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Brice Sperandio, Celine Gautier, Nicolas Pons, Stanislav Ehrlich, Pierre Renault, et al.. Three paralogous LysR-type transcriptional regulators control sulfur amino acid supply in *Streptococcus mutans*. *Journal of Bacteriology*, 2010, 192 (13), pp.3464-3473. 10.1128/JB.00119-10 . hal-01204450

HAL Id: hal-01204450

<https://hal.science/hal-01204450>

Submitted on 30 May 2020

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Three Paralogous LysR-Type Transcriptional Regulators Control Sulfur Amino Acid Supply in *Streptococcus mutans*^{∇†}

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Received 3 February 2010/Accepted 13 April 2010

The genome of *Streptococcus mutans* encodes 4 LysR-type transcriptional regulators (LTTRs), three of which, MetR, CysR (cysteine synthesis regulator), and HomR (homocysteine synthesis regulator), are phylogenetically related. MetR was previously shown to control methionine metabolic gene expression. Functional analysis of CysR and HomR was carried out by phenotypical studies and transcriptional analysis. CysR is required to activate the transcription of *cysK* encoding the cysteine biosynthesis enzyme, *tcyABC* and *gshT* genes encoding cysteine and glutathione transporter systems, and *homR*. HomR activates the transcription of *metBC* encoding methionine biosynthesis enzymes, *tcyDEFGH* involved in cysteine transport, and still uncharacterized thio-sulfate assimilation genes. Control of HomR by CysR provides evidence of a cascade regulation for sulfur amino acid metabolism in *S. mutans*. Two conserved motifs were found in the promoter regions of CysR and HomR target genes, suggesting their role in the regulator binding recognition site. Both CysR and HomR require *O*-acetylserine to activate transcription. A global sulfur amino acid supply gene regulatory pathway is proposed for *S. mutans*, including the cascade regulation consequent to transcriptional activation of HomR by CysR. Phylogenetic study of MetR, CysR, and HomR homologues and comparison of their potential regulatory patterns among the *Streptococcaceae* suggest their rapid evolution.

Adaptation of bacteria to new environments often requires acquisition of new functions. Mutation of genes is not considered the primary mechanism for adaptation since it often leads to abnormal products and, after further changes, could lead to loss of the initial function. Alternatively, lateral gene transfer is proposed to play a most important role in the acquisition of new functions, but it does not explain how functions initially arose in the donor. In this respect, gene duplication might be a valuable source for function evolution, since a duplicated gene may evolve without loss of a possibly essential function. Therefore, duplication events lead to the appraisal of a family of genes, which have related sequences but may exert different functions. Genome studies show that such cases occur frequently, and genes that retain their initial functions in different bacteria are often referred to as orthologs, whereas those that underwent changes after duplication are called paralogs (37). However, recent computational studies have suggested that bacterial transcriptional factors that may be orthologous by criteria such as alignment or synteny may have different functions and may regulate different genes due to a very rapid evolution (50). Therefore, study of orthologous and paralogous genes is an important focus of research to ensure correct assignment of their functions in the ever-increasing number of sequenced genomes.

The availability of many genomes of related species offers a

good opportunity to detect duplication events, as shown with yeast (34, 35, 48), enterobacteria (50), bacilli (15, 32, 60), and streptococci (9, 23, 41). *Streptococcus* is a genus that contains a wide variety of species, mostly commensal or pathogenic for humans and animals but also used as food starters. Within the host, streptococci occupy a broad range of ecological niches, such as mucosal surfaces and skin or muscle tissues, and the factors responsible for their niche adaptations remain poorly understood. The particular importance of these bacteria, both in severe pathologies and in food productions, prompted studies aiming to understand their adaptation versatility, in particular, at the genome level. Gene acquisition was shown to be an important feature in the evolution of their genome (3, 17, 36, 40, 45, 62), but also were other processes, such as recombination and positive selection within their core genomes (10, 11, 39).

In this paper, we study the function of two new members of the LysR-type transcriptional regulator (LTTR) family involved in sulfur amino acid metabolism in streptococci. Cysteine and methionine have crucial roles in different metabolisms in the cell, due in particular to the reactivity of their sulfur group, which plays an essential role in the catalytic sites of many enzymes and participates in ion and redox metabolisms (6). Interestingly, regulation of these pathways involves a large variety of mechanisms in different groups of bacteria so that it appears to evolve more rapidly than that of many other regulatory pathways. For example, *Bacillus subtilis* and *Lactococcus lactis*, which are used as models of the *Firmicutes*, do not share orthologous transcriptional regulatory factors for sulfur amino acid pathways. Furthermore, in *L. lactis*, a member of the *Streptococcaceae*, most of the *cys* and *met* genes are regulated by a single LTTR, CmbR (cysteine/methionine biosynthesis regulator, also named FhuR) (19, 58), which recognizes a 13-bp box located in their promoter regions (19, 26, 58). The

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† Supplemental material for this article may be found at <http://jlb.asm.org/>.

∇ Published ahead of print on 23 April 2010.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
<i>E. coli</i> strain TG1	<i>supE Δthi(lac-proAB) hsdD5 (F⁺ traD36 proAB lac^qZΔM15)</i>	25
<i>S. mutans</i> strains		
UA159	<i>S. mutans</i> wild-type strain (ATCC 700610)	ATCC
JIM8877	UA159 Δ <i>cysR</i>	This work
JIM8878	UA159 Δ <i>homR</i> , Em ^r	This work
JIM8879	UA159 Δ <i>gshT</i> , Em ^r	This work
JIM8880	UA159 Δ <i>cysK</i> , Em ^r	This work
JIM8882	UA159 Δ <i>tcyA</i> , Em ^r	This work
JIM8884	UA159 Δ <i>tcyE</i> , Em ^r	This work
JIM8886	UA159 Δ <i>tcyA ΔtcyE</i> , Em ^r Tet ^r	This work
JIM8890	UA159 containing pJIM5788 integrated at the <i>PhomR</i> locus, Em ^r	This work
JIM8892	UA159 containing pJIM5790 integrated at the <i>PtcyD</i> locus, Em ^r	This work
JIM8887	UA159 containing pJIM5786 integrated at the <i>PcysK</i> locus, Em ^r	This work
JIM8888	UA159 containing pJIM5787 integrated at the <i>PcysK</i> locus, Em ^r	This work
JIM8889	JIM8877 containing pJIM5786 integrated at the <i>PcysK</i> locus, Em ^r	This work
Plasmids		
pGEM-T easy	Ap ^r , M13ori pBR322ori, linear T-overhang vector	Promega
pGhost 9	Em ^r , ori ⁺ Δ <i>repA</i> , integrative promoter probe vector	43
pJIM4900	Em ^r , ori ⁺ Δ <i>repA</i> , integrative promoter probe vector containing <i>luxAB</i> genes	57
pJIM5785	Sall fusion of pGhost 9 and pGEM-T Easy containing 818-bp fragment upstream and 850-bp fragment downstream <i>cysR</i> gene ligated in BamHI	This work
pJIM5788	P_{HomR} - <i>lux</i> -SpeI site fusion of pJIM4900 and pGEM-T Easy containing P_{homR} on a 551-bp fragment	This work
pJIM5786	P_{cysK} - <i>lux</i> -BclI/BamHI site fusion of pJIM4900 and pGEM-T Easy containing P_{cysK} on a 591-bp fragment	This work
pJIM5787	P_{cysK} - <i>lux</i> -BclI/BamHI site fusion of pJIM4900 and pGEM-T Easy containing P_{cysK} carrying nucleotide substitution on the CysR box on a 591-bp fragment	This work
pJIM5790	P_{tcyD} - <i>lux</i> -SpeI site fusion of pJIM4900 and pGEM-T Easy containing P_{tcyD} on a 591-bp fragment	This work

