The *cmaR* gene of *Corynebacterium ammoniagenes* performs a novel regulatory role in the metabolism of sulfur-containing amino acids

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A novel regulatory gene, which performs an essential function in sulfur metabolism, has been identified in *Corynebacterium ammoniagenes* and was designated *cmaR* (cysteine and methionine regulator in *C. ammoniagenes*). The *cmaR*-disrupted strain ($\Delta cmaR$) lost the ability to grow on minimal medium, and was identified as a methionine and cysteine double auxotroph. The mutant strain proved unable to convert cysteine to methionine (and vice versa), and lost the ability to assimilate and reduce sulfate to sulfide. In the $\Delta cmaR$ strain, the mRNAs of the methionine biosynthetic genes *metYX*, *metB* and *metFE* were significantly reduced, and the activities of the methionine biosynthetic enzymes cystathionine γ -synthase, *O*-acetylhomoserine sulphydrylase, and cystathionine β -lyase were relatively low, thereby suggesting that the *cmaR* gene exerts a positive regulatory effect on methionine biosynthetic genes. In addition, with the exception of *cysK*, reduced transcription levels of the sulfur-assimilatory genes *cysXYZ* and *cysHDN* were noted in the *cmaR*-disrupted strain, which suggests that sulfur assimilation is also under the positive control of the *cmaR* gene. Furthermore, the expression of the *cmaR* gene itself was strongly induced via the addition of cysteine or methionine alone, but not the introduction of both amino acids together to the growth medium. In addition, the expression of the *cmaR* gene was enhanced in an *mcbR*-disrupted strain, which suggests that *cmaR* is under the negative control of *McbR*, which has been identified as a global regulator of sulfur metabolism. DNA binding of the purified CmaR protein to the promoter region of its target genes could be demonstrated *in vitro*. No metabolite effector was required for the protein to bind DNA. These results demonstrated that the *cmaR* gene of *C. ammoniagenes* plays a role similar to but distinct from that of the functional homologue *cysR* of *Corynebacterium glutamicum*.

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INTRODUCTION

Sulfur is indispensable to the growth of micro-organisms. It is a critical component of the amino acids cysteine and methionine. Moreover, cysteine-derived proteins such as

Abbreviations: OAH, *O*-acetyl-L-homoserine; OAS, *O*-acetyl-L-serine; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine.

The GenBank/EMBL/DDBJ accession numbers for the *cmaR*, *metYX*, *metFE*, *metB*, *cysXYZ*, *cysHDN*, *cysKE*, *mcbR* and *aecD* sequences of *Corynebacterium ammoniagenes* are FJ210287, FJ483537, FJ483538, FJ483539, FJ483540, FJ483541, FJ483542, FJ483543 and FJ483544, respectively.

A supplementary table, listing primers used in this study, is available with the online version of this paper.

thioredoxin participate in a number of critical cellular defence processes against oxidative stress. The sulfur moiety of cysteine is synthesized from sulfide, which is generated by the serial reduction of oxidized sulfur sources via a process referred to as 'sulfur assimilation'. The sulfur moiety of methionine is supplied by cysteine (transsulfuration) or sulfide (direct sulphydrylation) (Fig. 1). Although significant progress has been made with regard to our knowledge of methionine and cysteine biosynthesis in *Corynebacterium glutamicum* (Lee, 2005; Rückert & Kalinowski, 2008), current information regarding the interrelation and control of the cysteine and methionine biosynthetic pathways remains relatively limited. Recently, the reverse transsulfuration route, which is exploited in the

Fig. 1. Biosynthesis of methionine and cysteine in *C. glutamicum*. The genes encode the following enzymes: *cysDN*, ATP sulfurylase; *cysH*, adenosine 5'-phosphosulfate (APS) reductase; *cysIX*, sulfite reductase; *cysK*, O-acetylserine thiolase; *cysE*, serine acetyltransferase; *cysZ*, putative transporter protein; *aecD*, cysteine desulfhydrase and cystathionine β -lyase; *metX*, homoserine acetyltransferase; *metB*, cystathionine γ -synthase; *metY*, O-acetylhomoserine sulphydrylase; *metF*, $N^{5,10}$ -methylene H_4 reductase; *metE*, B_{12} -independent methionine synthase; *methH*, B_{12} -dependent methionine synthase; *metK*, S-adenosyl methionine synthase; *ssuD*, FMNH₂-dependent aliphatic sulfonate monooxygenase; *ssuCBA*, ABC-type aliphatic sulfonate transporter; *seuAB*, FMNH₂-dependent monooxygenase, sulfonate ester degrading; *seuC*, probable FMNH₂-dependent monooxygenase.

