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

María Fernández,* Michiel Kleerebezem,† Oscar P. Kuipers,‡ Roland J. Siezen, and Richard van Kranenburg§

Wageningen Centre for Food Sciences, Wageningen, and Department of Flavour, Nutrition and Ingredients, NIZO Food Research, Ede, The Netherlands

Received 19 July 2001/Accepted 11 October 2001

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 regulon of MetR and is required for the metE gene activation (47). Furthermore, vitamin B12 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
metC and cysK have 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, 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 function of CysK was confirmed by its overexpression and reduction seem to be present, although there is a putative sulfate transporter (yafB) of the SulP family (4).

FIG. 1. Cysteine and methionine biosynthesis pathways in E. coli and S. enterica serovar Typhimurium and the responsible genes. Activation by CysB (−β) or MetR (−γ) 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).

methylene 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 metCysK expression and is the first regulator of sulfur metabolism described for gram-positive bacteria.

MATERIALS AND METHODS

Bacteria, strains, plasmids, and media. The bacterial strains and plasmids used in this work are listed in Table 1. E. coli was grown in Luria-Bertani broth at 37°C (40). L. lactis cells were routinely grown in M17 broth (Difco Laboratories) supplemented with 0.5% glucose (GM17) or in chemically defined medium (CDM) (26) at 30°C, unless stated otherwise. CDM contains 0.82 mM cysteine (1× cysteine) and 0.84 mM methionine (1× methionine). To test the effect of the addition of various sulfur compounds, the sulfate salts of the CDM were replaced by equimolar amounts of chloride or nitrate salts, cysteine was omitted, the methionine concentration was reduced to 0.1× methionine (0.084 mM), and the medium was supplemented with 1 mM glutathione, 1 mM Na2SO4, 1 mM Na2SO3, 0.5 mM Na2S2O3, or 0.5 mM L-djenkolic acid. The addition of methionine was required for normal growth of L. lactis. The effect of OAS was evaluated by addition of a 1 mM concentration of OAS when the cells reached a turbidity of 0.6 to 0.9 at 600 nm followed by a 2-h incubation. When appropriate, the media contained tetracycline (5 μg ml−1 for E. coli or 2.5 μg ml−1 for L. lactis), ampicillin (100 μg ml−1), chloramphenicol (5 μg ml−1), or cethromycin (5 μg ml−1).

DNA isolation and manipulation. E. coli MC1061 was used as an intermediate host for cloning (40). Isolation of E. coli plasmid DNA and standard recombinant techniques were performed as described by Sambrook et al. (40). Large-scale
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 (Pmet-cysK) with lacZ, the promoter region, including the 5’ part of the metC gene, was cloned as a 0.6-kb EcoRI-ScaI fragment in the plasmid pUC19lacZi that was digested with NcoI and blunt ended with Klenow polymerase and digested with EcoRI. The plasmid pUC19lacZi is pUC19 digested with EcoRI and Smal ligated with a nucleotide linker made with the oligonucleotides 5’-ATTCGAGGATATTCATCGAGATCTG-3’ and 5’-ATCGAATATCACTCGGATCCCG-3’, introducing an NcoI site overlapping the start codon (underlined) and a BamHI site for cloning (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’-CTTCTA AAAAGGCGAATATCATGAGATCTG-3’ and 5’-ATCGAATATCACTCGGATCCCG-3’, introducing an NcoI site overlapping the start codon (underlined) and a BamHI site for cloning (underlined). The PCR fragment was cloned as a 3.0-kb NcoI-BamHI fragment in pUC19lacZ containing the chromycin resistance gene from pUC18Ery (49) digested with EcoRI-I, with the EcoRI site blunted by Klenow polymerase, generating the plasmid pNZ3948.

To complement the cmbR-knockout strain, the cmbR gene was cloned as an EcoRI-PstI fragment from pNZ3946 into the plasmid pNZ124, resulting in the plasmid pNZ3950. For the construction of a cmbR disruption strain, an internal fragment of the cmbR gene was generated by PCR using the primers 5’-GGCCGGGATCTTGCGATAAGATTCATCGAGATCTG-3’ and 5’-GGCCGGGATCCTAACGCGAATATCACTCGGATCCCG-3’, introducing EcoRI and BamHI sites (underlined) for cloning. The PCR fragment was cloned as a 0.8-kb EcoRI-BamHI fragment in pUC18Ery (49), generating the plasmid pNZ3949.