absence of CmbR binding sites in the other streptococcal genomes (26, 58) and the presence of a different motif upstream of several potential *cys* genes in their genomes (38) suggest that the control of sulfur amino acid metabolism may be different in the other members of the closely related *Streptococcaceae* family. Lastly, study of the streptococcal MetR regulator suggested that, although MetR and its regulatory binding site are conserved among streptococci, the regulatory scheme mediated by MetR is significantly different among several bacteria of this genus (38, 52, 57). Here, we report the role of two additional LTRs involved in sulfur amino acid transport and synthesis in streptococci. Their respective coinducer, putative binding sites, and regulatory functions were determined. The first, CysR (for cysteine synthesis regulator), appears to be orthologous to CmbR but controls cysteine synthesis genes only by the means of a specific regulatory motif. The second, HomR (for homocysteine synthesis regulator) is a CysR/MetR paralog that controls homocysteine synthesis genes. Finally, likely evolutionary schemes for reporting the diversity of cysteine and methionine regulatory pathways in streptococci are discussed in the light of their specific environmental niches.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture media, and growth conditions. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* TG1 was used for plasmid propagation. *E. coli* was grown in Luria-Bertani medium at 37°C (44). *Streptococcus mutans* strains were grown at 37°C in M17 glucose medium or in chemically defined medium (CDM) (61) containing glucose (20 g/liter) as a carbon source and all amino acids except methionine and cysteine. The CDM was supplemented with 1 mM sulfide (Na₂S; Sigma), 0.5 mM thiosulfate (Na₂S₂O₃; Merck), 1 mM L-glutathione oxidized (Sigma), or 1 mM L-cystine (Sigma), when indicated. CDM+M designates CDM with 0.67 mM L-methionine (Merck), CDM+C designates CDM with 1.65 mM L-cysteine (Sigma), and

CDM+CM designates CDM with L-cysteine and L-methionine. The effect of O-acetylserine (OAS; Sigma), thiosulfate (Merck), N-acetylserine (NAS; Sigma), sulfide (Merck), or DL-homocysteine (Sigma) on transcription was tested by the addition of 4 mM concentrations of these compounds to exponentially growing cells (optical density at 600 nm [OD₆₀₀] of 0.2) in CDM+CM. When required, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (0.04 g/liter), isopropyl 1-thio-β-D-galactopyranoside (IPTG; 0.04 g/liter), ampicillin (100 μg/ml for *E. coli*), erythromycin (8 μg/ml for *S. mutans* and 100 μg/ml for *E. coli*), and tetracycline (3 μg/ml for *S. mutans*) were added to the culture medium.

DNA manipulation procedures. DNA manipulations and *E. coli* transformation were carried out as described previously (58). All enzymes for DNA technology were used according to the manufacturer specifications. Transformation of *S. mutans* by natural competence was performed as described by Murchison et al. (47). Primers used in this work were synthesized by Sigma Genosis (see Table S1 in the supplemental material). DNA sequencing was performed on both strands using a fluorescent sequencing procedure.

Gene deletion in *S. mutans*. The chromosomal *homR* (SMU.930c; GeneID 1028282), *gshT* (SMU.1942c; GeneID 1029139), *tcyA* (SMU.459; GeneID 1027964), *tcyE* (SMU.933; GeneID 1028287), and *cysK* (SMU.496; GeneID 1027990) genes were deleted in *S. mutans* strain UA159 as described previously (57). Briefly, two DNA fragments of about 750 bp corresponding to the upstream and the downstream regions of each gene were amplified by PCR using specific primers that contained 5' sequences of 19 nucleotides complementary to an erythromycin cassette (see Table S1 in the supplemental material) devoid of a transcriptional regulatory region (e.g., promoter or terminator). A joining PCR was carried out using these PCR fragments and the erythromycin cassette by the use of the created overlap at their extremities. It yielded a template allowing the amplification of a fragment carrying the antibiotic cassette flanked by the upstream and downstream chromosomal regions of the respective genes. The PCR fragments were used directly to transform *S. mutans* strain UA159. The antibiotic resistance cassette was integrated at the corresponding locus by a double-crossover event leading to the deletion of the different genes. Its transcription was driven by the initiation and termination of regulatory signals from the deleted genes in order to avoid interference with surrounding gene expression. The chromosomal *cysR* gene (SMU.852; GeneID 1028225) was deleted as follows. DNA fragments of 818 and 850 bp carrying, respectively, the upstream and the downstream regions of the *cysR* gene were generated by PCR. These fragments contain a BamHI restriction site at their 3' and 5' ends, respectively, introduced in *cysR* up and

TABLE 2. Growth of *S. mutans* UA159 and its derivatives on different sulfur sources^a

Supplement(s) in CDM	Morphology of colonies of <i>S. mutans</i> UA159	Morphology of colonies of strain expressing:						
		Δ cysR	Δ homR	Δ cysK	Δ gshT	Δ tcyA	Δ tcyE	Δ tcyA Δ tcyE
Thiosulfate	+	–	–	–	+	+	+	+
Sulfide	+	–	+	–	+	+	+	+
Glutathione	+	–	+	+	–	+	+	+
L-cystine	+	+	NT	+	+	+/-	+/-	+/-
CM	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+
M	+/-	–	+/-	–	+/-	+/-	+/-	+/-
M + thiosulfate	+	–	+	–	+	+	+	+
M + sulfide	+	–	+	–	+	+	+	+
M + glutathione	+	–	+	+	–	+	+	+
M + L-cystine	+	+	NT	+	+	+	+	+

^a Washed precultures of *S. mutans* UA159 and its derivatives were streaked onto CDM plates supplemented with different sulfur sources, and the growth was evaluated as a function of colony morphology on agar plates. C, L-cysteine; M, L-methionine; CM, L-cysteine and L-methionine; +, normal colonies; +/-, pinpoint translucent colonies; –, no colonies; NT, not tested. Pinpoint colonies were approximately 10 times smaller than normal colonies, and growth on CDM+CM or M17 medium restored a normal colony morphology.

down primers (Table S1). The PCR-amplified products were digested with BamHI, ligated, and cloned into pGEM-T Easy cloning vector. This plasmid was fused to thermosensitive pGhost 9 in SalI to yield pJIM5785 (Table 1). In *S. mutans* UA159, the *cysR* gene was deleted using pJIM5785 by double-crossing over as described by Biswas et al. (8). The mutants obtained are listed in Table 1. Regions flanking the deletions were verified by sequencing to ensure the absence of PCR-induced mutations.