conversion of methionine to cysteine, has been identified in *Bacillus subtilis* and *Mycobacterium tuberculosis* (Hullo *et al.*, 2007; Wheeler *et al.*, 2005). Furthermore, in *Pseudomonas putida*, methionine is probably utilized to synthesize cysteine via the unusual intermediate methanesulfonate (Vermeij & Kertesz, 1999). In *Corynebacterium*, despite its importance as a possible methionine or cysteine producer, the method by which methionine and cysteine are metabolized as a sole sulfur source remains to be clearly elucidated. Therefore, not only experimental corroboration but also *in silico* prediction from full genome sequences has been utilized to identify the genes involved in sulfur metabolism (Haitani *et al.*, 2006; Hwang *et al.*, 1999, 2002; Kim *et al.*, 2001; Koch *et al.*, 2005a, b; Lee, 2005; Park *et al.*, 1998; Rückert *et al.*, 2003, 2005, 2008; Rey *et al.*, 2003, 2005; Wada *et al.*, 2002, 2004). Significant progress has been made toward understanding the regulatory mechanisms of sulfur metabolism in micro-organisms. In general, sulfur metabolism is strictly regulated by activators and repressors. Thus far, several different types of regulators that control sulfur metabolism in micro-organisms have been identified. In *Escherichia coli*, CysB activates the

transcription of genes associated with cysteine biosynthesis (Kredich, 1996). Under sulfate-starvation conditions, *cbl*, the transcription of which is also positively regulated by CysB, is expressed and, in turn, the gene product activates the transcription of genes involved in taurine and organic sulfur utilization (van der Ploeg *et al.*, 2001). In *B. subtilis*, CymR plays an important role (Even *et al.*, 2006). Under excess cysteine conditions, CymR forms a complex with CysK and, subsequently, the complex represses genes that participate in cysteine biosynthesis (Hullo *et al.*, 2007). O-Acetylserine is a key substance that controls the formation of the CymR–CysK complex (Tanous *et al.*, 2008). *Lactococcus lactis* employs a similar mechanism for the control of genes (Sperandio *et al.*, 2005). In addition, the positive regulatory protein CysL has also been identified in *B. subtilis* (Guillouard *et al.*, 2002). The regulatory mechanism of sulfur metabolism in *C. glutamicum* has recently been established (Rückert *et al.*, 2008). It involves a global repressor McbR and the two activators, SsuR and CysR. The activity of McbR is controlled by the intracellular ratio of S-adenosyl-L-homocysteine (SAH) and S-adenosyl-L-methionine (SAM) (Rey *et al.*, 2005;

Suda *et al.*, 2008). Under repressive conditions, the intracellular concentration of SAH, which is synthesized from methionine via SAM, is low. Subsequently, free McbR proteins repress genes by interacting with the regulatory region of the target genes, including *ssuR* and *cysR*. Thus, sulfur metabolism including methionine biosynthesis is repressed. However, if the SAH:SAM ratio is high, McbR is inactivated, *cysR* is released from repression, and its gene product CysR activates genes associated with sulfur assimilation to supply the sulfur moiety to metabolic pathways. Activation by CysR requires metabolic effectors such as *O*-acetyl-L-serine (OAS) and *O*-acetyl-L-homoserine (OAH) (Rückert *et al.*, 2008). SsuR, which is activated by CysR, is involved in the activation of genes responsible for organic sulfur utilization (Koch *et al.*, 2005a). This hierarchical regulatory model shows similarity to that of *E. coli* involving CysB and Cbl (Iwanicka-Nowicka & Hryniewicz, 1995; van der Ploeg *et al.*, 1997).

In this study, we assessed the function of the *cysR* homologue in *Corynebacterium ammoniagenes*, a Gram-positive nucleotide-producing bacterium (Liebl, 2006). Based on the phenotypic, biochemical and molecular biological data, we named the *cysR* homologue of *C. ammoniagenes* *cmaR* (cysteine and methionine regulator in *C. ammoniagenes*). We have shown that the *cmaR* gene encodes a positive regulatory protein for genes involved in sulfur assimilation and methionine biosynthesis in *C. ammoniagenes*. Moreover, we demonstrate that the *cmaR* gene performs an essential regulatory function in the conversion of methionine to cysteine, and vice versa. We also demonstrate that the mechanism by which the CmaR protein exerts its regulatory effect is via the control of the expression of the *cmaR* gene, rather than by modulating the activity of CmaR through effectors, as is the case for CysR. The clarification of the mechanism underlying the regulation of sulfur metabolism in this strain is expected to prove beneficial to the development of *C. ammoniagenes* strains that generate large quantities of sulfur-containing amino acids.