Nucleotide sequencing and analysis. Automatic double-stranded DNA sequence analysis was performed on both strands with an ALF Red DNA sequence kit (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 3.309 [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-32P]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'-dithio-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.

### TABLE 1. Strains and plasmids used in this work

| Strain or plasmid | Relevant characteristic(s) | Reference |
|-------------------|-----------------------------|-----------|
| **E. coli** MC1061 | Plasmid-free | 6 |
| **L. lactis** | | |
| MG1363ΔmetC | Ery′ derivative of MG1363 with a metC disruption by single crossover | 16 |
| MG1363::pNZ9342 | Ery′ derivative of MG1363 carrying a single chromosomal copy of the lacZ gene under the control of Pmet-cysK | This study |
| MG1363::pISS#1 | Ery′ Tet′ derivative of MG1363::pNZ9342 with pGibh::ISS1 integrated in cambR | This study |
| MG1363::pISS#2 | Ery′ Tet′ derivative of MG1363::pNZ9342 with pGibh::ISS2 integrated in cambR | This study |
| MG1363::pISS#3 | Ery′ Tet′ derivative of MG1363::pNZ9342 with pGibh::ISS3 integrated in cambR | This study |
| MG1363cambR | Ery′ derivative of MG1363 with a cambR disruption by single crossover | This study |
| MG1363cambT | Ery′ derivative of MG1363 with a cambT disruption by single crossover | This study |
| **Plasmids** | | |
| pGibh::ISS1 | Tet′, 5.2-kb pWV01 thermosensitive replicon | 28 |
| pNZ124 | Cm′, 2.8-kb pSHF1 replicon | 37 |
| pUC19NcoI | Amp′, 2.6-kb pUC19 derivative containing an additional fragment with an Ncol site overlapping an ATG start codon preceded by the L. lactis SK11 protP ribosome-binding site | This study |
| pNZ9340 | Cm′, 5.2-kb pNZ273 derivative containing the gusA gene fused to Pmet-cysK on a 0.6-kb Scal-EcoRI fragment | This study |
| pNZ9341 | Ery′, 7.1-kb pIL252 derivative carrying the gusA gene fused to Pmet-cysK on a 0.6-kb Scal-EcoRI fragment | This study |
| pNZ9342 | Ery′, 7.2-kb pUC19NcoI derivative containing the lacZ gene fused to Pmet-cysK and the Ery′ gene from pH253 | This study |
| pNZ9343 | Tet′, 5.5-kb pGibh::ISS1 derivative carrying a 0.3-kb HindIII chromosomal DNA fragment | This study |
| pNZ9344 | Tet′, 5.5-kb pGibh::ISS1 derivative lacking a 0.3-kb HindIII chromosomal DNA fragment | This study |
| pNZ9345 | Amp′, 6.3-kb pUC18 derivative carrying a 3.6-kb Sall-EcoRI fragment with cambR | This study |
| pNZ9346 | Amp′, 9.5-kb pUC18 derivative carrying a 6.8-kb SrfI-SalI fragment with cambR | This study |
| pNZ9348 | Ery′, 4.0-kb pUC18Ery derivative carrying a 0.3-kb HindIII-accl fragment of cambR gene from pNZ9344 | This study |
| pNZ9349 | Ery′, 4.6-kb pUC18Ery derivative carrying a 0.8-kb PCR fragment of cambT | This study |
| pNZ9350 | Cm′, 4.6-kb pNZ124 derivative carrying a 1.8-kb EcoRI-PstI fragment from with cambR | This study |