Construction of lux transcriptional fusions and determination of Lux activity in *S. mutans*. The *luxAB* reporter genes were placed under the control of the *cysK*, *tcyD*, and *homR* promoter regions and integrated at their respective loci as described previously (57). Briefly, chromosomal DNA from *S. mutans* UA159 was used as a template to amplify DNA fragments containing wild-type *tcyD*, *homR*, and *cysK* or the mutant *cysK* promoter variant. PCR fragments were cloned into the pGEM-T Easy vector, sequenced, and introduced into the streptococcal integration vector pJIM4900 upstream of the *luxAB* genes (Table 1). The resulting plasmids were integrated by a single-crossover event at the *tcyD*, *homR* and *cysK* loci in the *S. mutans* UA159 chromosome. Strains carrying a single copy of the integrated plasmids were identified by PCR with specific primers. The resulting strains (Table 1) contained the corresponding transcriptional fusion as well as an intact copy of the gene. Luciferase assays were carried out on a Lumat LB9501 apparatus (Berthold Technologies, Bad Wildbad, Germany) (28).

RNA isolation, transcriptional start mapping, and real-time qRT-PCR. Total RNA was isolated as described by Sperandio et al. (57). Residual chromosomal DNA was removed by treating total RNA preparations with RNase-free DNase I (Roche) according to the manufacturer's protocol. The RNA concentration was determined by absorbance at 260 nm, and the quality of RNA preparations was checked as described previously (30). Transcriptional start sites of the *cysK*, *gshT*, *homR*, *tcyA*, and *tcyD* genes were determined using the 5'/3' rapid amplification of cDNA ends (RACE) kit (Roche). Real-time quantitative RT-PCR (qRT-PCR) was carried out using first-strand cDNA as the template. The primers used are listed in Table S1 in the supplemental material. Synthesis of cDNAs and quantitative PCR were carried out as described previously (57). For each gene, qRT-PCR experiments were performed in triplicate. Results were normalized by using the translational elongation factor Tu coding gene. Statistical significance between the mean ratios of genes was evaluated by Student's *t* test by using R (*R: A language and environment for statistical computing; reference index*; R Foundation for Statistical Computing, Vienna, Austria; ISBN 3-900051-07-0; <http://www.R-project.org>). A *P* value of <0.05 was considered significant.

Bioinformatics analysis. Research of orthologous proteins for LTRRs and sulfur amino acid supply proteins were obtained from iMoMi databases (49). Phylogenetic analyses were performed by using PHYLOGENY pipeline facilities (www.phylogeny.fr) (16). Further studies, including analysis of additional streptococcal genome sequences, were performed using the Scissors and iMoMi database (49), MUSCLE for multiple alignment (18), and PhyML for phylogeny (maximum likelihood). A search for potential regulatory motifs was performed in regions –250 to +50 relative to translational gene starts by using the MEME algorithm implemented in the iMoMi database (<http://locus.jouy.inra.fr/imomi>). WebLogo (<http://weblogo.berkeley.edu>) was used to generate CysR and HomR motifs from sequence alignments.

RESULTS

Identification of two LTRRs involved in sulfur amino acid supply in streptococci. In previous studies, CmbR (FhuR) and MetR were shown to control sulfur amino acid metabolism genes in *L. lactis* and methionine genes in *S. mutans* and *Streptococcus agalactiae*, respectively (12, 19, 56–58). A phylogenetic analysis of these proteins and their homologues from 14 *Streptococcus* genomes, presented in Fig. S1 in the supplemental material, shows that they can be clustered in three distinct groups whose representatives in *S. mutans* were named MetR, CysR, and HomR (see below). Each *Streptococcus* species encodes one protein in the CysR and MetR clusters, which contain the previously studied *L. lactis* CmbR and *S. mutans* MetR regulators, respectively. Members of the third group, the HomR cluster, are found only in *S. mutans*, *Streptococcus salivarius*, *Streptococcus infantarius*, and *Streptococcus thermophilus*. Proteins of the MetR, CysR, and HomR clusters share 57 to 88% identity with members of the same clusters, 27 to 36% with members of the other clusters, and only about 15% with the other streptococcal LTRRs. This relatedness suggests that these regulators may have diverged recently and prompts the question of their exact role in the *Streptococcaceae*.

To address this question, a functional study of mutants for genes coding for a representative protein of each cluster was carried out with *S. mutans*. With this bacterium, we have previously shown that MetR is specifically involved in the control of methionine synthesis and uptake (57). In contrast, the function of CysR and HomR (formerly SMU.852 and SMU.930c, respectively) is not known yet, although their homology with CmbR (57% and 36% of identity, respectively) suggests a role in sulfur metabolism. To test this hypothesis, Δ cysR and Δ homR mutants were constructed and their growth was tested in the presence of various sulfur compounds (Table 2). Compared to the UA159 parental strain, the Δ cysR and Δ homR mutants were unable to grow on CDM containing thiosulfate as the sole sulfur source, indicating their involvement in the regulation of the assimilation of this compound. In addition, the Δ cysR strain growth was completely abolished in the presence of sulfide or glutathione. These data indicate that both

TABLE 3. Effect of OAS on the expression of several sulfur amino acid supply genes in *S. mutans* UA159 wild-type strain and $\Delta cysR$ and $\Delta homR$ mutants^a

Gene	Organization ^b	CysR box	HomR box	Mean ratio \pm SD of gene expression levels in: ^c		
				UA159	$\Delta cysR$ mutant	$\Delta homR$ mutant
<i>cysK</i>	<i>cysK</i>	+	-	13.9 \pm 0.3	1.4 \pm 0.1*	25.7 \pm 0.7*
<i>gshT</i>	<i>gshT</i>	+	-	6.3 \pm 0.4	1.1 \pm 0.05*	8.7 \pm 0.4*
<i>tcyA</i>	<i>tcyABC</i>	+	-	2.4 \pm 0.03	0.9 \pm 0.02*	7.0 \pm 0.3*
<i>homR</i>	<i>homR</i>	+	-	16.6 \pm 1.7	2.4 \pm 0.2*	NA
<i>tcyD</i>	<i>tcyDEFGH</i>	-	+	31.8 \pm 3.3	8.5 \pm 0.1*	1.2 \pm 0.4*
<i>metB</i>	<i>metBC</i>	-	+	21.0 \pm 0.1	3.3 \pm 0.02*	1.1 \pm 0.1*
<i>cysR</i>	<i>cysR-lspA-SMU.854</i>	-	-	1.0 \pm 0.1	NA	1.0 \pm 0.1
<i>cysE</i>	<i>pnpA-SMU.156-cysE</i>	-	-	1.01 \pm 0.02	1.06 \pm 0.03	1.0 \pm 0.01
<i>cysD</i>	<i>cysD-SMU.1172c</i>	-	-	0.9 \pm 0.02	0.9 \pm 0.02	1.0 \pm 0.1
<i>metE</i>	<i>metEF</i>	-	-	0.8 \pm 0.03	1.2 \pm 0.3	1.0 \pm 0.1

^a Gene expression in *S. mutans* UA159 and the $\Delta cysR$ and $\Delta homR$ mutants was evaluated by qRT-PCR. The effect of OAS on gene expression was evaluated 45 min after the addition of 4 mM OAS to exponentially growing cells (OD₆₀₀ of 0.2) in CDM+CM.

^b Gene clusters were deduced from sequence analysis and transcriptional start site mapping.

^c Ratios correspond to expression levels in CDM+CM+OAS (OAS induced) relative to CDM+CM (uninduced). NA, not applicable; *, ratios in $\Delta cysR$ and $\Delta homR$ mutants significantly different ($P < 0.05$ by Student's *t* test) from those in the UA159 wild-type strain.

