Regulation of the *metC-cysK* Operon, Involved in Sulfur Metabolism in *Lactococcus lactis*

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Sulfur metabolism in gram-positive bacteria is poorly characterized. Information on the molecular mechanisms of regulation of genes involved in sulfur metabolism is limited, and no regulator genes have been identified. Here we describe the regulation of the lactococcal *metC-cysK* operon, encoding a cystathionine β-lyase (*metC*) and cysteine synthase (*cysK*). Its expression was shown to be negatively affected by high concentrations of cysteine, methionine, and glutathione in the culture medium, while sulfur limitation resulted in a high level of expression. Other sulfur sources tested showed no significant effect on *metC-cysK* gene expression. In addition we found that *O*-acetyl-L-serine, the substrate of cysteine synthase, was an inducer of the *metC-cysK* operon. Using a random mutagenesis approach, we identified two genes, *cmbR* and *cmbT*, involved in regulation of *metC-cysK* expression. The *cmbT* gene is predicted to encode a transport protein, but its precise role in regulation remains unclear. Disruption of *cmbT* resulted in a two- to threefold reduction of *metC-cysK* transcription. A 5.7-kb region containing the *cmbR* gene was cloned and sequenced. The encoded CmbR protein is homologous to the LysR family of regulator proteins and is an activator of the *metC-cysK* operon. In analogy to CysB from *Escherichia coli*, we propose that CmbR requires acetylserine to be able to bind the activation sites and subsequently activate transcription of the *metC-cysK* operon.

In many bacteria, cysteine biosynthesis is the predominant mechanism by which inorganic sulfur is reduced and incorporated into organic compounds. The pathway involves transport and reduction of inorganic sulfate to sulfide in one branch and the synthesis of *O*-acetyl-L-serine (OAS) in another. The subsequent reaction of sulfide with OAS results in cysteine synthesis (Fig. 1). For *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, at least 22 genes have been identified that are required for the transport and reduction of sulfate and its incorporation into cysteine (21). Full expression of these genes requires a positively regulatory protein encoded by *cysB*, the presence of the inducer *N*-acetyl-L-serine (NAS), and sulfur limitation (21, 35).

Methionine biosynthesis is closely linked to cysteine biosynthesis (Fig. 1). For *E. coli* and *S. enterica* serovar Typhimurium, transcription of all the *met* genes involved in methionine biosynthesis, except *metH,* is under negative control of the MetJ repressor, with *S*-adenosylmethionine, the pathway's end product, acting as a corepressor (39). Expression of the *metE, metA, metF, metH,* and *glyA* genes is also under positive control of the MetR activator (9, 27, 30, 31, 46, 51). Homocysteine may modulate the regulator role of MetR and is required for the *metE* gene activation (47). Furthermore, vitamin B_{12} is involved in *metE* repression, probably by depletion of the coactivator homocysteine (53).

CysB and MetR are members of the LysR family of prokaryotic transcriptional regulatory proteins. Common family features are the size (between 300 and 350 amino acids), the formation of either homodimers or homotetramers, the presence of a helix-turn-helix DNA binding motif in the N-terminal region, and the requirement for a small molecule that acts as a coinducer (41). Both CysB and MetR activate gene expression at one or more loci while negatively regulating the expression of their own genes. The interactions of CysB with responsive promoter regions are well characterized. CysB binds as a tetramer, bending the DNA, and interaction with the inducer results in a conformational change of CysB, allowing it to interact with activation sites of the *cysJ*, *cysK*, or *cysP* promoters (19, 32). Several amino acid residues of CysB involved in DNA binding, response to the inducer, or oligomerization have been identified through mutagenesis (25).

Little is known about the organization and regulation of the sulfur assimilation and methionine biosynthesis pathway in gram-positive bacteria. In *Bacillus subtilis*, *Clostridium acetobutylicum*, and *Staphylococcus aureus*, several genes involved in the biosynthesis of methionine and cysteine are regulated by a global transcription termination control system called the S box regulon (17). Recently Mansilla et al. (29) showed that in *B. subtilis*, the *cysH* operon that contains an S box motif in the leader region is not regulated by a transcription terminator control system. The expression of this operon is controlled at the transcription initiation level by a repressor; the expression of the operon is induced by OAS and repressed by cysteine.

For *Lactococcus lactis*, a lactic acid bacterium commonly used as a starter in the dairy industry, the biosynthesis of