METHODS

Bacterial strains, culture conditions, and auxotrophic test. *E. coli* DH10B (Grant *et al.*, 1990) was grown in Luria broth (LB) at 37 °C. *C. ammoniagenes* ATCC 6872 was used as the wild-type and grown at 30 °C in MB (Follettie *et al.*, 1993) or FMCA minimal medium containing the following (g l⁻¹): glucose, 20; urea, 3; NH₄Cl, 2; KH₂PO₄, 1; K₂HPO₄, 3; asparagine, 5; L-cysteine, 0.04; MnSO₄·H₂O, 0.001; ZnSO₄·7H₂O, 0.001; CuSO₄·2H₂O, 2 × 10⁻⁴; calcium pantothenate, 0.02; CaCl₂, 0.01; MgSO₄, 0.3; FeSO₄·7H₂O, 0.01; biotin, 6 × 10⁻⁵; thiamine-HCl, 0.01. For the preparation of sulfur-free medium (FMCAS), cysteine was omitted from FMCA, and MgSO₄, MnSO₄, ZnSO₄, CuSO₄ and FeSO₄ were substituted by equimolar quantities of MgCl₂, MnCl₂, ZnCl₂, CuCl₂ and FeCl₂, respectively. Antibiotics were added at the following concentrations (µg ml⁻¹): ampicillin, 50; chloramphenicol, 30 for *E. coli* and 10 for *C. ammoniagenes*; kanamycin, 50 for *E. coli* and 30 for *C. ammoniagenes*. Sulfur sources were added at the appropriate concentrations, as shown in the figures and tables. For the amino acid auxotrophy test, wild-type and HL1168 were streaked onto the

FMCA solid medium containing a combination of amino acids (50 µg ml⁻¹) as described elsewhere (Adams & Kaiser, 1998).

Construction of plasmids and strains. The *C. ammoniagenes* Δ *cmaR* mutant strain was constructed according to the method described by Schäfer *et al.* (1994). The deletion construct for the *cmaR* gene was generated via crossover PCR (Link *et al.*, 1997). The primary PCR products amplified with *cmaR*_delF1/*cmaR*_delR1 and *cmaR*_delF2/*cmaR*_delR2 (for primer sequences, see Supplementary Table S1) were employed as templates for secondary PCR. Following *Hind*III treatment, the PCR fragment was introduced into *Hind*III-digested pK19*mobsacB* (Schäfer *et al.*, 1994). The resultant plasmid, pSL415, was transformed into *E. coli* ET12567 (MacNeil *et al.*, 1992) and the plasmids from the strain were electro-transformed into *C. ammoniagenes*. Subsequent steps were conducted as described previously (Hwang *et al.*, 2002; Schäfer *et al.*, 1994). After validating chromosomal deletion via PCR, the mutant strain was designated HL1168. Fundamentally identical procedures were employed to construct the *mcbR*-deficient strain, with the exception of the primers, which were *mcbR*_delF1/*mcbR*_delR1 and *mcbR*_delF2/*mcbR*_delR2. The Δ *cmaR* Δ *mcbR* double mutant strain was constructed by deleting the *cmaR* gene in the Δ *mcbR* strain. In order to construct the *cmaR*-restored strain, the *cmaR* gene amplified with *cmaR*_compF1 and *cmaR*_compR1 was digested with *Kpn*I and ligated to *Kpn*I-digested pMT1 (Follettie *et al.*, 1993). The resultant plasmid, pSL433, was then introduced into HL1168 in order to generate HL1178. To construct a transcriptional fusion of *cmaR* with *cat*, the promoter region of the *cmaR* gene was amplified with the primers *cmaRCAT*_F1 and *cmaRCAT*_R1, using Pyrobest *Pfu* polymerase (Takara). After digestion with *Bam*HI, the fragment was ligated into *Bam*HI-treated pSK1CAT (Park *et al.*, 2004) for the construction of pSL457. The plasmid was then electro-transformed into the *C. ammoniagenes* wild-type strain in order to generate HL1257. Plasmid pSL466 was constructed via the amplification of the *cmaR* coding region with the *cmaR*_pET_F1 and *cmaR*_pET_R2N primers and subsequently ligating the *Xho*I/*Nde*I-digested PCR product with *Xho*I/*Nde*I-treated pET22b (Merck Biosciences).

RNA work. Total RNA was isolated from cells at the exponential phase with OD₆₀₀ ~3–4, and first-strand cDNA synthesis was conducted as described previously (Park *et al.*, 2007). In order to determine the transcriptional start site, 5' RACE (rapid amplification of cDNA ends) was conducted with a 5'/3' RACE kit, 2nd generation (Roche Diagnostics), in accordance with the manufacturer's instructions. Real-time RT-PCR was conducted as described by Choi *et al.* (2009). The standard deviation of the expression of each gene was calculated with Sequence Detection Software version 1.3.1 (Applied Biosystems). A CFX96 Real-Time PCR Detection system (Bio-Rad) was used to compare transcription levels observed in different bacterial strains (Table 2). Relative expression and the standard errors were calculated with CFX Manager software, which employs the $\Delta\Delta C_t$ method. Normalization was performed with 16S rRNA.