CysR and HomR play a role in the control of sulfur amino acid supply in *S. mutans*.

Role of OAS as a regulatory signal for sulfur amino acid supply genes in *S. mutans*. Transcriptional activation by LTRs requires coeffectors, which are typically intermediates of the pathways they regulate (42, 55, 57). Previously, we have shown that *O*-acetyl serine (OAS) was a coeffector of CmbR (58), making this metabolite a good candidate to be an effector of LTRs involved in cysteine supply control, such as CysR and HomR. Therefore, we tested the effect of OAS on the expression of genes coding for enzymes involved in the synthesis of cysteine (CysE and CysK) or methionine (CysD, MetA, MetBC, and MetEF), for potential L-cystine uptake systems (TcyABC [formerly SMU.459-460-461], TcyDEFGH

[SMU.932-933-934-935-936], and GshT [SMU.1942c]), and CysR and HomR regulators.

The transcriptional levels of these genes in the wild-type UA159 *S. mutans* strain growing in CDM+CM with/without OAS were compared by qRT-PCR (Table 3). The expression levels of *cysK*, *gshT*, *tcyA*, *homR*, *tcyD*, and *metB* increased 2- to 30-fold after OAS addition in the medium, whereas those of *cysR*, *cysE*, *cysD*, *metA*, and *metE* remained unchanged. The effect of other sulfur amino acid metabolic intermediates, such as NAS, sulfide, or thiosulfate, was tested on *homR* and *tcyD*, but no change was detected (Fig. 1). These results show that OAS is a key metabolite for regulation of sulfur amino acid supply in *S. mutans* and a good candidate as an effector for CysR or HomR.

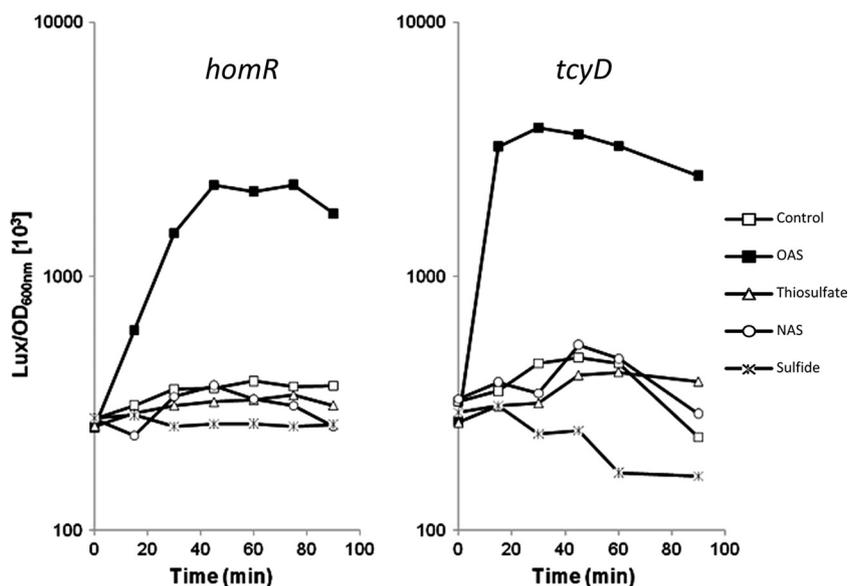


FIG. 1. Effect of OAS, thiosulfate, NAS, or sulfide on *homR* and *tcyD* expression. Luciferase activities (10^3 Lux/OD₆₀₀) were measured for JIM8890 and JIM8892 strains carrying P_{homR} -lux and P_{tcyD} -lux fusions, respectively, grown in CDM+CM (□) supplemented with OAS (■), thiosulfate (Δ), NAS (○), or sulfide (×). The x axis shows time of incubation relative to the addition of the different sulfur compounds (time zero). One curve representative of the results from at least three experiments is presented.

Respective effect of CysR, HomR, and OAS in the control of sulfur amino acid genes. To test the possible role of OAS as an effector of CysR or HomR regulators, induction levels mediated by OAS on the previously studied set of genes were measured in the Δ *cysR* and Δ *homR* mutant strains (Table 3). No change was detected in the transcription level of the genes whose expression is not affected by OAS addition, such as *cysE*, *cysD*, *metA*, and *metE*. In contrast, the activation observed for *cysK*, *gshT*, and *tcyA* transcription was abolished in the Δ *cysR* mutant, whereas it remained effective in the Δ *homR* background, indicating the requirement of CysR in their OAS-dependent activation. Moreover, the induction of *homR*, *tcyD*, and *metB* transcription by OAS decreased in the Δ *cysR* mutant (2.5 to 8.5 versus 18 to 32), suggesting a role for CysR in their regulation. Finally, the activation of *tcyD* and *metB* transcription was abolished in the Δ *homR* mutant, indicating that HomR is involved in their activation. To conclude, CysR and HomR are both involved in OAS-dependent transcription activation but target different sets of genes. CysR would directly regulate *cysK*, *gshT*, and *tcyABC* transcription, while HomR would control that of *metBC* and *tcyDEFGH*. The positive effect of CysR on the HomR-dependent gene could be indirect, through a control of *homR* transcription.

Regulatory CysR sites. In order to identify a putative regulatory site of CysR, a search for conserved sequence elements in upstream regions of potentially CysR-regulated genes in *Streptococcus* genomes was carried out using the MEME algorithm (7, 49). As an initial step, this search was performed by phylogenetic footprinting using, as a set of genes, the *Streptococcus* orthologs of *cysK*, *tcyABC*, *homR*, and *gshT*, which are controlled by CysR in *S. mutans*. A 13-bp motif, named the CysR box, organized as an interrupted dyad symmetry element, with TATCACNGTGATA as a consensus, is present in the upstream region of all *cysK*, *gshT*, *tcyA*, and *homR* orthologs and most *tcyB* orthologs (9 out of 13) (Fig. 2). In the vicinity of the CysR box, two other well-conserved boxes were identified: box1, which may be a direct repeat of the right arm of the CysR box (GTGATA), and box2 (TAT), which corresponds to the inverted sequence of the 3' half of box1. The relative position of these boxes is perfectly conserved: box1 and box2 are placed 9 nucleotides upstream and downstream of the CysR box, respectively. Finally, the CysR box is centered in a larger inverted repeat sequence covering a 37-nucleotide DNA region from the 3' half of box1 (ATA) to the 5' half of box2 (TAT) (Fig. 2, dotted inverted arrows).

As a second step, additional regulatory sites were searched over the entire *Streptococcus* genome with the consensus sequence previously computed (ATA-N₉-TATCACNGTGATA-N₉-TAT) with PATSCAN software, allowing for two changes (see Table S2 in the supplemental material). The number of genes found by this procedure varied from a single gene (*cysK* in *Streptococcus equi* and *Streptococcus mitis*) to 12 genes in *S. thermophilus* LMG18311. Among the newly identified genes, *cysD* of *S. thermophilus* and *S. salivarius* is the only gene directly related to sulfur amino acid supply (Fig. 2; see also Table S2). No target in addition to those already pointed out was identified in *S. mutans*.