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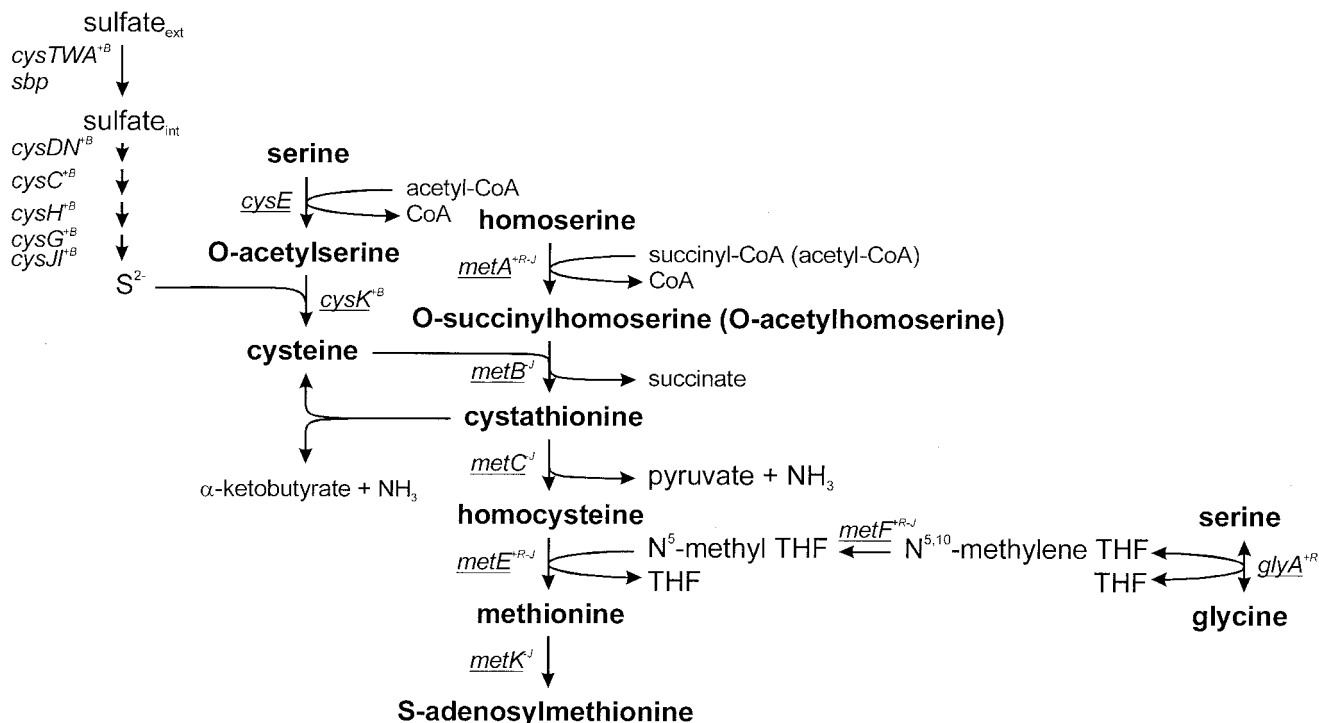§ Present address: PURAC Biochem, Gorinchem, The Netherlands.

FIG. 1. Cysteine and methionine biosynthesis pathways in *E. coli* and *S. enterica* serovar Typhimurium and the responsible genes. Activation by CysB ($^{+B}$) or MetR ($^{+R}$) and repression by MetJ ($^-$) are indicated in superscript. The genes from this pathway with homologues in the *L. lactis* IL1403 genome sequence are underlined. The conversion of cystathionine to cysteine is not described for *E. coli*. CBL from *L. lactis* is able to catalyze this reaction in vitro (1).

methionine and cysteine has been poorly characterized. This microorganism has been described as prototrophic for cysteine and auxotrophic for methionine (8). Nevertheless, it was expected that the genes coding for the enzymes involved in methionine biosynthesis are present, since some autotrophic strains are known (8). This was corroborated by the analysis of the *L. lactis* IL1403 genome sequence (4). Homologues for all the genes involved in methionine biosynthesis in *E. coli* (*metA*, *metB*, *metC*, *metE*, *metF*, and *glyA*) are present together with *cysE* and two *cysK* homologues from the cysteine biosynthesis pathway (Fig. 1). In contrast, no homologues of the *E. coli* genes responsible for sulfate uptake and reduction seem to be present, although there is a putative sulfate transporter (*yafB*) of the SulP family (4).

Previously we characterized the *metC* gene encoding a cystathionine β-lyase (CBL) that has both β- and γ-lyase activity (1, 12). In vitro this CBL is able to convert cystathionine to cysteine or homocysteine (Fig. 1). The latter conversion is the penultimate step in methionine biosynthesis. The *metC* gene forms an operon together with *cysK*, encoding a cysteine synthase (12). The function of CysK was confirmed by its overproduction, resulting in increased cysteine synthase activity and the construction of a double-crossover *cysK* knockout in *L. lactis* MG1363 that resulted in auxotrophy for cysteine (R. van Kranenburg and M. Fernández, unpublished results). The alternative *cysK* gene seems to be inactive under these conditions. The organization of this *metC-cysK* gene cluster and its function in sulfur metabolism suggests a putative regulation by cysteine and/or methionine. Dias and Weimer (11) report that cystathionine lyase (CL) activity is influenced by the methio-

nine and cysteine concentrations in the culture medium, confirming this hypothesis.

In this paper we demonstrate that methionine, cysteine, glutathione, and OAS have an effect on *metC-cysK* gene expression. We identify two genes, *cmbT* (cysteine and methionine biosynthesis transporter) and *cmbR* (cysteine and methionine biosynthesis regulator), that are involved in regulation of *metC-cysK* transcription. CmbR is a LysR-type regulator protein that is essential for *metCcysK* expression and is the first regulator of sulfur metabolism described for gram-positive bacteria.