Enzymic assays. Corynebacterial cells were cultivated to late exponential phase and the crude extracts were prepared as previously described (Kim *et al.*, 2004). The chloramphenicol acetyltransferase (CAT) assay was conducted in accordance with the method described by Kim *et al.* (2005). The enzymic activities of *O*-acetylhomoserine sulphydrylation, cystathionine γ -synthase, and cystathionine β -lyase were determined as previously reported (Yeom *et al.*, 2004).

Purification of His₆-CmaR and gel mobility shift assays. *E. coli* BL21 (DE3) (Merck Biosciences) harbouring pSL466 was cultivated in LB. Protein expression was induced by treating cells with 0.5 mM IPTG at OD₆₀₀ 0.4. The cells were cultivated for an additional 3 h to fully induce protein expression. Purification of the fusion protein was conducted using HisTrap FF (GE Healthcare). The purified fusion

protein was then dialysed and concentrated with Amicon Ultra (Millipore). Gel mobility shift assays were conducted as reported elsewhere (Suda *et al.*, 2008). For the binding, 100 nM DNA and 5 μ M purified protein were used. Each DNA fragment, as shown in Fig. 4, was amplified with the primers listed in Supplementary Table S1.

RESULTS AND DISCUSSION

The *cmaR* gene and characteristics of the *cmaR*-deficient strain

We have long been interested in biosynthetic pathways involving sulfur in *C. ammoniagenes*. In recent years, the transcriptional regulation of biosynthetic metabolism has become a prominent topic with regard to the genus *Corynebacterium*. Among the candidate regulators of sulfur metabolism, we have been most interested in *cysR* (NCgl0120) of *C. glutamicum*. It was recently studied by Rückert and co-workers, who revealed the role of the gene in encoding the dual function regulator of sulfur metabolism (Rückert *et al.*, 2008). Subsequently, we attempted to identify the relevant genes in *C. ammoniagenes*, which is widely employed as an industrial nucleotide producer. An ORF encoding a CysR homologue was detected in the *C. ammoniagenes* genome database (unpublished data). However, despite the close phylogenetic relationship of *C. ammoniagenes* with *C. glutamicum*, the putative protein from *C. ammoniagenes*, which is composed of 400 amino acids with a deduced molecular mass of 42.7 kDa, evidenced only a 52.5 % identity with the CysR of *C. glutamicum*. As the first step in studying the function of the *C. ammoniagenes* ORF, we determined the transcriptional start point of the *cysR* homologue using 5' RACE. Adenine was identified as the transcriptional start point of the gene, and it clearly overlapped with the adenine of the presumed translational start (Fig. 5c). This result demonstrates that the *cysR* homologue is transcribed without the 5' leader sequence. Moll *et al.* (2002) have reported that leaderless transcripts that do not harbour the Shine–Dalgarno sequence on their mRNA tend to occur more frequently in Gram-positive eubacteria than in Gram-negative bacteria. In addition, Patek *et al.* (2003) have compiled a list of *C. glutamicum* genes that harbour leaderless mRNA. Furthermore, it has recently been reported that *cysR* of *C. glutamicum* is also transcribed as a leaderless transcript (Rückert *et al.*, 2008). Upstream of the transcriptional start site, we were able to locate a putative McbR-binding sequence within the promoter region of the gene (Fig. 5c), thereby suggesting the involvement of an *mcbR* homologue in the regulation of the expression of the *C. ammoniagenes* ORF (see below). In *C. glutamicum*, the *cysR* gene (NCgl0120) is thought to be under negative regulation by McbR, as evidenced by the increased transcription of the gene in the *mcbR* mutant strain (Rey *et al.*, 2005).

In an effort to assess the function of the *cysR* homologue, the pertinent gene in *C. ammoniagenes* was deleted via a