To further assess regulatory features of CysR-regulated genes, we determined the transcriptional start sites of *cysK*, *gshT*, *homR*, and *tcyA* genes from *S. mutans* by 5' RACE (Fig.

2, +1). Transcription is initiated 21, 22, 24, and 25 bp upstream of the translational start point of *gshT*, *cysK*, *tcyA*, and *homR* genes, respectively. CysR boxes are thus located 48 to 56 bp upstream of the transcriptional start sites of regulated genes, fitting the general rule for LTRR operators (55). The CysR box is therefore a good candidate to be part of the CysR regulatory site and to participate in the control of cysteine synthesis in streptococci. Finally, in order to demonstrate the regulatory role of the CysR box, the effect of modification in the CysR box at positions corresponding to the conserved bases (underlined in the following sequence) for LTRRs (T-N₁₁-A) was examined on the most strongly CysR-dependent *S. mutans* gene, *cysK*, using *cysK-lux* transcriptional fusion (Table 4). The expression of *cysK* was approximately 30-fold lower when *cysR* was deleted or when the CysR box was substituted, demonstrating the role of CysR and of the CysR box.

Regulatory HomR sites. Our transcriptional analysis showed that the expression of *S. mutans metBC* and *tcyDEFGH* clusters is specifically affected in a Δ *homR* mutant (Table 3). To identify a putative HomR DNA-binding site, conserved sequence elements were searched in the upstream regions of the *metBC* and *tcyDEFGH* orthologs from *Streptococcus* species that contain HomR. This analysis revealed the presence of a potential LTRR motif (T-N₁₁-A) in the upstream region of *metB* orthologs and the *S. mutans tcyD* gene, with TADCYAACYATMA as a consensus (Fig. 3). The motif, named HomR box, is located 43 bp upstream of the transcriptional start site of *tcyD*, a correct location for an LTRR recognition site. Two conserved elements, similar to box1 and box2 previously defined in the CysR-regulated genes, were found 9 and 8 nucleotides upstream and downstream of the HomR box, respectively. Finally, no additional HomR motifs upstream of genes related to sulfur amino acid metabolism were identified in the genome of *S. infantarius*, *S. mutans*, *S. salivarius*, and *S. thermophilus* with the computed motif ATA-N₉-TATADCYAACYATMA-N₈-WAT, in allowing for one change.

Functional analysis of substrate-binding proteins regulated by CysR and HomR. The results presented above indicated that CysR and HomR activate the expression of three transcriptional units (*tcyABC*, *tcyDEFGH*, and *gshT*) encoding components of ABC transporters sharing similarities with L-cystine uptake systems. TcyA, TcyE, and GshT exhibit 29 to 32% identity to YtmJ and YckK L-cystine binding proteins of *B. subtilis* (13). Transcription of *tcyABC* and *gshT* is activated by CysR, while that of *tcyDEFGH* by HomR. In order to clarify the function of these genes and better define the metabolic role of CysR and HomR, we have performed a functional analysis of mutants for the *tcyA*, *tcyE*, and *gshT* genes encoding substrate-binding proteins of ABC transporters in *S. mutans* (Table 2).

The growth of the Δ *tcyA* and Δ *tcyE* mutant strains was significantly reduced in the presence of L-cystine as a sole sulfur source, suggesting that these genes encode transport systems involved in the uptake of this molecule. The Δ *gshT* mutant strain was unable to grow in CDM containing glutathione (GSH) as a sole sulfur source, indicating that GshT is required for GSH assimilation. *gshT* is located upstream of the *atmBCDE* cluster involved in methionine uptake and thiosulfate assimilation (57). Since the Δ *atmB* and Δ *atmBCDE* mutants and strain UA159 display similar growth levels in the