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant characteristic(s)[a]	Reference
Strains		
E. coli MC1061		6
L. lactis		
MG1363	Plasmid-free	16
MG1363Δ*metC*	Ery[r] derivative of MG1363 with a *metC* disruption by single crossover	12
MG1363::pNZ9342	Ery[r] derivative of MG1363 carrying a single chromosomal copy of the *lacZ* gene under the control of P$_{metC\text{-}cysK}$	This study
MG1363::ISS*1*#1	Ery[r] Tet[r] derivative of MG1363::pNZ9342 with pGh8::ISS*1* integrated in *cmbR*	This study
MG1363::ISS*1*#2	Ery[r] Tet[r] derivative of MG1363::pNZ9342 with pGh8::ISS*1* integrated in *cmbR*	This study
MG1363::ISS*1*#3	Ery[r] Tet[r] derivative of MG1363::pNZ9342 with pGh8::ISS*1* integrated in *cmbR*	This study
MG1363*cmbR*	Ery[r] derivative of MG1363 with a *cmbR* disruption by single crossover	This study
MG1363*cmbT*	Ery[r] derivative of MG1363 with a *cmbT* disruption by single crossover	This study
Plasmids		
pGh8:ISS*1*	Tet[r], 5.2-kb pWV01 thermosensitive replicon	28
pNZ124	Cm[r], 2.8-kb pSH71 replicon	37
pUC19*Nco*I	Amp[r], 2.6-kb pUC19 derivative containing an additional fragment with an *Nco*I site overlapping an ATG start codon preceded by the *L. lactis* SK11 *prtP* ribosome-binding site	This study
pNZ9340	Cm[r], 5.2-kb pNZ273 derivative containing the *gusA* gene fused to P$_{metC\text{-}cysK}$ on a 0.6-kb *Sca*I-*Eco*RI fragment	This study
pNZ9341	Ery[r], 7.1-kb pIL252 derivative carrying the *gusA* gene fused to P$_{metC\text{-}cysK}$ on a 0.6-kb *Sca*I-*Eco*RI fragment	This study
pNZ9342	Ery[r], 7.2-kb pUC19*Nco*I derivative containing the *lacZ* gene fused to P$_{metC\text{-}cysK}$ and the Ery[r] gene from pIL253	This study
pNZ9343	Tet[r], 5.5-kb pGh8:ISS*1* derivative carrying a 0.3-kb *Eco*RI chromosomal DNA fragment	This study
pNZ9344	Tet[r], 5.5-kb pGh8:ISS*1* derivative carrying a 0.3-kb *Hin*dIII chromosomal DNA fragment	This study
pNZ9345	Tet[r], 5.7-kb pGh8:ISS*1* derivative carrying a 0.5-kb *Hin*dIII chromosomal DNA fragment	This study
pNZ9346	Amp[r], 6.3-kb pUC18 derivative carrying a 3.6-kb *Sal*I-*Eco*RI fragment with *cmbR*	This study
pNZ9347	Amp[r], 9.5-kb pUC18 derivative carrying a 6.8-kb *Sst*I-*Sal*I fragment with *cmbR*	This study
pNZ9348	Ery[r], 4.0-kb pUC18Ery derivative carrying a 0.3-kb *Hin*dIII-*Acc*I fragment of *cmbR* gene from pNZ9344	This study
pNZ9349	Ery[r], 4.6-kb pUC18Ery derivative carrying a 0.8-kb PCR fragment of *cmbT*	This study
pNZ9350	Cm[r], 4.6-kb pNZ124 derivative carrying a 1.8-kb *Eco*RI-*Pst*I fragment from with *cmbR*	This study

[a] Amp[r], ampicillin resistant; Ery[r], erythromycin resistant; Tet[r], tetracycline resistant; Cm[r], chloramphenicol resistant.

isolation of *E. coli* plasmid DNA for nucleotide sequence analysis was performed using the Jet Star system (Genomed GmbH, Bad Deynhausen, Germany), according to the instructions of the manufacturer. Isolation and transformation of *L. lactis* plasmid DNA were performed as described previously (10). Southern blots were hybridized with the different probes at 65°C and washed with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C before autoradiography.

Construction of strain MG1363::pNZ9342. To obtain a transcriptional fusion of the *metC-cysK* promoter (P$_{metC\text{-}cysK}$) with *lacZ*, the promoter region, including the 5′ part of the *metC* gene, was cloned as a 0.6-kb *Eco*RI-*Sca*I fragment in the plasmid pUC19*Nco*I that was digested with *Nde*I made blunt with Klenow polymerase and digested with *Eco*RI. The plasmid pUC19*Nco*I is pUC19 digested with *Eco*RI and *Sma*I ligated with a nucleotide linker made with the oligonucleotides 5′-AATTC**AGGAGG**ATATT**CCATG**-3′ and 5′-CATGGAATATCCT CCTG-3′. The linker contains the upstream sequence of the lactococcal *prtP* gene, including the ribosome binding site and *prtP* start codon (boldface), and generates after ligation an *Nco*I site overlapping the start codon (underlined) that can be used to make translational fusions. The *lacZ* gene from *Streptococcus thermophilus* CNRZ 302 was generated by PCR using the primers 5′-CCTTCA AAAAAGGAGAATAAT**CCATGG**ACATG-3′ and 5′-ATC**GGATCC**TAATT TAGTGGTTCAATCATGAAGCTTAATTATAGCTATCTGCTGAGC-3′, introducing an *Nco*I site overlapping the start codon (underlined) and a *Bam*HI site for cloning (underlined). The PCR fragment was cloned as a 3.0-kb *Nco*I-*Bam*HI fragment in pUC19*Nco*I containing P$_{metC\text{-}cysK}$. The erythromycin resistance gene from pUC18Ery (49) was cloned as a *Bam*HI-*Pst*I fragment in the resulting plasmid, generating the plasmid pNZ9342. This plasmid cannot replicate in *L. lactis* and was integrated into the chromosome by single-crossover recombination on the P$_{metC\text{-}cysK}$ region. PCR analysis and Southern blotting were used to confirm the site of integration.

Construction of plasmids. To study the expression of the *metC-cysK* operon, the *gusA* gene was cloned under control of P$_{metC\text{-}cysK}$ in a low-copy-number plasmid. First, the P$_{metC\text{-}cysK}$ region was cloned upstream of the promoterless *gusA* gene as a 0.6-kb *Eco*RI-*Sca*I fragment in pNZ273 (37). From the resulting plasmid (pNZ9340), a 2.5-kb *Bam*HI-*Xho*I DNA fragment containing P$_{metC\text{-}cysK}$-*gusA* was cloned into the low-copy-number plasmid pIL252 (44), generating the plasmid pNZ9341.

For the construction of a *cmbR* disruption strain, an internal fragment of the *cmbR* gene was isolated as a 0.3-kb *Hin*dIII-*Acc*I fragment from the plasmid pNZ9344 in which the *Hin*dIII site was made blunt by Klenow polymerase. This fragment was cloned into the plasmid pUC18Ery (49) digested with *Eco*RI-*Acc*I, with the *Eco*RI site blunted by Klenow polymerase, generating the plasmid pNZ9348.

To complement the *cmbR*-knockout strain, the *cmbR* gene was cloned as an *Eco*RI-*Pst*I fragment from pNZ9346 into the plasmid pNZ124, resulting in the plasmid pNZ9350.