gene-disruption technique, as described in Methods. During a series of phenotypic tests, we determined that HL1168, the *cysR* homologue-deleted mutant, evidenced an interesting feature, namely that it was unable to grow on minimal medium. By complementing the HL1168 strain with the vector pSL433, we could corroborate that this growth defect was attributable to a deficiency of the *cysR* homologue. Subsequently, via an amino acid auxotrophy test, we identified HL1168 as a methionine and cysteine double auxotroph (Fig. 2a). In order to determine whether the cysteine auxotrophy of HL1168 was caused by the inability to reduce inorganic sulfur sources, we assessed growth with intermediates of the sulfur assimilation pathway, using (NH₄)₂SO₄, Na₂SO₃, Na₂S₂O₃ and Na₂S as the sole sulfur sources. Among these, only Na₂S was demonstrated to support effectively the growth of the auxotroph (Fig. 2a); this implies that the *cysR* homologue is necessary for the assimilation of sulfur from sulfate to sulfide, and *O*-acetylserine thiolase, which is encoded by *cysK* (Fig. 1), did not appear to be affected by the mutation. Subsequently, we conducted another growth test to elucidate the methionine biosynthetic steps affected by the mutation. Intermediates, including *O*-acetylhomoserine, cystathionine and homocysteine, were shown to be ineffective in supporting the growth of HL1168 (data not shown). These data show that the *cysR* homologue is a requisite for methionine biosynthesis, as well as sulfate reduction. Thus, we named the *cysR* homologue *cmaR* (cysteine and methionine regulator in *C. ammoniagenes*). In addition, we determined that the wild-type *C. ammoniagenes* strain had a weak ability to use methionine as the sole sulfur source (Fig. 2a), as is the case with *C. glutamicum* (Rückert & Kalinowski, 2008). Revealing the cause of such poor growth on methionine will require the elucidation of catabolic pathways for methionine in *C. glutamicum* and *C. ammoniagenes*. Although no experimental evidence is available, Rückert *et al.* (2003), based on the genome data, suggested the presence of the reverse transsulfuration pathway in *C. glutamicum*.

Thus far, several positive and negative regulators of sulfur metabolism have been identified in several bacterial species (Burguière *et al.*, 2005; Fernandez *et al.*, 2002; Guillouard *et al.*, 2002; van der Ploeg *et al.*, 2001). Against our expectation, these proteins evidenced a low level of identity with CmaR (7 % with *E. coli* CysB, 5 % with *B. subtilis* YtlI, 6 % with *B. subtilis* CysL and 3 % with *L. lactis* CmbR). Similarly, regulators known to control sulfur metabolism in *C. glutamicum* evidenced no structural homology to known regulators from other genera (Koch *et al.*, 2005a; Rückert *et al.*, 2008; Rey *et al.*, 2003). This result may indicate that the regulatory mechanism of sulfur metabolism operates in a distinctive manner in the *Corynebacteria*, and even among *Corynebacterium* species more generally.

In an effort to evaluate the functions of *cmaR*, we conducted more precise growth tests over a range of methionine concentrations. As can be observed in Fig. 2(b),

Fig. 2. Growth of cells on various sulfur sources. (a) Wild-type (black bars) and HL1168 (grey bars) strains were grown in minimal medium with the indicated sulfur sources. HL1168 (b) or wild-type (c) was grown in minimal medium containing 500 μ M cysteine and varying amounts of methionine. Symbols: ●, no sulfur source; ○, 500 μ M methionine; ▼, 200 μ M methionine; △, 50 μ M methionine; ■, 20 μ M methionine; □, 0 μ M methionine.

HL1168, the Δ *cmaR* mutant strain, displayed only marginal growth in the absence of sulfur sources or in the presence of cysteine as the sole sulfur source. However, it did grow when methionine was added to the medium in the presence of a fixed amount of cysteine (0.5 mM). The final cell yield was proportional to the quantity of added methionine. However, in the wild-type strain (Fig. 2c), the added methionine affected the growth rate, but not the final yield. An increase in the methionine concentration from 50 to 500 μ M improved the doubling time from 2.5 to 0.7 h. In the absence of methionine, a slow growth rate with a doubling time of 5.4 h was noted. Collectively, these data demonstrate that *cmaR* is required for the conversion of cysteine to methionine in *C. ammoniagenes*, and shows a clear distinction between *cmaR* and *cysR* in functional aspects.

Activity of selected methionine biosynthetic enzymes

In order to evaluate the possible cause of the defective growth of HL1168 when cysteine was utilized as the sole

sulfur source, we monitored the activities of methionine biosynthetic enzymes, including *O*-acetylhomoserine sulfhydrylase (OAHSH, encoded by *metY*), cystathionine γ -synthase (CGS, encoded by *metB*) and cystathionine β -lyase (CBL, encoded by *aecD*). As is shown in Table 1, the activities of OAHSH and CBL were reduced by 20 % in the Δ *cmaR* strain. Surprisingly, CGS activity was reduced by more than 50 % in the Δ *cmaR* strain. Furthermore, when the added methionine concentration was lowered from 0.5 to 0.25 mM to fully induce methionine biosynthetic genes, the CGS activity achieved a level of only 23 % as compared with the activity observed with the wild-type strain. The reduction of methionine biosynthetic enzyme activity resulting from *cmaR* deficiency (Table 1) was beyond the level observed with repression (Yeom *et al.*, 2004). On the basis of these results, we concluded that the growth defect of the mutant strain was caused, in part, by defects in the methionine biosynthetic pathway. Additionally, as is the case in *C. glutamicum* (Lee & Hwang, 2003), we determined that *C. ammoniagenes* also utilizes *O*-acetylhomoserine as the substrate for OAHSH and CGS (data not shown).