	box1	CysR-box	box2	+1
<i>cysK</i> Smu	GTTCCCGTGATGATGATTTTCCT	TATCACGGTGATAAATAATTTATAT	TTTACAAGCTTATTTTTTCAGCCT	TATAC
Sag	CTGAAACCGATAAATAATAGCT	TATCACT-TGATAAAGAATTGATAT	TTTACTTTAATCAATGTGAGTTT	TATAAT
Sdy	GAAAGATGATAGAACTTTTAT	TATCAGAGTGATAGTAAATCTGTAT	TTTACTTTGGACTAGTATCCTCT	TATAC
Seq	TGAACAGTGATAGCGTCTCTT	TATCAGTACGATAGTAAATGGCTAT	TTTACTTTGTCGATTATTAGCTAT	TATAC
Sgo	TTTCTTGATATAAAAAAAGCT	TATGATAGCCATAGCTTTTTTATAT	TTTACAAATCCAAATTTTCATTT	TATAC
Sinf	TGCCTTGTGATAAGATTTCCCT	TATCACCATGATAAAGAAATATATAT	TTTACAACAGTCAATTTGAGCAT	TATAC
Smi	ACCTGTGATATAAAAAATCCT	TATCACTTCGATAGGATTTCTATAT	TTTACAATGGTATAGATAGCGT	TATAC
Spn	ATCCTATGATATAGAAAATCCT	TATCGCTTCGATAGGATTTCTATAT	TTTACAAATGGTATGGATAGCGT	TATAC
Spy	ACAAAAGTGATAGGCTTTTAT	TATCAGAGTGATAGTAAATCTGTAT	TTTACTTTAATAATATCTCTCCT	TATAC
Ssa	TGTTTTGACATAAAGAAAAGCT	TATGGCTTCATAGCTTTTTTATAT	TTTACAAGCTCTTATTTTCATTT	TATAC
Ssu	TCCTATCTGATAGTCAAACGT	TATCAGAGTGATAAAGAAGATGAT	TTTACAAGTTGAGCCTACCCTCGT	TATAC
Sth	TACCTGTCTTAAAAAAGCT	TATCGCGAGATAGTTTTTTATAT	TATCCTTTTGTACTTCTACTAT	TATAAT
Sub	TCCTATCTGATAGTCAAACAT	TATCAGAGTGATAAAGAAGATGAT	TTTACAAGTTAGGCTACCCTCGT	TATAC
	AGGCCAATGATAAGCCTTTCC	TATCACGCCAATAAGATTAACTAT	TTTACTTTTCATCTTAAGAGACT	TATAC
<i>gshT</i> Smu	ATATTCGTGATAGAAATTTTCT	TATCACCGTGATAGAAATATATAA	TAGGCAAGCTTTTCAATTAGT	GATAAGAT
Sag	ATCCATATAATAGCAAAAATCCT	CTCAG-ATGATAGAGATAAGTGT	TAGTTAAAAACTTAAAGATAT	TATGATAT
Sdy	AAAAAGTAAATGCAAAAGTG	TATCAGCTTGATAGGAATTAATAT	TAGTTAACTCTAATAAAAAGT	GGTAAAC
Seq	TAGTAGTGTATCGTACATGGT	GCTGCTTTAGCAGAGTTCAATCAT	TAGTATCTTATGATAAAAAAT	GATAAACT
Sgo	GAGCTCTCAATAAATAAAAT	TATACCTTGATAAATAATATATAT	TAGGCAAGCTTAGGGATATTT	GTATATAT
Sinf	CCTAGGGTGATAAGATTTCTT	TATCGCGGTGATAGAAATTAAGTAT	TTGTTAAGAAGCTTATCTGATG	TAAAGT
Spn	AAATTAACCATAAAAAAATCCT	TATCAGAGATAGATATCATATAA	TGGCGTAAATGCCTTTTCTCT	TAAAGT
Spy	TAAAGAACAATAGTAAATCAGT	TACAACTTGATAGGAATCAATTAT	TAGTGAACCTAATAAAAATG	TAAACT
Ssa	TCAAAGCTGATAAAGAAAAGCT	TACACCTCCATAAAAAATATATAA	TGGTCAAGTTTCTCCTTATTT	GATATGATA
Ssal	TTTTAGTGTATGATTTTCTT	TATCACCCTGATATGTTTAAAGTAT	TAGTTAAGCTTAGTGAATTC	GTATACAA
Sth	TTTTAGTGTATGATTTTCTT	TATCACTTTGATATATTAAGTAT	TAGTTAAGCTTAGTGAATTC	GTATACAA
Sub	TTTTGTGTGATAGAGAATCTAT	TATCATAGCGATAGTGAATCACTAT	TAGTCAAGTACGATTCAGAGAT	GTAAAA
<i>tcyA</i> Smu	TGTGGAGTGATAGAAAATAT	TATCACAGTGATAGGAAAAACTAT	TATACAGAGCTTGTGTATCGT	GATAGACT
Sag	TTTTTATTGATATAAAATCTT	TATCACC-TGATAAGGAAATACTAT	TATACAGATAGATTTTAGAGT	GTATAGACT
Sdy	GAGGATGTGATT-AATATTTT	GATCAGTTTGATAGTTAAATGGTAT	TAGGCAAGCTCTTTTATTTT	GTATAGACT
Seq	GCTTGTGTTGATACTATTTTCT	TATTGCCTTGATAGAGAAATGATAT	TATTCAAATTTATCATTTTTT	GTAAAA
Sinf	GATCTCATGATAGTTTTTCT	TATCAAGTCTATAGAAAATATATA	TGGCAGGCGCCTCTAAGTT	TGGTAGACT
Ssal	GATTCCCTATAACCTTTATCT	TATCACC-TCTTAGGATTTTATAT	TAGACGAGTTTGTAGGAAGAT	GATAAAT
Sth	ACATGAGTGGCAACCTTTTCT	TATCACC-ACTTAGGTTTTTATAT	TAGACAAGTTGGGATAAAGAT	GTAACCT
Sub	CGTTTTGCATATAAATAGCTA	AATTACCTTGATAGAAAATGGTAT	TATTCAAAGGCAGGCTTCT	GTAAAA
<i>tcyB</i> Sdy	GCTGTCGTGATAAGTTTTGCT	TATTGCTATAATATAGATTTACTAT	TTTACAATGTGGGTGAAGAGT	GTATAGAAT
Seq	TCTTTCGCGATAGGCTTTTCT	TATCATTATCATGCGGATTTACTAT	TTTACATGACATCTCAAGAA	GTATAAAA
Sgo	ATACCCTTGATAAATAATTTATA	CTGTTTCGATATAAATAATGGTGT	GATAGACTAGAGTATAC--	TGCTCTAT
Spn	GGGACTGAAATAGTTTTTATC	TATCTCTTTGATAGATAAATAATATA	AACCTCCCTGTTCAAAGAT	GATAAAT
Spy	GAAAGTATCATAGCTTTTCT	TATTGCTATAATACGGATTTACTAT	TTTACATGTGGATCTAAGAGT	GTATAGAA
Ssa	CTTTTAGTGATATACTTTTCT	TATCGCTGTGATAAAGAAAATATAT	TAGTCTTTATGATAGATAT	GATAGACT
Ssal	CTATCAGTGATACGATTTTCT	TATCACTATGATAAAGAAAAGCTAT	TTCACAGACTTTCTGAGTCG	TAAAA
Sth	TTATCAGTGATATGATTTTCT	TATCACAATGATAAAGAAAAGCTAT	TTCACAGACTTTCTGAGTCG	TAAAA
Sub	ACTTCTGTGATAGGCTTTAAC	TATTGCTTCAATAACTTCTTCTAT	TTTACAATGATTTATGGTAA	TGCTAAAC
<i>homR</i> Smu	TAGTAGGCCATAAGAAAAGT	TATCACTCCTTTGTTTTTTC	TATCGGTTTTTGGCGAT-N16	TGTTATAAT
Sin	TCACCCGCTATAGGATTTTTT	TATCAAGGCTTTAGATATTCT	TATCAGAATTTTGGCTGAT-N16	TCTATAAT
Ssal	GACTTAATCATAGAAATTTT	CATATCAGGTCCTCATATTTCC	TATCAGAATTTTGGCTGAT-N16	TGTTATAAT
Sth	CCTGACTTTATAAAAATTTCA	TATCAGGTCCTCATATTTCC	TATCAGGATTTTACTGAT-N16	TGTTATAAT
<i>cysD</i> Sth	GTCCTTTTATAAGCCCTCCC	TATCACATTGATAAAAAATTTCTAT	TAGACAGCTAGATTTTCTAGGT	TAGAAT
Ssal	GTCCTTTTATAAGCCCTCCC	TATCACATTGATAAAAAATTTCTAT	TAGACAGTTAGCTTTTATGGGT	TAGAAT

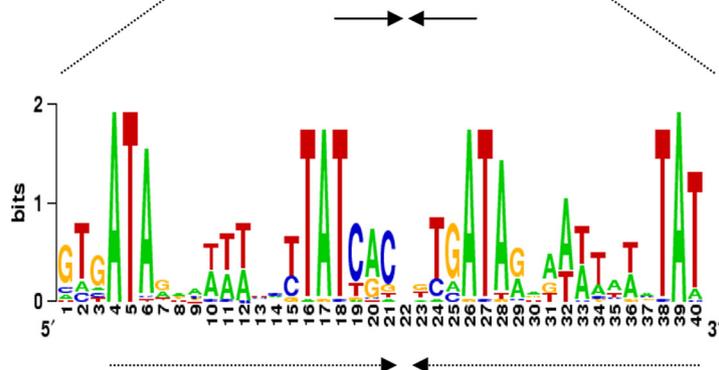


FIG. 2. Alignment of CysR-regulated gene promoter regions of *S. mutans* and of their orthologs in other streptococci. Conserved regions (box1, CysR box, box2) are shaded, and inverted regions (arrows) are indicated below. Transcription start sites (+1) of *cysK*, *gshT*, *tcyA*, and *homR* of *S. mutans* were identified by 5' RACE. The deduced -35, -10, and extended -10 regions are boxed. Logo of the region containing the CysR box was generated from the sequence alignments of promoter regions of *cysK*, *gshT*, *tcyA*, and *tcyB* orthologs with WebLogo (<http://weblogo.berkeley.edu>).

TABLE 4. Effect of CysR and CysR-box mutations on *cysK* expression in *S. mutans*

Strain	CysR box ^a	Luciferase activity ^b
UA159 P _{<i>cysK</i>::lux}	TATCACGGTGATA	9,733 ± 180
UA159 P _{<i>cysK</i>::lux}	GATCACGGTGATG	423 ± 15
Δ <i>cysR</i> P _{<i>cysK</i>::lux} mutant	TATCACGGTGATA	315 ± 11

^a Nucleotide substitutions on the CysR box of the *cysK* gene are underlined.

