Nm^r cassette	[15]
pIC333	pBR322 *ori*, repTs, Sp^r Ery^r	E. Bremer
pME4823	pUC19, *cysJ′-kan-′cysI*	this study
pME4833	pBluescript containing *cysJI* promoter region	this study
pME4834	pDH32M containing *cysJI* promoter region	this study
pME4857	$cysK_{ST}$ in pRB374	this study
pME4864	$cysK_{BS}$ in pRB374	this study
pME4881	*cysK′–gusA* in pMLK83	this study
pME4886	*cysK′–gusA* (truncated) in pMLK83	this study
pME4899	pBluescript II KS, *ytkP::kan*	this study
Primer	Sequence (5′–3′)	Used for
BcysK2	GCGAACC**T**G**C**AGTTTTGGC	cloning of *B. subtilis cysK*
BscysK3	AATA**AGCT**TTACAAATAGTCGG	cloning of *B. subtilis cysK*
cysKf3	**GCT**TT**G**CAT**G**CAGTTAAGGACAG	cloning of *S. typhimurium cysK*
cysKr	TTA**GG**AT**C**CTGGCATCACTG	cloning of *S. typhimurium cysK*

Nucleotides in boldface indicate changes from the original sequence to introduce restriction sites.

2.2. Plasmids, primers and bacterial strains

The strains, plasmids and primers used in this study are listed in Table 1. *B. subtilis* and *E. coli* strains were grown at 30 or 37°C in LB medium or in sulfur free M63 medium [9]. Sulfur sources were added to a final concentration of 250 μM. If required, amino acids were added at the following concentrations: L-histidine at 100 μg ml^{-1}, L-threonine at 100 μg ml^{-1} and L-tryptophan at 40 μg ml^{-1}. For *B. subtilis*, antibiotics were added at the following concentrations when necessary: chloramphenicol, 5 μg ml^{-1}; neomycin, 5 μg ml^{-1}; erythromycin, 0.5 μg ml^{-1}; spectinomycin, 100 μg ml^{-1}. For *E. coli*, antibiotics were used at the following concentrations: kanamycin (50 μg ml^{-1}), ampicillin (100 μg ml^{-1}) and chloramphenicol (35 μg ml^{-1}), spectinomycin (100 μg ml^{-1}). Solid media contained 1.5% agar (Serva). The sequences of the primers used for amplification of *B. subtilis* genes were based on the genome sequence [10].

2.3. DNA and RNA manipulation

For plasmid isolation, restriction enzyme digestion, ligation and transformation of *E. coli*, standard procedures

were used [11]. *B. subtilis* was transformed as described [12]. Chromosomal DNA from *B. subtilis* was isolated using the CTAB method [11]. Total RNA was isolated from strain 1A1 grown in minimal medium with sulfate or glutathione as sulfur source to an optical density at 600 nm of approximately 0.5 as described [13]. Primer extension analysis was according to Babst et al. [14].

2.4. Construction of deletion insertions in cysJI and in ytkP

For the construction of a *cysJI* knock-out, a neomycin resistance cassette from pBEST501 [15] was cloned in between two fragments located within the *cysJI* genes which had been generated by PCR. The resulting plasmid, pME4823, was transformed into the wild-type *B. subtilis* strain 1A1 by selecting for neomycin resistance. PCR analysis of one of the resulting colonies (designated SB12) showed that a correct replacement had occurred.

For the construction of strains containing a deletion/insertion of *ytkP*, a similar strategy was used. Two fragments, encompassing the 5′ end of *ytkP* and the 3′ end of *ytkP*, were amplified by PCR and cloned into pBluescript II KS. A neomycin resistance cassette was then cloned in between the two fragments to give plasmid pME4899. Transformation of linearized pME4899 into *B. subtilis* and selection for neomycin resistance yielded *ytkP* mutants.

2.5. Transposon mutagenesis

Transposon mutagenesis was carried out using pIC333, which contains a temperature sensitive origin of replication and harbors a derivative of Tn*10* (Steinmetz and Richter, unpublished). Strain MS11 (*ssuD′–lacZ*), which does not produce sufficient β-galactosidase to form blue colonies on LB plates containing X-gal (40 μg ml^{-1}), was transformed with plasmid pIC333 and grown at 30°C on LB containing spectinomycin. Transformants were grown overnight in LB containing spectinomycin at 30°C, diluted 1:100 in the same medium and grown for 3 h at 30°C. The temperature was then shifted to 37°C and incubation was continued for another 4 h. A total of about 15 000 mutants were screened for the formation of blue colonies on LB plates containing X-gal. Chromosomal DNA from mutants was isolated and digested with *Eco*RI and *Hin*dIII. The DNA was ligated under conditions that favor circularization of fragments and transformed to *E. coli* by selection for spectinomycin resistance. Plasmids were isolated from the resulting transformants, and the sequence flanking the transposon insertion site was determined.