Table 1. Activities of selected enzymes of the methionine biosynthetic pathway

Crude extracts were prepared as described in Methods. Average values from two independent experiments are shown. WT, wild-type.

Enzyme*	Activity (nmol min ⁻¹ mg ⁻¹)					
	Cysteine (0.5 mM)	Methionine (0.5 mM)	Cysteine (0.5 mM), methionine (0.5 mM)		Cysteine (0.5 mM), methionine (0.25 mM)	
	WT	WT	WT	$\Delta cmaR$	WT	$\Delta cmaR$
OAHSH (<i>metY</i>)	15 ± 2.4	12 ± 1.9	16 ± 0.8	12 ± 1.3	14 ± 2.1	12 ± 1.5
CGS (<i>metB</i>)	49 ± 1.9	35 ± 2.1	35 ± 3.0	19 ± 1.2	48 ± 0.7	11 ± 0.7
CBL (<i>aecD</i>)	79 ± 5.9	30 ± 2.3	36 ± 1.8	30 ± 0.4	73 ± 5.8	57 ± 4.6

*Abbreviations: OAHSH, O-acetylhomoserine sulphydrylase; CGS, cystathionine γ -synthase; CBL, cystathionine β -lyase.

Transcription of methionine biosynthetic and sulfur assimilatory genes

In order to determine whether low levels of enzyme activity of methionine biosynthetic genes in the mutant strain were caused by impaired transcription of the corresponding genes, we determined the quantity of mRNA expressed from the methionine biosynthetic genes via real-time RT-PCR. Genes in the methionine pathway, including *metYX*, *metFE*, *metB* and *aecD*, and in sulfur assimilatory routes, such as *cysIXYZ*, *cysHDN* and *cysK*, were selected for the experiment. First of all, the organization of *cys* genes in *C. ammoniagenes* was analysed. Interestingly, in contrast to the sulfur assimilatory genes in *C. glutamicum*, which consisted of one operon (Rückert *et al.*, 2005), the corresponding genes in *C. ammoniagenes* were divided into *cysHDN* and *cysIXYZ* operons (data not shown). Furthermore, unlike the situation in *C. glutamicum*, the *metF* and *metE* genes of *C. ammoniagenes* were organized into an operon. In addition to the genes mentioned above, we also determined that the *metYX* genes of *C. ammoniagenes* constitute an operon, in addition to *cysKE* (data not shown).

As is shown in Table 2, the transcription levels of *metYX*, *metB* and *cysIXYZ* in the *cmaR*-deficient strain were reduced by 80, 50 and 90 %, respectively. The low transcription levels of the genes in the $\Delta cmaR$ strain were also observed at lower concentrations of methionine (Table 2). The gradual induction of the transcriptional level in *metFE* and *cysHDN* with decreasing methionine concentrations may be attributable to the release of repression by methionine in the wild-type strain. Although 80 % of MetY activity was noted in the mutant strain (Table 1), the quantity of *metY* mRNA observed in the mutant strain was at a level of only 15–20 %, as compared with that of the wild-type strain (Table 2). Although the cause of such a discrepancy remains to be clearly determined, we assume that it could have been caused by differences in experimental conditions, including the growth state of the cells at the time of cell harvest, as has been observed in *C. glutamicum* (Glanemann *et al.*, 2003). In contrast, the

quantity of *aecD* and *cysK* transcripts detected in the *cmaR*-deficient strain was comparable to that of the wild-type strain. The constitutive expression of *cysK* was consistent with the growth results on sulfide and methionine in HL1168 (Fig. 2a). It is worthwhile to note that the *cysK* gene of *C. glutamicum* is activated by CysR and repressed by McbR (Rückert *et al.*, 2008; Rey *et al.*, 2003). Repression of *cysK* as well as other genes by McbR was also observed in *C. ammoniagenes* (Table 2). In *C. glutamicum*, the role of the *aecD* gene is not restricted solely to methionine biosynthesis. For example, the protein product of the *aecD* gene functions as a cysteine desulphydrase, performing a role in the degradation of cysteine to pyruvate, ammonia and sulfide (Wada *et al.*, 2002). In addition, it may function as a general β -C-S lyase via α -ammonia and β -C-S elimination activity (Rossol & Pühler, 1992). This may be one of the reasons that *aecD* was not regulated by *cmaR*. Collectively, these data show that not only the methionine biosynthetic route but also the sulfur assimilatory pathway are under the transcriptional control of *cmaR*.