*a* Amp′, ampicillin resistant; Ery′, erythromycin resistant; Tet′, tetracycline resistant; Cm′, chloramphenicol resistant.
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 PmetC-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 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. 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.
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:ISS1 (28). Rapid screening of the mutants lacking PметC-cysK activity was facilitated by the use of the strain MG1363::pNZ9342, in which lacZ is under the control of PметC-cysK (see Materials and Methods). Random integrants of pGh8:ISS1 in MG1363::pNZ9342 were analyzed on GM17 plates containing 5-bromo-4-chloro-3-indolyl-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:ISS1 was integrated into lacZ, while in the other three, pGh8:ISS1 was inserted in another part of the chromosome. The latter three were designated MG1363::ISS1/#1, MG1363::ISS1/#2, and MG1363::ISS1/#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:ISS1 insertion site were cloned using the unique EcoRI and HindIII sites adjacent to the ISS1 element as described by Maguin et al. (28). From strain MG1363::ISS1/#1, both 0.3-kb flanking regions were cloned, while from MG1363::ISS1/#2, only the 0.6-kb HindIII 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::ISS1/#3 a 1.9-kb HindIII flanking region was cloned, and nucleotide sequence analysis showed that pGh8:ISS1 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 biosynthesis 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 SalI-EcoRI chromosomal DNA fragment (pNZ9346) and an overlapping 6.8-kb SalI-SalI 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 (ΔG = −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 LL1403 (4), and it shares strongest homology with the gene product of spa0824 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 of the protein is 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. 3. Effects of sulfur sources and OAS on CL activity in L. lactis](http://jb.asm.org/)

![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.](http://jb.asm.org/)
plasmid, which is unable to replicate in \textit{L. lactis}, was introduced into strain MG1363. Candidate single-crossover mutants in the \textit{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\textit{cmbT}. The CL activity in this strain was assayed and compared to that of MG1363::ISS1#3 (Fig. 4). Furthermore, MG1363\textit{cmbT} is auxotrophic for cysteine, as it is able to grow on CDM without cysteine, indicating that \textit{cysK} expression, although reduced, is sufficient to sustain growth. Northern blot analysis showed that disruption of \textit{cmbT} has an effect on the transcription of the \textit{metC-cysK} operon (Fig. 6). These results confirm that \textit{cmbT} is involved in regulation of \textit{metC-cysK} transcription.

\textbf{\textit{cmbR} is essential for regulation.} With an approach similar to that for \textit{cmbT}, a directed knockout strain of \textit{cmbR} was constructed to confirm its role in regulation. For this purpose, a 0.3-kb \textit{HindIII}-\textit{AccI} 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\textit{cmbR}. The CL activity in this strain was assayed and compared to that of MG1363. A dramatic decrease in the activity was observed for MG1363\textit{cmbR}, similar to those of MG1363::ISS1#1 and MG1363::ISS1#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 \textit{metC-cysK} operon is completely abolished in MG1363\textit{cmbR} (Fig. 6). Complementation of MG1363\textit{cmbR} was achieved by introducing the plasmid pNZ9350 carrying the complete \textit{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 \textit{cmbR} is involved in activation of the \textit{metC-cysK} gene expression.

To test if OAS is an inducer of \textit{CmbR}, we measured CL activities of MG1363, MG1363\textit{cmbT}, and MG1363\textit{cmbR} grown in GM17 medium with and without OAS. CL activity increased 4.5-fold for MG1363 upon addition of OAS and...
4-fold for MG1363cmbT. In contrast, MG1363cmbR 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 metC-cysK operon. It is a member of the family of LysR-type regulators that includes CysB and MetR, and it activates metC-cysK 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 MG1363cmbR. 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 metC-cysK promoter. Nevertheless, the F 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. MG1363cmbT 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 transcription 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 *yid* 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*-acetylhomoserine sulphydrylase), 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 *flud* gene of the adjacent *flu* 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 fluR as a probe indicate that CmbR is also involved in regulation of the flu operon. In the L. lactis IL1403 genome sequence, CmbR is annotated as FluR, the regulator of the flu operon (4). The flu 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 embT, 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 flu genes.

ACKNOWLEDGMENTS

We thank Jeroen Hugenholtz and Igor Mierau for critically reading the manuscript.

Maria Fernández was supported by a scholarship of the Ministerio de Educación y Cultura of Spain. This work was partially supported by EU research grant FAIR CT97-3173.