2.6. Construction of cysJ′–lacZ and cysK′–gusA fusions

For the construction of a transcriptional *cysJ′–lacZ* fusion, a 311-bp fragment encompassing 222 bp of the region upstream of the start of *cysJ* was amplified by PCR. The product was cloned in pBluescript KS and in pDH32M [16] to give pME4833 and pME4834 respectively. A transcriptional *cysK′–gusA* fusion was constructed by cloning a 440-bp fragment, encompassing 317 bp of the region upstream of the *cysK* start codon, into pMLK83 [17] to give pME4881. A truncated *cysK′–gusA* fusion, which contained 84 bp of the region upstream of the *cysK* start codon, was constructed on plasmid pME4886. The plasmids containing the fusions were introduced in *B. subtilis* strain 1A1 by transformation [18] by selection for chloramphenicol or neomycin resistance. Double crossover events were confirmed by testing for an *amyE* negative phenotype. β-Galactosidase and β-glucuronidase activities were measured according to Miller [19].

2.7. Cloning of B. subtilis and Salmonella typhimurium cysK genes and complementation experiments

The *cysK* gene from *B. subtilis* was cloned by PCR amplification using the forward primer BcysK2 and the reverse primer BscysK3 (Table 1) with chromosomal DNA from strain BD99 as template. The PCR fragment was digested with *Hin*dIII and *Pst*I and cloned in pRB374 to give pME4864.

The *S. typhimurium cysK* gene was amplified using PCR with the forward primer cysKf3 and the reverse primer cysKr using plasmid pRSM40 as template. A *Pae*I–*Bam*HI fragment was cloned in pRB374 to give pME4857. *E. coli* strain NK3 (*cysKcysM*) and *B. subtilis* strain SB11 (*cysK*::Tn*10*) were used as recipient in complementation studies to verify the functionality of $cysK_{BS}$ and $cysK_{ST}$.

3. Results

3.1. Functional analysis of the B. subtilis cysJI genes

In *E. coli*, reduction of sulfite to sulfide is catalyzed by sulfite reductase, encoded by the *cysI* and *cysJ* genes [2]. The *cysJ* gene product accepts electrons from NADPH, which are subsequently transferred to the hemoprotein CysI, and from there to sulfite to give sulfide. In *B. subtilis*, open reading frames with sequence similarity to the *E. coli cysI* and *cysJ* genes are encoded by *yvgQ* (51% identity over 567 amino acid residues) and *yvgR* (42% identity over 600 amino acid residues) respectively, which are positioned at 293 min on the chromosome. Sequence analysis showed that YvgQ contains a siroheme motif, which is conserved in sulfite reductases. The YvgR protein contains putative FAD and NADH binding sites.

A mutant with a deletion in *yvgRQ* and a simultaneous insertion of a neomycin resistance cassette was constructed as described in Section 2. The resulting mutant, strain SB12, was unable to utilize sulfate, sulfite or butanesulfonate as a source of sulfur and grew poorly with sulfide, but it could still grow with thiosulfate, cysteine or methionine.

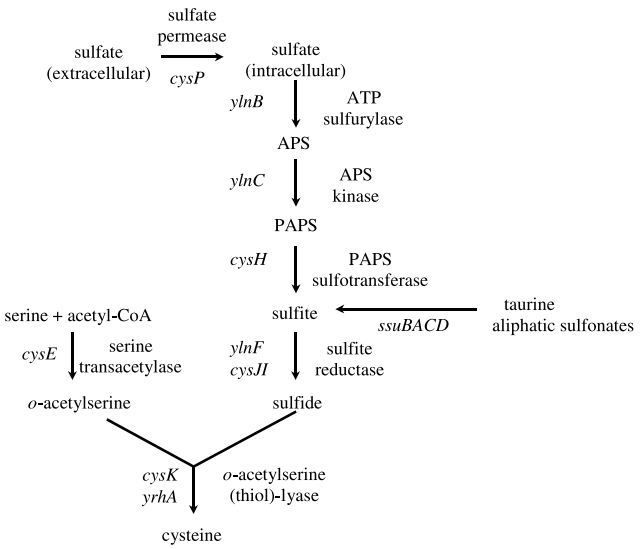

Fig. 1. Cysteine biosynthesis from sulfate and aliphatic sulfonates in *B. subtilis*.