For the construction of a *cmbT* disruption strain, an internal fragment of the *cmbT* gene was generated by PCR using the primers 5′-GGCGCG**GGAATTC**T TGCGATTACGATTCCTCTCTC-3′ and 5′-CGCGGC**GGATCC**GTAATCG GCTTTCTTGCCCC-3′, introducing *Eco*RI and *Bam*HI sites (underlined) for cloning. The PCR fragment was cloned as a 0.8-kb *Eco*RI-*Bam*HI fragment in pUC18Ery (49), generating the plasmid pNZ9349.

Nucleotide sequencing and analysis. Automatic double-stranded DNA sequence analysis was performed on both strands with an ALF*red* DNA sequencer (Amersham Pharmacia Biotech). Sequencing reactions were accomplished by using the AutoRead sequencing kit, initiated by using Cy5-labeled universal and reverse primers and continued with synthetic primers in combination with Cy5-13-dATP according to the instructions of the manufacturer (Amersham Pharmacia Biotech). Sequence data were assembled and analyzed using the pc/gene program, version 6.70 (IntelliGenetics). The protein sequences were compared with the swiss-prot all library (6 June 2001) using the Fasta3 WWW service (version 3.3t09 [18 May 2001]) at the European Bioinformatics Institute (36).

RNA isolation and Northern blotting. Total RNA was isolated from exponentially growing MG1363 cultures by the Macaloid method described by Kuipers et al. (24). For Northern blot analysis, RNA was separated on a 1% formaldehyde agarose gel, blotted, and hybridized as described previously (50). The probes used for hybridization were radiolabeled with [α-^{32}P]dATP by nick translation. The blots were washed with 0.1× SSC at 65°C prior to autoradiography.

Enzymatic assays. For the different enzymatic assays, the cells were grown in M17 or CDM either with different methionine and cysteine concentrations or with different sulfur sources. CL activity was monitored using cystathionine as a substrate. Enzyme activity was measured in the cell extracts (CFE) by determination of free-thiol group formation with DTNB (5,5′-dithiobis 2-nitrobenzoic acid) as described by Uren (48). Pyridoxal-5′-phosphate was added to the reaction mixture at a final concentration of 20 μM. The data in the figures are from single experiments. The variation of specific activities was less than 10% between different experiments. The relative activities of the different conditions were always alike.

Cysteine synthase activity was measured by the method of Becker et al. (3). The amount of cysteine formed was determined as described by Gaitonde (15).

β-Glucuronidase (GUS) assays were performed with *para*-nitrophenyl-β-D-glucuronic acid as a substrate, monitoring the optical density at 412 nm as described by Platteeuw et al. (37).

Protein concentrations were determined by a protein assay (Bio-Rad) based on the method of Bradford, with bovine serum albumin as the standard (5).

A qualitative β-galactosidase plate assay was used for blue/white screening of *L. lactis* colonies by including 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside at a final concentration of 0.5 mM in GM17 plates.

Nucleotide sequence accession number. The nucleotide sequence reported in this study was submitted to GenBank under the accession no. AF240531.

RESULTS

Regulation of gene expression of the *metC-cysK* operon. The genes encoding CBL (*metC*) and cysteine synthase (*cysK*) are involved in methionine and cysteine biosynthesis pathways and form a single transcriptional unit (12). CL activity in *L. lactis* has been reported to be affected by the concentrations of cysteine and methionine in the culture medium (11). To test if this effect is caused by regulation of gene expression, both the CL activity and the expression level of the *metC-cysK* operon were determined using cells grown in CDM supplemented with different concentrations of methionine and cysteine. When the levels of methionine and cysteine were increased, the CL activity decreased (Fig. 2A). The effect of cysteine was more pronounced, as addition of 1× cysteine reduced the activity to that of a *metC* knockout strain, while addition of 10× methionine resulted in a reduction to two-thirds of the activity of cells grown in CDM supplemented with 0.1× methionine. The cysteine synthase activities were also determined, and these showed the same mode of regulation by methionine and cysteine (data not shown). Northern blot analysis revealed that the effects of cysteine and methionine are at the transcriptional level (Fig. 2B).

To quantify the effects of cysteine and methionine on *metC-cysK* transcription, a transcriptional fusion of P*metC-cysK* with the *gusA* reporter gene was constructed on a low-copy-number plasmid. The resulting plasmid, pNZ9341, was introduced in MG1363, and the GUS activities of cells grown in CDM with different amounts of cysteine and methionine were determined. Addition of 1× cysteine to CDM resulted in a 19-fold reduction of GUS activity compared to that for CDM containing no cysteine (Fig. 2C). Cells grown in CDM supplemented with 10× methionine still retained 61% of the GUS activity of those grown in 0.1× methionine, confirming the results of the Northern blot analysis.

The influence of other sulfur sources on the CL activity was tested (Fig. 3). As described above, sulfur starvation (CDM with 0.1× methionine and no cysteine) resulted in a high level of CL activity. Addition of sulfate, sulfite, thiosulfate, and djenkolate to the medium resulted in a similarly high activity level. In contrast, cysteine and glutathione almost completely abolished CL activity. Sulfide affected the growth rate of *L. lactis* dramatically and was therefore not included in the experiments.