Control of *cmaR* expression

We have demonstrated above that *cmaR* can positively control methionine biosynthesis and sulfur assimilation. In order to determine whether the level of *cmaR* expression is influenced by available sulfur sources, we conducted experiments employing real-time RT-PCR (Fig. 3). When methionine and cysteine were added together to the growth medium, the transcription of the *cmaR* gene was repressed almost completely. However, when methionine or cysteine was employed as the sole sulfur source, the transcription of the *cmaR* gene was drastically increased. Maximal expression levels were noted when methionine was utilized as the sole sulfur source. By reducing the concentration of methionine from 0.5 to 0.05 mM, we were able to further increase the transcription of the *cmaR* gene. These data demonstrate that the *cmaR* gene is induced to execute a regulatory role in the conversion of methionine to cysteine, or vice versa. Furthermore, these data demonstrate that the

Table 2. Determination of relative gene expression level by real-time PCR

The expression level obtained in the wild-type under culture conditions of 0.5 mM cysteine and 0.5 mM methionine was arbitrarily set to 1. Mean values from two independent experiments are shown. Standard errors ($\pm$) are shown in parentheses. WT, wild-type.

Gene	Cysteine (0.5 mM)											
	Methionine (0.5 mM)				Methionine (0.25 mM)				Methionine (0.1 mM)			
	WT	$\Delta cmaR$	$\Delta mcbR$	$\Delta cmaR$ $\Delta mcbR$	WT	$\Delta cmaR$	$\Delta mcbR$	$\Delta cmaR$ $\Delta mcbR$	WT	$\Delta cmaR$	$\Delta mcbR$	$\Delta cmaR$ $\Delta mcbR$
<i>metYX</i>	1 (0.23)	0.19 (0.04)	101 (15.9)	0.62 (0.18)	1.48 (0.31)	0.22 (0.03)	84.6 (4.90)	0.63 (0.07)	1.11 (0.18)	0.22 (0.04)	91.1 (16.1)	0.77 (0.14)
<i>metB</i>	1 (0.20)	0.33 (0.04)	22.3 (3.44)	5.37 (1.56)	0.90 (0.18)	0.45 (0.04)	18.3 (2.35)	6.56 (1.27)	1.00 (0.16)	0.55 (0.10)	10.5 (1.86)	7.90 (0.64)
<i>aecD</i>	1 (0.09)	1.48 (0.26)	3.72 (0.61)	1.46 (0.23)	1.22 (0.07)	1.79 (0.11)	5.93 (0.35)	1.30 (0.16)	1.53 (0.14)	1.54 (0.04)	3.09 (0.58)	1.55 (0.18)
<i>metFE</i>	1 (0.06)	0.89 (0.07)	352 (39.7)	5.33 (1.57)	1.68 (0.22)	1.12 (0.07)	373 (18.4)	5.54 (0.64)	3.31 (0.28)	0.99 (0.12)	300 (35.1)	5.70 (0.56)
<i>cysK</i>	1 (0.18)	0.95 (0.11)	11.1 (1.57)	5.45 (1.62)	1.00 (0.20)	0.90 (0.06)	12.0 (1.85)	5.58 (0.93)	0.72 (0.09)	0.71 (0.13)	6.29 (1.33)	5.42 (0.80)
<i>cysIXYZ</i>	1 (0.20)	0.11 (0.02)	46.5 (7.72)	0.73 (0.22)	1.46 (0.42)	0.10 (0.02)	45.9 (3.60)	0.77 (0.09)	1.49 (0.18)	0.11 (0.02)	33.8 (8.88)	0.82 (0.07)
<i>cysHDN</i>	1 (0.20)	0.66 (0.07)	24.0 (3.86)	1.19 (0.37)	1.13 (0.20)	0.64 (0.11)	38.8 (2.06)	1.15 (0.13)	1.23 (0.16)	0.56 (0.13)	23.0 (4.10)	1.02 (0.21)

low-level expression of the methionine and sulfur assimilatory genes in the $\Delta cmaR$ strain is attributable to the absence of the *cmaR* gene, which probably encodes an activator. The mechanism by which methionine and/or cysteine regulates *cmaR* gene transcription remains to be clearly elucidated.

Thus far, we have evaluated the relative level of *cmaR* transcription via real-time RT-PCR. In order to determine the absolute level of *cmaR* expression, we constructed a

vector ($P_{cmaR}::CAT$) harbouring a *cat* gene fused to the downstream portion of the *cmaR* promoter. As is shown in Table 3, the promoter of *cmaR* was shown to be relatively weak. Only one-tenth of the activity was observed with the P_{cmaR} promoter as compared with the activity observed with the strong promoter P_{180} . It has been well-established that genes encoding transcriptional regulatory proteins are generally expressed at low levels. However, in contrast to the data obtained with real-time RT-PCR, maximal *cmaR* gene expression was noted when cysteine was employed as the sole sulfur source. This may be attributable to the intrinsic limitations of the assay employing *cat* genes as reporter genes (Shaw, 1975). Further research will be required to determine why such discrepancies arise.