These data suggested that *yvgRQ* indeed encodes sulfite reductase. We have therefore renamed *yvgR* and *yvgQ* into *cysJ* and *cysI* respectively. The results also indicate that, unlike in *E. coli* [2], a direct pathway from methionine to cysteine exists since growth with methionine was not affected.

3.2. Regulation of expression of cysJI

To study whether expression of the *B. subtilis cysJI* genes is regulated by the sulfur source used for growth, a strain containing a transcriptional *cysJ′–lacZ* fusion integrated at *amyE* was constructed (strain SB36) and β-galactosidase was measured in cells grown with different sulfur sources (Table 2). The levels of β-galactosidase were low compared to those found with *ssuD′–lacZ* fusions [7] or *cysH′–lacZ* fusions [5]. β-Galactosidase levels were higher in sulfate or butanesulfonate grown cells than in cells grown with other sulfur sources.

The transcriptional start of *cysJI* was mapped by primer extension analysis using RNA isolated from strain 1A1 grown with sulfate as sulfur source. It was located 46 bp upstream of the translation initiation codon of *cysJ* (results not shown). The band was weak, which confirmed the low levels of β-galactosidase obtained with the *cysJ′–lacZ* fusion.

Table 2
Expression β-galactosidase from a transcriptional *cysJ′–lacZ* fusion

Sulfur source	β-Galactosidase activity (Miller units)
Sulfate	17.4 ± 5.2
Cystine	1.2 ± 1.4
Sulfate+cystine	8.2 ± 1.4
Methionine	5.8 ± 0.6
Methionine+sulfate	6.6 ± 0.9
Butanesulfonate	12.7 ± 2.3
Glutathione	7.5 ± 2.1

An overnight culture of *B. subtilis* strain SB36, containing a chromosomal *cysJ′–lacZ* fusion, was 100-fold diluted in fresh medium containing the indicated sulfur source and β-galactosidase was measured in the mid-exponential phase of growth.

3.3. Isolation and characterization of a cysK mutant

In an attempt to identify proteins involved in regulation of the *ssu* operon, transposon mutagenesis with a Tn*10* derivative was used to isolate mutants that exhibited constitutive expression of β-galactosidase from a transcriptional *ssuD′–lacZ* fusion. Although regulatory mutants were not obtained, a mutant containing a transposon insertion at bp 341 of the *cysK* gene exhibited constitutive expression of β-galactosidase [8]. The *cysK* gene from *B. subtilis* has not been previously characterized, but it may be assumed that it encodes cysteine synthase (*O*-acetylserine (thiol)-lyase), since it shows 43% amino acid sequence identity to the *cysK* gene from *E. coli*. The *cysK* mutant, strain MS11-6, could still grow with sulfate, butanesulfonate and sulfite, but its growth rates were lower than those of the wild-type strain (not shown). Thus, *cysK* might be involved in the biosynthesis of cysteine from sulfate, but *B. subtilis* contains probably more than one gene encoding this enzyme activity. There are still two more *cysK* paralogs present on the *B. subtilis* chromosome, *yrhA* and *ytkP*. The function of these paralogs is not known, but either both or one of them apparently can only partly take over the function of *cysK*, since the *cysK* mutant grew slowly with sulfate and butanesulfonate. We constructed several mutants that contained knock-outs in *yrhA*, *ytkP* and *cysK* and tested their growth properties. All strains containing single mutations were able to grow with sulfate as sulfur source. The *yrhA cysK* double mutant SB50 as well as the *yrhA cysK ytkP* triple mutant SB53 could not grow anymore with sulfate or with thio-