NAS (often provided as OAS) has been described as a coinducer of the *cys* regulon in *E. coli* and *S. enterica* serovar Typhimurium (22). To test if OAS has an effect on the regulation of the *metC-cysK* operon, CL activity was measured using CFEs from cells grown in a medium with or without
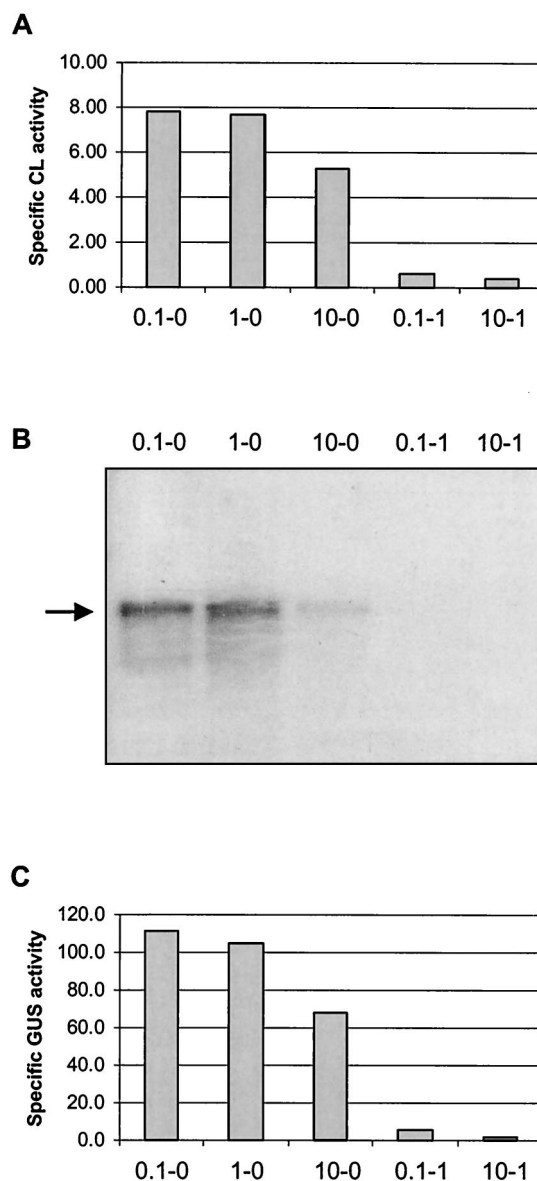

FIG. 2. (A) Specific cystathionine lyase activity in CFE of *L. lactis* MG1363 cells grown in CDM with various methionine and cysteine concentrations. 0.1-0, 0.1× methionine, no cysteine; 1-0, 1× methionine, no cysteine; 10-0, 10× methionine, no cysteine; 0.1-1, 0.1× methionine, 1× cysteine; 10-1, 10× methionine, 1× cysteine. The specific activities are expressed as nanomoles of mercaptide formed per minute per milligram of protein. (B) Transcriptional analysis of the *metC-cysK* operon. Total RNA was extracted from *L. lactis* MG1363 cells grown in CDM with various methionine and cysteine concentrations (see panel A). The blot was hybridized with an internal gene fragment of MG1363 *metC*. The arrow indicates the position of the *metC-cysK* transcript. (C) Specific GUS activities in CFE of *L. lactis* MG1363 harboring pNZ9341 grown in CDM with various methionine and cysteine concentrations (see panel A). The specific activities are expressed as nanomoles of *para*-nitrophenyl-β-D-glucuronic acid converted per minute per milligram of protein.

addition of OAS in the early log phase (see Materials and Methods). An increase in the CL activity in the presence of OAS was observed for all media tested except for those containing thiosulfate and L-djenkolic acid (Fig. 3). Northern blot analysis of RNA from cells grown in these conditions revealed
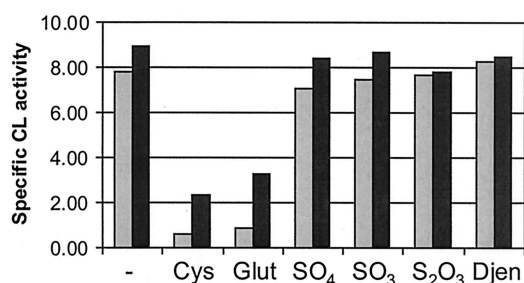
FIG. 3. Effects of sulfur sources and OAS on CL activity in *L. lactis*. Dark bars represent cells grown in the presence of OAS. Cys, cysteine; Glut, glutathione; SO_4, sulfate; SO_3, sulfite; S_2O_3, thiosulfate; Djen, djenkolic acid.

that the induction by OAS takes place at the transcriptional level (data not shown).

Identification of genes involved in regulation. To test if the regulation of *metC-cysK* transcription results from activity of an activator or repressor, a high-copy-number plasmid (pNZ9340) carrying the promoter region was introduced into MG1363. Cells harboring pNZ9340 grown in GM17 showed a fivefold-lower CL activity than MG1363 without the plasmid. This is likely to be the result of a lower level of *metC-cysK* expression caused by the titration of a putative activator by the multiple copies of the promoter.

For the identification of the activator gene, a random mutagenesis strategy was followed using the thermosensitive plasmid pGh8:ISS*1* (28). Rapid screening of the mutants lacking P*metC-cysK* activity was facilitated by the use of the strain MG1363::pNZ9342, in which *lacZ* is under the control of P*metC-cysK* (see Materials and Methods). Random integrants of pGh8:ISS*1* in MG1363::pNZ9342 were analyzed on GM17 plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. From approximately 22,000 colonies, only five showed a white phenotype. Southern blot analysis using *lacZ* as a probe revealed that in two of those, pGh8:ISS*1* was integrated into *lacZ*, while in the other three, pGh8:ISS*1* was inserted in another part of the chromosome. The latter three were designated MG1363::ISS*1*#1, MG1363::ISS*1*#2, and MG1363::ISS*1*#3. The CL activities of these mutants were tested and showed a clear decrease in the CL activity from that of the wild-type strain (Fig. 4).

The chromosomal DNA regions flanking the pGh8:ISS*1* insertion site were cloned using the unique *Eco*RI and *Hin*dIII sites adjacent to the ISS*1* element as described by Maguin et al. (28). From strain MG1363::ISS*1*#1, both 0.3-kb flanking regions were cloned, while from MG1363::ISS*1*#2, only the 0.6-kb *Hin*dIII flanking region could be cloned (Fig. 5A). Nucleotide sequence analysis revealed that in the two mutants the integration took place in the same gene, which was designated *cmbR* (cysteine and methionine biosynthesis regulator). From strain MG1363::ISS*1*#3 a 1.9-kb *Hin*dIII flanking region was cloned, and nucleotide sequence analysis showed that pGh8: ISS*1* had integrated into a 1,362-bp open reading frame (ORF) putatively encoding an efflux protein with homology to members of the 2.A.1.3.X drug/H^+ antiporter-2 subfamily of the major facilitator superfamily (38) (Fig. 5B). This gene, located upstream of the *pyrKDbF* operon and previously reported as *orfA* (2), was designated *cmbT* (cysteine and methionine bio-

synthesis transporter). The *pyrKDbF* operon has a promoter upstream of *pyrK* (2), ruling out possible polar effects of the integration in *cmbT*.