REFERENCES

1. Alting, A. C., W. J. M. Engels, S. van Schaikwik, and F. A. E. E xt erase. 1995. Purification and characterization of cystathionine β-lyase from Lactococcus lactis subsp. cremoris IL1403 and its possible role in flavor development in cheese. Appl. Environ. Microbiol. 61:4037–4042.

2. Andersen, P. S., J. Martinussen, and K. Hammer. 1996. Sequence analysis and identification of the pyrKDB operon from Lactococcus lactis including a novel gene, pyrK, involved in pyrimidine biosynthesis. J. Bacteriol. 178:5005–5012.

3. Becker, M. A., N. M. Kredich, and G. M. Tomkins. 1969. The purification and characterization of O-acetylserine sulfhydrylase-A from Salinomona typhimurium. J. Biol. Chem. 244:2418–2427.

4. Bolotin, A., P. Wincker, M. A. Becker, M. N. Kredich, and G. M. Tomkins. 1996. Stoichiometry of binding of Cysb to the cysH11, cysK, and cysP promoter regions of Salinomona typhimurium. J. Bacteriol. 178:3673–3682.

5. Boskiniemi, M., S. Melin, and M. Norgren. 1998. Identification of two genes, cysX and cysY, with promoter regulatory function on capsule expression in pathogenic Neisseria species. FEBS Lett. 421:158–161.

6. Kredich, N. M. 1996. Biosynthesis of cysteine. p. 514–527. In F. C. Neidhart, R. Curtiss, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Schaechter, and H. Umbarger (ed.), Escherichia coli and Salmonella typhimurium: cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, D.C.

7. Kredich, N. M. 1992. The molecular basis for positive regulation of cys promoters in Salinomona typhimurium and Escherichia coli. Mol. Microbiol. 6:2747–2753.

8. Kredich, N. M. 1971. Regulation of L-cysteine biosynthesis in Salinomona typhimurium. J. Biol. Chem. 246:3474–3484.

9. Kuipers, O. P., M. M. Beert huyzen, R. J. Slezen, and M. W. De Vos. 1993. Characterization of the nisin gene cluster nisABCIPR in Lactococcus lactis subsp. cremoris NIZO 57. Identification of two regions important for DNA binding, inducer response, oligomerization and protective functions. J. Bacteriol. 175:2098–2107.

10. Looijesteijn, P. J., and J. Hugenholtz. 1999. Uncoupling of growth and tyrosine-tyramine production by lac-taric acid production of cysteine in Lactococcus lactis subsp. cremoris NIZO 840 and optimisation of its synthesis. J. Biosci. Bioeng. 88:178–182.

11. Lorenz, E., and G. V. Stauffer. 1996. MetR-mediated repression of the glyA gene in Escherichia coli. FEMS Microbiol. Lett. 144:229–233.

12. Massagi, E. H., H. Prevost, S. D. Ehrlich, and A. G. Green. 1997. Efficient inser- tional mutagenesis in lactococci and other gram-positive bacteria. J. Bacte- riol. 179:931–935.

13. Mansilla, M. C., D. Albanesi, and D. de Mendoza. 2000. Transcriptional control of the sulfur-regulated cystX operon, containing genes involved in l-cysteine biosynthesis in Bacillus subtilis. J. Bacteriol. 182:5885–5892.

14. Mares, R., M. L. Urbanowski, and G. V. Stauffer. 1992. Regulation of the Salinomona typhimurium metC gene by the metE protein and homocysteine. J. Bacteriol. 174:390–397.

15. Maxon, E. M., B. Redfield, Y. X. Cai, R. Shoeman, K. Fujita, W. Fisher, G. Stauffer, H. Weissbach, and N. Brot. 1989. Regulation of methionine synthase in Escherichia coli: effect of the MetR protein on the expression of the metAB and metEF genes. Proc. Natl. Acad. Sci. USA 86:857–861.

16. Monroe, R. S., I. Ostrowski, M. M. Hryniwcz, and N. M. Kredich. 1990. In vitro interactions of CysB protein with the cysK and cysJH promoter regions of Salinomona typhimurium. J. Bacteriol. 172:6919–6929.