^b Luciferase activity (10³ Lux/OD₆₀₀) was measured for strains JIM8887 (UA159 P_{*cysK*::lux}), JIM8888 (UA159 P_{*cysK*::lux} carrying nucleotide substitutions in CysR box), and JIM8889 (Δ*cysR* P_{*cysK*::lux}) cultivated in CDM+CM. The luciferase activities shown are the averages ± standard deviations of three independent cultures at an OD₆₀₀ of 0.4.

presence of GSH (data not shown), GshT is likely a substrate-binding protein necessary for GSH uptake.

DISCUSSION

In this work, CysR and HomR, two new LTRRs involved in sulfur amino acid metabolism in *S. mutans* were characterized. These regulators respond to OAS as a coeffector but control different sets of genes. CysR activates the transcription of *cysK*, *tcyABC*, and *gshT*, which are required for *de novo* cysteine synthesis and transport of L-cystine and glutathione, a cysteine-containing tripeptide, respectively. HomR positively regulates the transcription of *metBC* and *tcyDEFGH*, involved in homocysteine synthesis and L-cystine uptake, respectively. Furthermore, the absence of growth of a *homR* mutant in a medium containing thiosulfate as the sole sulfur source indicates that HomR controls additional genes remaining to be determined. Interestingly, *homR* expression is activated by CysR, leading to a regulatory cascade. The fact that the two regulators are responding to the same coeffector suggests that this cascade is a fine-tuning system to control HomR targets.

On the basis of this and previous work we propose a global

regulatory pathway for sulfur amino acid supply in *S. mutans* (Fig. 4). Its upper part, corresponding to cysteine synthesis, is activated by CysR and OAS, and its intermediate part, allowing homocysteine supply, is under the control of HomR and OAS and indirectly of CysR. Finally, the lower part, leading to methionine synthesis, is activated by MetR, with homocysteine as a coeffector (57), and by an *S*-adenosylmethionine (SAM)-responsive riboswitch mechanism (22). While the three regulators participate in the control of cysteine or of its precursor's uptake, the transport of methionine and of its precursor homocysteine is regulated only by MetR. This regulatory network, composed essentially of 3 paralogous LTRRs, differs significantly from those described for other Gram-positive bacteria, such as *B. subtilis*, *Corynebacterium glutamicum*, and *Clostridium acetobutylicum*, in which a large diversity of molecular mechanisms controls sulfur metabolism (2, 4, 5, 14, 27, 31, 51–54, 59).

Orthologs of the *S. mutans* CysR and MetR regulators are encoded by all *Streptococcus* and *Lactococcus* genomes, while HomR is found only in *S. mutans*, *S. infantarius*, *S. salivarius*, and *S. thermophilus* (Fig. S1 in the supplemental material). As deduced from specific motifs found for these regulators, common lines for sulfur amino acid gene regulation are conserved among the *Streptococcaceae*, even if differences do exist. MetR displays the most conserved regulatory pattern and recognizes a motif present in the upstream region of *Streptococcus* and *Lactococcus metE*, encoding the last step for methionine synthesis. In few species, MetR may display a limited number of additional targets (52, 57). In contrast, *L. lactis* CmbR and *S. mutans* CysR, which both belong to the CysR cluster, display significantly different regulatory patterns (58; this study). CmbR positively regulates 18 genes (7 transcriptional units), including most cysteine and methionine metabolic and transporter genes, while *S. mutans* CysR controls 6 genes (4 transcriptional units) involved only in cysteine supply. The phylo-

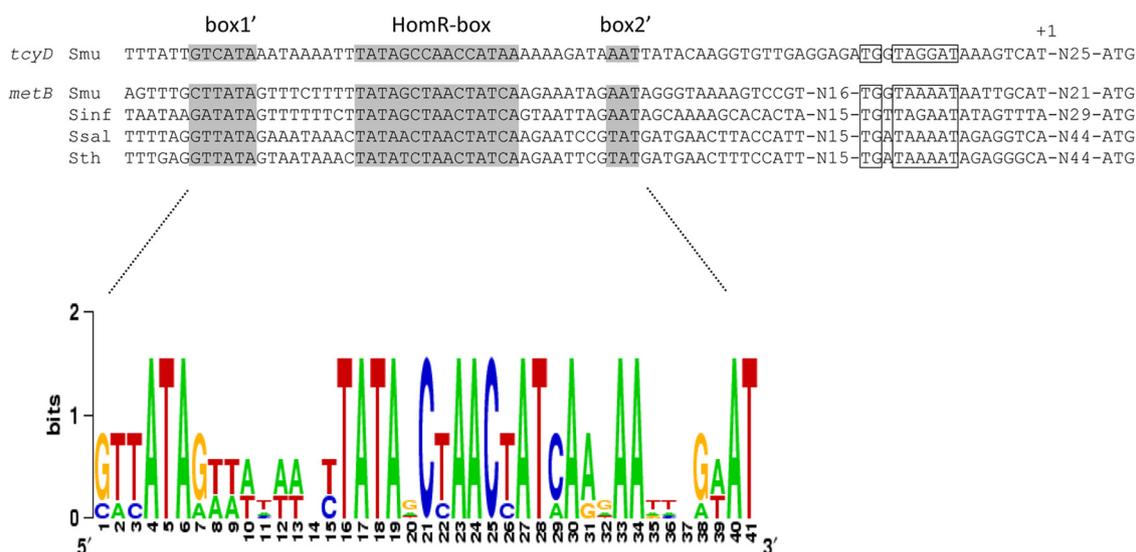


FIG. 3. Alignment of HomR-regulated gene promoter regions of *S. mutans* and of their orthologs in other streptococci. Conserved regions (box1', HomR box, box2') are shaded. Transcription start site (+1) of *tcyD* of *S. mutans* was identified by 5' RACE, and the deduced extended -10 regions are boxed. Logo of the region containing box1' to box2' (including the HomR box) was generated from the sequence alignments with WebLogo (<http://weblogo.berkeley.edu>).