Cloning of the *cmbR* gene. The 0.3-kb internal *cmbR* fragment from pNZ9344 was used as a probe to clone the complete MG1363 *cmbR* gene on a 3.6-kb *Sal*I-*Eco*RI chromosomal DNA fragment (pNZ9346) and an overlapping 6.8-kb *Sst*I-*Sal*I fragment (pNZ9347). Sequence analysis of 5.7 kb of the cloned fragments revealed the presence of six ORFs (Fig. 5). The *cmbR* gene encodes a putative protein of 301 amino acids. It is preceded by a reasonable consensus ribosome binding site for *L. lactis* (AAGGA) (7) and a putative −35 and −10 region. An inverted repeat sequence, which could form a hairpin structure ($\Delta G = -18.1$ kcal mol^{-1}) and thus function as a terminator, was identified four nucleotides downstream of the stop codon. Northern blot analysis revealed that the *cmbR* gene is expressed as a 1-kb monocistronic transcript (data not shown).

The predicted CmbR protein is homologous to LysR-type regulators. It differs by only two amino acids from FhuR of *L. lactis* IL1403 (4), and it shares strongest homology with the gene product of *spy0824* from *Streptococcus pyogenes* (52% identity) (13) and CpsY from *Streptococcus agalactiae* (30% identity) (20) and moderate homology with CysB from *E. coli* (24% identity) (34). The N-terminal region containing the DNA-binding domain is the most conserved.

The three ORFs upstream of *cmbR* encode proteins homologous to FhuB (31% identity), FhuG (38% identity), and FhuD (33% identity) of *Bacillus subtilis*, involved in ferrichrome transport (42). The ORF downstream of *cmbR* codes for a putative transmembrane protein with homology (51% identity with YtbD of *B. subtilis*) to members of the major facilitator superfamily (classified as 2.A.1.X.X) (38). ORF6 encodes a protein that is homologous (59% identity with YvgN from *B. subtilis*) to (putative) dehydrogenases and oxidoreductases.

cmbT is involved in regulation. A directed knockout strain of *cmbT* was constructed to confirm its role in regulation. A 0.8-kb internal gene fragment was generated by PCR and cloned in pUC18Ery, generating the plasmid pNZ9349. This
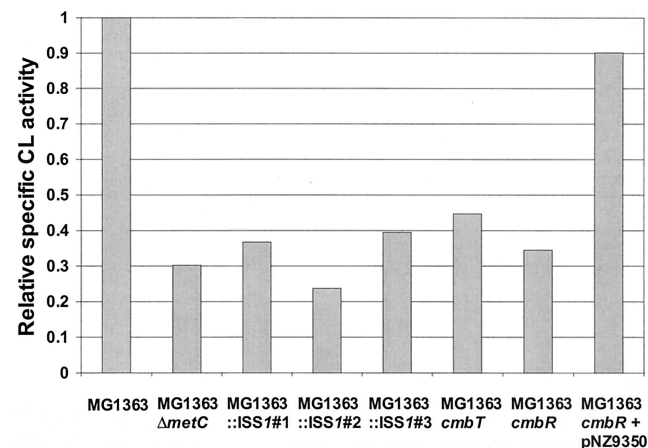

FIG. 4. Specific CL activities in CFE of *L. lactis* strains grown in GM17 relative to that of MG1363. The specific activities are expressed as nanomoles of mercaptide formed per minute per milligram of protein.
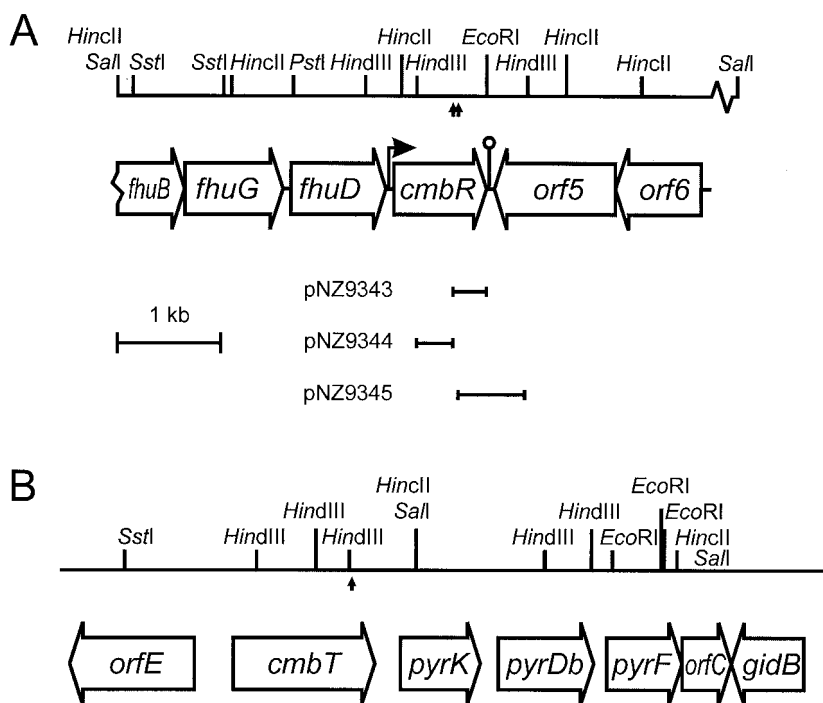
FIG. 5. Physical and genetic map of fragments of the *L. lactis* MG1363 chromosome containing *cmbR* (A) or *cmbT* (B) (2). For *Hin*dIII, only sites relevant for cloning are listed (A). The upward arrows indicate the sites of insertion of ISS*1* in MG1363::ISS*1#*1, MG1363::ISS*1#*2, and MG1363::ISS*1#*3. The flanking regions cloned from MG1363::ISS*1#*1 (pNZ9343 and pNZ9344) and MG1363::ISS*1#*2 (pNZ9345) are indicated.