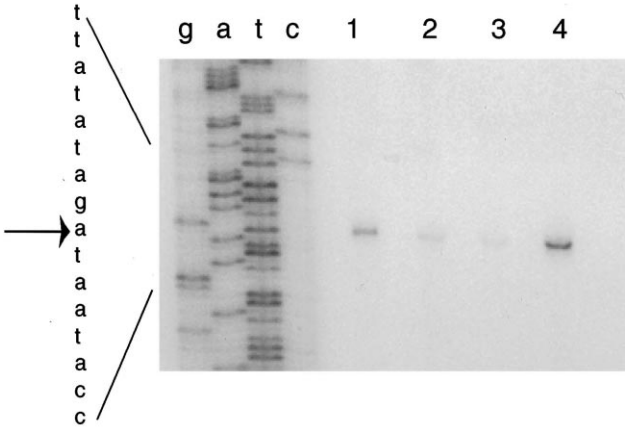

Fig. 2. Determination of the *cysK* transcription start site. Approximately equal amounts of RNA were isolated from cells grown with butanesulfonate (lane 1), sulfate (lane 2), cystine (lane 3) or glutathione (lane 4) and were reverse-transcribed using the primer indicated in Fig. 3. The sequencing ladder was obtained using the same primer. The transcription start is indicated by an arrow.

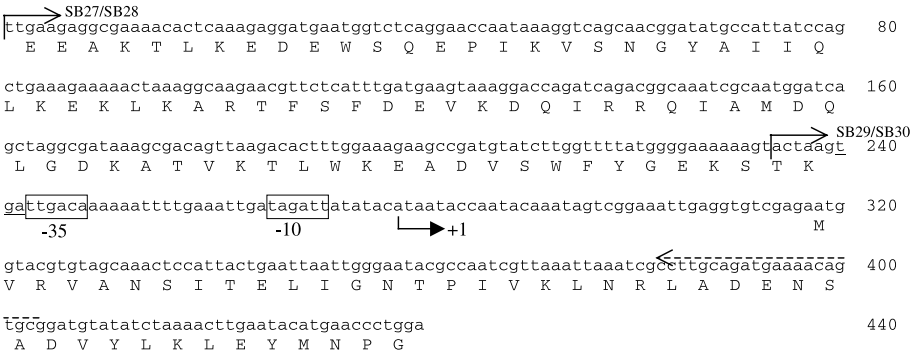

Fig. 3. Promoter region of the *cysK* gene. The −35 and −10 regions of the *cysK* promoter are boxed and the transcription start site is indicated. The stop codon of the *yacD* gene is underlined. The 5′ ends of the promoter regions contained in strains harboring *cysK′–gusA* fusions are indicated by arrows. The 3′ end of the *cysk* portion of these fusions coincides with the last nucleotide shown. The primer used for primer extension analysis (Fig. 2) is indicated by a stippled arrow.

sulfate. This indicates that *yrhA* encodes cysteine synthase activity as well.

We used complementation analysis to confirm the function of *B. subtilis cysK*. The gene was cloned in plasmid pRB374 under the control of the *vegII* promoter. The resulting plasmid, pME4864, could complement growth with sulfate as sulfur source of *E. coli* strain NK3, a *cysK cysM* mutant lacking *O*-acetylserine (thiol)-lyase activity. This suggests that *B. subtilis cysK* indeed encodes this enzyme. Using the same plasmid, the *cysK* mutation in *B. subtilis* strains MS11-6 or SB11 could not be complemented however. Plasmid pME4857, which contains the *S. typhimurium cysK* gene, could also complement *E. coli* NK3, but not *B. subtilis* MS11-6 or SB11 (results not shown). It is unlikely that the transposon insertion has a polar effect on expression of genes downstream of *cysK*, since there is a transcription terminator present immediately after the *cysK* stop codon. It is possible that the *S. typhimurium* and *B. subtilis cysK* genes were somehow not well expressed from the plasmid in *B. subtilis*.

3.4. Regulation of expression of cysK

We used strains SB27 and SB29, which contain chromosomally located transcriptional *cysK′–gusA* fusions in a wild-type background (Fig. 3), to investigate the conditions under which the *cysK* gene was expressed. In sulfate or cystine grown SB27, β-glucuronidase activities were about 5-fold lower than in cells grown with methionine, butanesulfonate or glutathione (Table 3). Strain SB29, which harbors a fusion containing just 9 bp from the region upstream of the −35 region, produced lower levels of β-glucuronidase, but repression by sulfate and cystine was retained. This indicates that this fusion lacked a region necessary for full expression, but we were unable to find a sequence which could function as enhancer.

Addition of OAS to the medium resulted in a 2-fold increase in activity in cystine grown cells, but had only a marginal effect on expression of the *cysK′–gusA* fusion in sulfate grown cells.