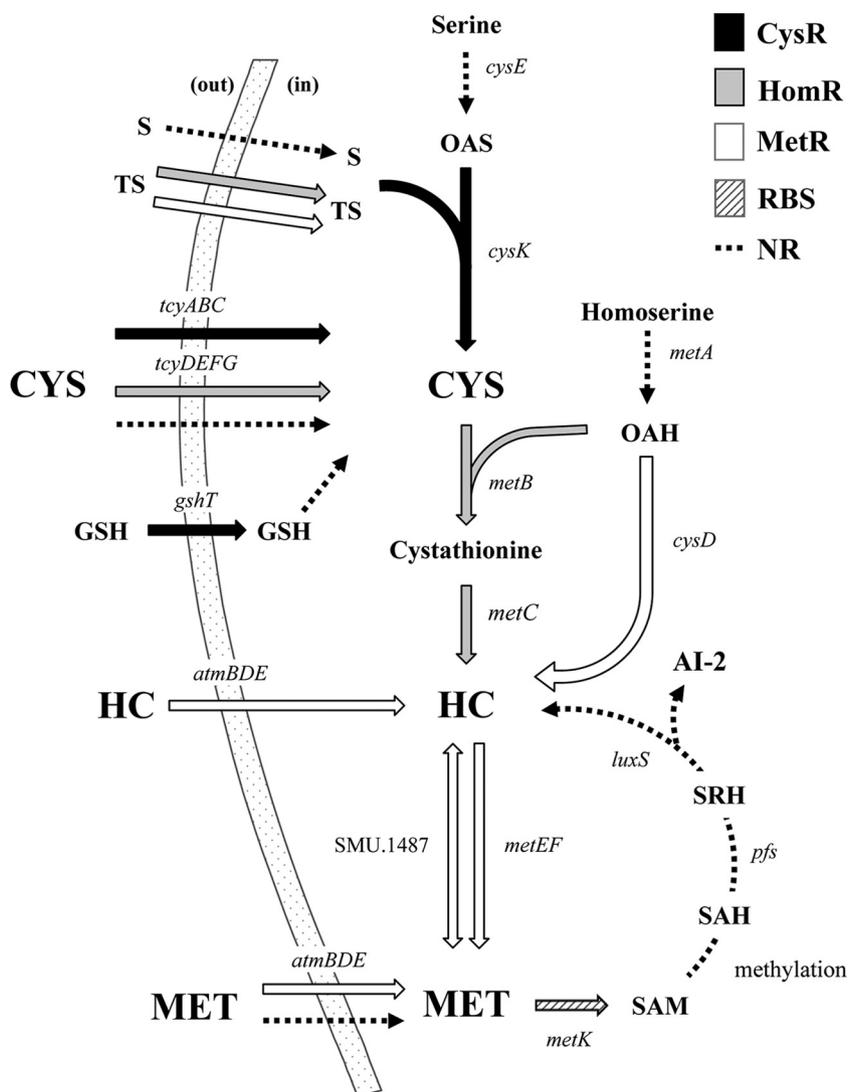


FIG. 4. Overall regulatory scheme for control of sulfur amino acid supply in *S. mutans*. Colored arrows represent the steps controlled by CysR (black), HomR (gray), MetR (white), and riboswitch (hatched). Discontinuous arrows indicate steps for which no regulation has been yet evidenced. Genes of *S. mutans* proposed to encode proteins involved in the corresponding reaction are indicated. OAS, *O*-acetylserine; OAH, *O*-acetylhomoserine; CYS, cysteine; HC, homocysteine; MET, methionine; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; SRH, *S*-ribosylhomocysteine; TS, thiosulfate; S, sulfide; GSH, glutathione; RBS, riboswitch; NR, not regulated.

genic analysis presented here and several aspects of their regulatory pattern suggest that these regulators are orthologs. For example, CmbR and CysR respond to OAS as a coeffector and control several orthologous genes involved in cysteine metabolism, such as *cysK*, the most strongly regulated genes in both species (19, 58; this work). However, the LTTR sites recognized by these regulators exhibit a different consensus motif (TATCACNGTGATA for CysR and TAAAWWTTT YTWA for FhuR/CmbR). The CysR motif is partly similar to a motif found by a comparative genomic approach (TGATA-N₉-TATCA-N₂₋₄-TGATA) and that was proposed to be the CmbR motif, although absent in *L. lactis* (38). The absence of potential CysR and CmbR boxes in lactococci and streptococci, respectively, confirms the divergence between the recognition sites of the 2 regulators (E. Guédon, unpublished data). Since functional and phylogenetic analysis clearly links

CmbR to the CysR cluster, we propose that CmbR separated from the CysR cluster and now recognizes a new and less conserved motif, allowing an enlarged regulatory spectrum. However, it is also possible that the CysR ancestor in streptococci evolved to recognize a strict consensus and a smaller number of targets. Both hypotheses are in agreement with the fact that regulatory functions are often evolving faster than other functions in microorganisms (50).

Besides this major divergence between lactococci and streptococci, further data suggest that CysR regulation is rapidly evolving within streptococci. CysR orthologs and CysR boxes are present in all *Streptococcus* genomes, suggesting a conserved control by CysR of *cysK*, *tsyABC*, and *gshT* orthologs when present. Nevertheless, in *S. agalactiae*, upstream of the *cysK*, *gshT*, and *tsyA* genes, a base is missing from the CysR boxes, suggesting a loss of functionality. A lack of activation of

cysK transcription would be in agreement with the requirement of cysteine for *S. agalactiae* growth (46). Conversely, the CysR box is overrepresented in the *S. thermophilus* genome in which, in addition to the above-mentioned sulfur metabolic genes, CysR boxes are found upstream of genes coding for two substrate-binding proteins of oligopeptide ABC transporters (24), two amino acid transporters (including a *gshT* paralogous gene with 96% identity), a protein associated with bacteriocin production (21), and a potential redox-sensitive transcriptional regulator. Since cysteine or cystine is not detectable as a free amino acid in milk, cysteine-containing peptides may represent the main source of cysteine for *S. thermophilus* in this environment (33). These facts suggest modification of the CysR target set in relation to *S. thermophilus* adaptation to its ecological niche.

In addition to MetR and CysR, several streptococci, such as *S. mutans*, *S. infantarius*, *S. salivarius*, and *S. thermophilus*, contain HomR, a third LTTR involved in sulfur amino acid metabolism. Phylogenetic analysis of HomR indicates that it is related to CysR, although it forms a cluster separate from CysR orthologs. The involvement of OAS as an effector in both groups indicates a functional conservation. Beside these conserved features, CysR and HomR differ by their recognition sites (TATCACNGTGATA and TADCYAACATMA, respectively) and target genes. In this work, we have shown that *S. mutans* HomR is required for the expression of two homocysteine synthesis enzymes (MetB and MetC) and an L-cysteine transport system (TcyDEFG) and is likely required for the expression of thiosulfate assimilation genes. These data indicate that HomR is a CysR paralog that drifted to control specific genes, likely to increase environmental fitness of these bacteria.

Conclusion. In the *Streptococcaceae*, sulfur amino acid supply is controlled by 2 or 3 related LTTRs. Their main regulatory features appear to be conserved among this family of bacteria, but several differences could be evidenced. First, HomR, which is present in a limited number of species, may be the result of duplication from *cysR* followed by a drift and specialization to few targets. Second, CmbR, the lactococcal CysR ortholog, may have diverged from a CysR ancestor to recognize a new motif for DNA binding and regulate an enlarged set of genes. Lastly, regulation functions of CysR in different streptococci display subtle differences that could reflect adaptation of regulatory function to cope with their different lifestyles. In this genus, several species display versatile metabolic ability; these include *S. mutans* (1, 20) and *S. salivarius* (P. Renault, unpublished data) living in an oral environment or those of more limited ability, such as *S. agalactiae*, in which CysR may have lost its regulatory role. Previous studies indicated that streptococci evolved by gene acquisition, recombination, and positive selection in their core genomes. This study underlines the rapid drift of regulatory function in the *Streptococcaceae* as an additional paradigm of evolution, allowing their adaptation to different niches within hosts and shaping their behaviors toward being commensal or pathogenic.

ACKNOWLEDGMENTS

We sincerely thank Tarek Msadek for critical reading of the manuscript, Jean-Baptiste Denis for statistical analysis of qRT-PCR data, and Isabelle Martin-Verstraete for helpful discussions. We also thank

Valentin Loux for the annotation of the *S. salivarius* JIM8777 genome with AGMIAL.

B. Sperandio had a grant from the Ministère de la Recherche et de l'Education Nationale.

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