plasmid, which is unable to replicate in *L. lactis*, was introduced into strain MG1363. Candidate single-crossover mutants in the *cmbT* locus were isolated as erythromycin-resistant colonies. Plasmid integration was confirmed by Southern blot and PCR analyses. One colony was used for further analysis and designated MG1363*cmbT*. The CL activity in this strain was assayed and compared to that of MG1363. For MG1363*cmbT*, the decrease in CL activity was comparable to that of MG1363::ISS*1#*3 (Fig. 4). Furthermore, MG1363*cmbT* is autotrophic for cysteine, as it is able to grow on CDM without cysteine, indicating that *cysK* expression, although reduced, is sufficient to sustain growth. Northern blot analysis showed that disruption of *cmbT* has an effect on the transcription of the *metC-cysK* operon (Fig. 6). These results confirm that *cmbT* is involved in regulation of *metC-cysK* transcription.

cmbR is essential for regulation. With an approach similar to that for *cmbT*, a directed knockout strain of *cmbR* was constructed to confirm its role in regulation. For this purpose, a 0.3-kb *Hin*dIII-*Acc*I fragment of the plasmid pNZ9344 was cloned into pUC18Ery, generating the plasmid pNZ9348, which was introduced into the strain MG1363. Plasmid integration of the erythromycin-resistant single-crossover mutants was confirmed by Southern blot and PCR analyses. One colony was selected for further analyses and designated MG1363*cmb*R. The CL activity in this strain was assayed and compared to that of MG1363. A dramatic decrease in the activity was observed for MG1363*cmb*R, similar to those of MG1363::ISS*1#*1 and MG1363::ISS*1#*2 (Fig. 4). The cysteine synthase activity was also affected, since the knockout strain is auxotrophic for cysteine. Northern blot analysis showed that expression of the *metCcysK* operon is com-

pletely abolished in MG1363*cmbR* (Fig. 6). Complementation of MG1363*cmbR* was achieved by introducing the plasmid pNZ9350 carrying the complete *cmbR* gene under the control of its own promoter. The complementation strain recovered the ability to grow in a medium without cysteine, and the CL activity was comparable to that in the wild-type strain (Fig. 4). These results prove that *cmbR* is involved in activation of the *metC-cysK* gene expression.

To test if OAS is an inducer of CmbR, we measured CL activities of MG1363, MG1363*cmbT*, and MG1363*cmbR* grown in GM17 medium with and without OAS. CL activity increased 4.5-fold for MG1363 upon addition of OAS and
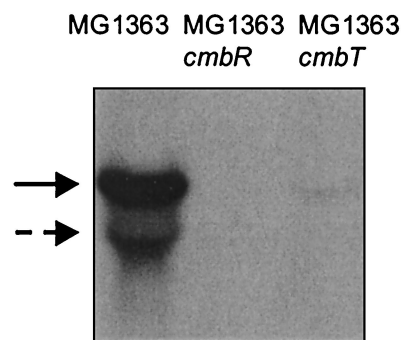

FIG. 6. Northern blot hybridization of RNA isolated from MG1363 (lane 1), MG1363*cmbR* (lane 2), and MG1363*cmbT* (lane 3), hybridized with an internal gene fragment of MG1363 *metC*. The cells were grown in GM17. The solid arrow indicates the position of the *metC-cysK* transcript, and the dashed arrow indicates that of *metC* alone.

4-fold for MG1363*cmbT*. In contrast, MG1363*cmbR* showed equal low CL activities when grown in medium with or without addition of OAS, indicating that OAS requires intact CmbR to activate CL activity.

DISCUSSION

We studied the regulation of the expression of the *metC-cysK* operon, encoding CBL and cysteine synthase (12). Previously Dias and Weimer (11) showed that the CL activity in *L. lactis* is influenced by the concentrations of methionine and cysteine in the culture medium. CBL is expected to be responsible for the majority of this CL activity, since disruption of *metC* results in a major reduction of CL activity (12). Furthermore, the organization of *metC* and *cysK*, involved in methionine and cysteine biosynthesis, respectively, in a single operon suggests a coordinated regulation of at least parts of both biosynthetic pathways. Our results demonstrate that cysteine and, to a lesser extent, methionine affect both CL and cysteine synthase activities and that this is caused by regulation of expression of the *metC-cysK* operon (Fig. 2). The effect of addition of other sulfur sources to medium containing no cysteine and a small amount of methionine was also evaluated. Addition of glutathione had an effect similar to addition of cysteine and reduced CL activity and *metC-cysK* expression. In contrast, addition of sulfate, sulfite, thiosulfate, or djenkolic acid had no clear effect on CL activity (Fig. 3). For *E. coli*, maximal expression of the *cys* genes is seen during growth with limiting sulfur sources, such as glutathione or djenkolic acid, while sulfate, sulfite, and thiosulfate partially reduce expression of the *cys* genes, and sulfide, cysteine, and cystine fully repress them (21, 23). For *B. subtilis*, expression of the *cysH* operon, encoding part of the pathway for transport and reduction of inorganic sulfate to sulfide, is repressed by cysteine, methionine, and thiosulfate and induced by glutathione (29). Our results demonstrate that for *L. lactis*, sulfur limitation results in maximum expression of the *metC-cysK* operon, similar to the *cys* regulon in *E. coli* and *S. enterica* serovar Typhimurium and the *cysH* operon in *B. subtilis*, but that the response to sulfate, sulfite, thiosulfate, and glutathione is different. An important difference with *E. coli* or *B. subtilis* is that *L. lactis* seems to lack the genes required for the sulfate reduction pathway (4). Although it contains a putative sulfate transporter (4), it is not likely to be able to use sulfate or sulfite as sulfur sources.