17. Nielsen, F. S., P. S. Andersen, and K. F. Jensen. 1996. The B form of dihydroorotate dehydrogenase from Lactococcus lactis consists of two different subunits, encoded by the pyrDB and pyrK genes, and contains FMN, FAD, and [FeS] redox centers. J. Bacteriol. 178:2589–2595.

18. Ostrowski, J. G., J. G. Jagas, and N. M. Kredich. 1987. DNA sequences of the cysY regions of Salinomona typhimurium and Escherichia coli: regulation by CysB protein and N-acetyl-l-serine. J. Bacteriol. 171:888–897.

19. Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA 85:6444–6448.

20. Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA 85:6444–6448.

21. regs\_data\_2019\_04\_30\_download.f104177e79eb96ce6908b8f82b2750e59
37. Platteeuw, C., G. Simons, and W. M. de Vos. 1993. Use of the Escherichia coli β-glucuronidase (gusA) gene as a reporter for analyzing of promoters in lactic acid bacteria. Appl. Environ. Microbiol. 60:587–593.
38. Saier, M. H., Jr. 2000. Families of transmembrane sugar transport proteins. Mol. Microbiol. 35:699–710.
39. Saint-Girons, I., C. Parsot, M. M. Zakin, O. Barzu, and G. N. Cohen. 1988. Methionine biosynthesis in Enterobacteriaceae: biochemical, regulatory, and evolutionary aspects. Crit. Rev. Biochem. 23:51–S42.
40. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
41. Schell, M. A. 1993. Molecular biology of the LysR family of transcriptional regulators. Annu. Rev. Microbiol. 47:597–626.
42. Schneider, R., and K. Hantke. 1993. Iron-hydroxamate uptake systems in Bacillus subtilis: identification of a lipoprotein as part of a binding protein-dependent transport system. Mol. Microbiol. 8:111–121.
43. Scott, C., J. R. Guest, and J. Green. 2000. Characterization of the Lactococcus lactis transcription factor FlpA and demonstration of an in vitro switch. Mol. Microbiol. 35:1383–1393.
44. Simon, D., and A. Chopin. 1988. Construction of a vector plasmid family and its use for molecular cloning in Streptococcus lactis. Biochimie 70:559–566.
45. Torrents, E., G. Buist, A. Liu, R. Eliasson, J. Kok, L. Gilbert, A. Graslund, and P. Reichard. 2000. The anaerobic (class III) ribonucleotide reductase from Lactococcus lactis. J. Biol. Chem. 275:2463–2471.
46. Urbanowski, M. L., and G. V. Stauffer. 1989. Genetic and biochemical analysis of the MetR activator-binding site in the metE metR control region of Salmonella typhimurium. J. Bacteriol. 171:5620–5629.
47. Urbanowski, M. L., and G. V. Stauffer. 1987. Regulation of the metR gene of Salmonella typhimurium. J. Bacteriol. 169:5841–5844.
48. Uren, J. R. 1987. Cystathionine β-lyase from Escherichia coli. Methods Enzymol. 143:483–486.
49. van Kranenburg, R., J. D. Marugg, I. I. van Swam, N. J. Willem, and W. M. de Vos. 1997. Molecular characterization of the plasmid-encoded eps gene cluster essential for exopolysaccharide biosynthesis in Lactococcus lactis. Mol. Microbiol. 24:387–397.
50. van Rooijen, R. J., and W. M. de Vos. 1990. Molecular cloning, transcriptional analysis, and nucleotide sequence of lacR, a gene encoding the repressor of the lactose phosphotransferase system of Lactococcus lactis. J. Biol. Chem. 265:18499–18503.
51. Weissbach, H., and N. Brot. 1991. Regulation of methionine synthesis in Escherichia coli. Mol. Microbiol. 5:1593–1597.
52. Wu, W. F., M. L. Urbanowski, and G. V. Stauffer. 1995. Characterization of a second MetR-binding site in the metE metR regulatory region of Salmonella typhimurium. J. Bacteriol. 177:1834–1839.
53. Wu, W. F., M. L. Urbanowski, and G. V. Stauffer. 1992. Role of the MetR regulatory system in vitamin B12-mediated repression of the Salmonella typhimurium metE gene. J. Bacteriol. 174:4833–4837.