When the *cysK′–gusA* fusions were introduced in the *cysK* mutant, the resulting strains SB28 and SB30 produced β-glucuronidase constitutively. These results indicate that either CysK has also a regulatory function, or that intermediates that accumulate when CysK is inactive give rise to higher expression levels.

Using primer extension analysis, the transcriptional start of *cysK* was determined (Fig. 2). One band was observed, which was strongest when RNA from cells grown with glutathione or butanesulfonate was used. The inten-

Table 3
Expression of β-glucuronidase from transcriptional *cysK′–gusA* fusions in a wild-type strain and in a *cysK* mutant

Sulfur source	β-Glucuronidase activity (Miller units)			
	SB27 (wild-type)	SB28 (*cysK*)	SB29 (wild-type)	SB30 (*cysK*)
Sulfate	99 ± 20	1085 ± 107	3 ± 0.8	74 ± 9
Cystine	111 ± 16	975 ± 30	3 ± 0.2	65 ± 2
Methionine	544 ± 19	1001 ± 104	nd[a]	nd
Butanesulfonate	506 ± 19	965 ± 28	19 ± 4	59 ± 4
Glutathione	677 ± 107	980 ± 62	35 ± 8	48 ± 1
Sulfate+OAS	114 ± 5	nd	nd	nd
Cystine+OAS	208 ± 1	nd	nd	nd

Overnight cultures of *B. subtilis* strains containing chromosomal *cysK′–gusA* fusions were diluted 100-fold in fresh medium containing the indicated sulfur source and grown to the mid-exponential phase. When indicated, OAS was added to a final concentration of 0.5 mM 2 h before measurement of β-glucuronidase activities.

[a]Not done.

sity of the bands was in agreement with the results obtained from measurements of β-glucuronidase activities from transcriptional *cysK′–gusA* fusions. The −35 region of the *cysK* promoter is located immediately downstream of the stop codon of the *yacD* gene (Fig. 3), whose function is unknown.

4. Discussion

Until now, four operons and one gene involved in the biosynthesis of cysteine from sulfate and from sulfonates have been identified. The *cysE* gene encodes serine transacetylase, the enzyme responsible for synthesis of OAS, and resides in one operon with *gltX* and *cysS* [6]. The *cysH* operon is required for uptake and reduction of sulfate to sulfite [5]. The *ssu* operon encodes transport and liberation of sulfite from aliphatic sulfonates [7]. In this study we have investigated the function and regulation of the *cysJI* operon, which is required for reduction of sulfite to sulfide, and that of the *cysK* gene, which encodes the final step in cysteine biosynthesis. The function of *cysK* can be taken over by its paralog *yrhA*. This is reminiscent to the situation in *E. coli*, which contains two proteins with *O*-acetylserine (thiol)-lyase activity, encoded by *cysK* and *cysM* [2]. The *E. coli* CysK protein is more active with sulfide, while the CysM protein has a higher affinity for thiosulfate [2]. Further analysis of the proteins encoded by *cysK* and *yrhA* should give insight in their substrate specificity.

In *E. coli*, expression of the *cys* genes and the *ssu* genes is under coordinate control of the transcriptional regulator CysB [2]. Whether the *B. subtilis cys* and *ssu* genes are also controlled by one regulatory system is as yet unknown, but we have been unable to find sequence similarity between the promoter regions of the *cysH*, the *cysJI*, the *cysK* and the *ssu* genes.

Similar as with the *cysH* and *ssu* operons, expression of *cysK* was repressed by sulfate and cysteine. The mechanism by which repression by these compounds is mediated remains unclear, but OAS or a closely related molecule plays an important role. In *E. coli*, OAS functions as co-inducer of the transcriptional regulatory protein CysB [2]. The level of OAS is a measure of sulfur availability for the cell, since cysteine exerts feedback inhibition on serine transacetylase. Sulfur limitation results in increased synthesis of OAS, which induces transcription of the *cys* genes. Although it is not known whether in *B. subtilis* serine transacetylase is inhibited by OAS, the expression of the *cysE* gene is regulated by transcription attenuation through binding of uncharged cysteinyl-tRNA to the leader RNA, thereby promoting formation of an anti-terminator, which results in transcription readthrough [6]. Cysteine could thus indirectly regulate the amount of OAS, which in turn could act as effector in regulation of the *cys* and *ssu* genes.

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