The effect of NAS as a coinducer for the *cys* regulon is well established with *E. coli* and *S. enterica* serovar Typhimurium (22). In these organisms, sulfide and thiosulfate counteract the effect of NAS on transcription. Recently, Mansilla et al. (29) showed that OAS also has a positive effect on transcription of the *cysH* operon in *B. subtilis* but that this is independent of sulfur starvation and insensitive to cysteine repression. In *L. lactis*, OAS induces expression of *metC-cysK* for all sulfur sources tested except for thiosulfate and djenkolic acid (Fig. 3). The positive effect of OAS is dependent on the sulfur source and is highest under sulfur starvation, resembling more the regulation of the *cys* regulon in *E. coli* than that of the *cysH* operon in *B. subtilis*.

CmbR is essential for expression of the *metCcysK* operon. It is a member of the family of LysR-type regulators that includes

CysB and MetR, and it activates *metCcysK* transcription. Since addition of OAS also activates transcription, we assume that CmbR, like CysB, requires NAS as an inducer to allow binding to the activation site. This is corroborated by the lack of effect of addition of OAS on CL activity of MG1363*cmbR*. The activator binding sequences described for the *cys* or *met* promoters in *E. coli* and *S. enterica* serovar Typhimurium (18, 52) were not found in the *metCcysK* promoter. Nevertheless, the $P_{metC-cysK}$ region contains three inverted repeat sequences and two direct repeats that are located upstream of the −35 region. A role of these repeat sequences in regulation has yet to be established. In our current studies we focus on the interaction of CmbR with the responsive promoter elements and try to define the CmbR binding sites.

The role of CmbT remains unclear. MG1363*cmbT* still responds to OAS, and we believe that the effect of CmbT is probably indirect, since it is predicted to be a transport protein. In that case the knockout strain would be unable to transport certain compounds, which has a negative effect on the activation of *metC-cysK* expression. These may be compounds that have an effect on the intracellular level of acetylserine, thereby affecting CmbR-dependent activation of *metC-cysK* transcription.

Knowledge of regulation of sulfur metabolism in gram-positive organisms is limited. The S box regulon described for *B. subtilis*, *C. acetobutylicum*, and *S. aureus* consists of putative transcriptional units, including *metC* but not *cysK*, containing a highly conserved motif in the leader region (17). For one of the members of the S box regulon, the methionine-regulated *yitJ* gene predicted to be involved in methionine biosynthesis in *B. subtilis*, it has been demonstrated that the mode of regulation is determined by the leader region rather than by the promoter activity (17). In contrast, regulation of the *cysH* operon, which is also part of the S box regulon, is independent of the leader region but depends on the promoter (29). A model for regulation of expression of this operon includes the activity of a putative negative regulator CysR, which prevents transcription in the absence of OAS (29). Although the lactococcal *metC-cysK* operon is not part of an S box regulon, there is an alternative combined regulation of the methionine and cysteine pathways in *L. lactis*, since this operon is dependent on CmbR, which is the first regulator of sulfur metabolism identified in gram-positive bacteria.

To test if CmbR is a regulator for other genes involved in sulfur metabolism, Northern blot analyses were performed with probes of the MG1363 *metA* gene (encoding an *O*-acetyl-homoserine sulfhydrylase), *metB* gene (encoding a serine acetyltransferase; annotated as *cysD* for IL1403), *cysE* gene (encoding a serine acetyltransferase), *glyA* gene (encoding a serine hydroxymethyltransferase), and the *fhuD* gene of the adjacent *fhu* gene cluster. Our preliminary results indicate that transcription of *metB*, *cysE*, and *glyA* is not influenced by the concentrations of methionine and cysteine in the growth media nor is it dependent on *cmbR*. Although barely detectable, the level of transcription of *metA*, which in strain IL1403 forms an operon with *metB* encoding cystathionine γ-synthase (4), was lower for cells grown on CDM with high concentrations of cysteine and methionine than for those grown on CDM with a low concentration of methionine and no cysteine. Disruption of *cmbR* resulted in a similar reduction of *metA* transcription,

which was equally low for cells grown on CDM containing low and high concentrations of methionine and cysteine. These preliminary results indicate that CmbR may be a regulator of the *metAB* operon. In addition, the results with *fhuD* as a probe indicate that CmbR is also involved in regulation of the *fhu* genes. In the *L. lactis* IL1403 genome sequence, CmbR is annotated as FhuR, the regulator of the *fhu* operon (4). The *fhu* genes are predicted to be involved in ferrichrome uptake, providing Fe^{3+}. Both Fe^{3+} and S^{2-} are required for Fe-S cluster synthesis, and in *E. coli* CBL and cysteine synthase are able to generate sulfide, enabling Fe-S cluster synthesis (14). Since lactococcal CBL is known to be able to use cysteine as a substrate, generating sulfide (1), it is tempting to speculate that the *metC-cysK* gene cluster may also have a role in Fe-S cluster synthesis, suggesting that CmbR is a regulator for this process. Fe-S clusters have been found in several lactococcal enzymes, including the transcription factor FlpA (43), the anaerobic ribonucleotide reductase activase (45), and the B form of dihydroorotate dehydrogenase formed by PyrDB and PyrK (33). Interestingly, the genes for the latter are genetically linked to *cmbT*, which is the other regulatory gene found in this study.

In conclusion, we have demonstrated that expression of the *metC-cysK* operon is activated by sulfur limitation. CmbT is involved in regulation, but its precise role remains obscure. CmbR is the activator that is essential for activation of *metC-cysK* transcription and may act as a regulator of *metAB* and the *fhu* genes.

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