DNA-binding activity of CmaR

After the identification of the involvement of *cmaR* in controlling the expression of genes involved in sulfur metabolism, we determined the ability of the purified CmaR to bind to the promoter region of its target genes. First of all, the CmaR protein was purified as described in Methods. Unlike CysR of *C. glutamicum* (Rückert *et al.*, 2008), CmaR could be purified without the incorporation of detergent into the column elution buffer, thereby suggesting the existence of intrinsic differences between the two proteins. Subsequently, we conducted gel mobility shift assays with a list of DNA fragments. As is shown in Fig. 4, retarded bands were noted for DNA fragments harbouring the promoter regions of *cysIXY*, *cysHDN*, *metFE*, *metYX* and *metB*. No band shift was observed with the promoter and the ORF region of the *gapB* gene, which

Fig. 3. Expression level of *cmaR* as determined by real-time RT-PCR conducted as described in Methods. The level of transcription achieved under the conditions of methionine and cysteine addition was arbitrarily set to 1 and utilized as a calibrator of relative quantification. Error bars, SD.

Fig. 4. DNA binding of the purified CmaR protein to the promoter regions of genes involved in sulfur metabolism. Gel mobility shift assays were conducted as described in Methods. Depending on the gene, one of the two different fragments of approximately 100 or 200 bp in length measured from the translational start site was employed in each binding reaction. Bands were separated by 6% Tris-borate EDTA (TBE)-PAGE and visualized after staining with ethidium bromide. The translational start site was arbitrarily set to +1. Panels: (a), *cysIXY*; (b), *metYX*; (c), *metB*; (d), *cysHDN*; (e), *metFE*; (f), *cysKE*; (g), *aecD*; (h), *cmaR*; (i), *gapB*.

Fig. 5. Effect of *mcbR* deletion on the expression of *metB* (a) and *cmaR* (b), and the location of CmaR-binding sequences on the *cmaR*-regulated genes (c). Sulfur sources were added to a final concentration of 1 mM. The level of transcription in the wild-type (white bars) or $\Delta mcbR$ strain (grey bars) obtained when 1 mM sulfate (SO_4) was added to the growth medium was arbitrarily set to 1 and utilized as a calibrator of relative quantification. Error bars, SD. Transcriptional start sites were determined via 5' RACE (c). Solid and dashed lines indicate McbR-binding sites and putative CmaR-binding sequences, respectively. See text for details. Cys, cysteine; Met, methionine; CM, cysteine + methionine.

Table 3. Expression of *cmaR*

C. ammoniagenes cells were cultivated in FMCAS medium. Preparation of cell extracts and determination of CAT activities were performed as described in Methods.

Plasmid*	Properties	Sulfur source (1 mM each)	CAT activity† ($\mu\text{mol mg}^{-1} \text{min}^{-1}$)
pSK1CAT	Empty vector	Cys + Met	0.03 ± 0.01
pSK1CAT::P ₁₈₀	Overexpression of CAT	Cys + Met	24.9 ± 4.20
pSL457	P _{cmaR} ::CAT	Cysteine	2.8 ± 0.20
		Methionine	1.1 ± 0.05
		Cys + Met	0.6 ± 0.02

*Plasmids pSK1CAT and pSK1CAT::P₁₈₀ have been described previously by Park *et al.* (2004).

†Mean values from three independent experiments are shown.

was utilized as a negative control (Fig. 4i). In accordance with the transcriptional and enzymic data (Tables 1 and 2), no shifted bands were observed with *aecD*. These data demonstrate that the CmaR protein functions as a transcriptional activator which controls the expression of genes involved in sulfur metabolism, as well as methionine biosynthesis. In order to determine the CmaR-binding site on each DNA, we conducted an experiment with two different fragments of approximately 100 and 200 bp in length, as measured from the translational start site (Fig. 4). For the *metYX*, *metB* and *cysHDN* genes, a band shift was noted with smaller DNA fragments. For *metFE*, band shifts were only noted with longer fragments. For *cysIXY*, although a major shift was observed with longer fragments, a weak shift was also noted with smaller fragments, thereby suggesting the presence of a CmaR-binding site near the upstream boundary of the smaller fragments. Contrary to the enzymic and transcriptional data, we observed the binding of the CmaR protein to the promoter region of *cysKE* (Fig. 4f). Judging from the weakness of the band shift, we speculated that the shift was induced by the non-specific interaction of CmaR with the DNA fragments. Rückert *et al.* (2008) also noted a non-specific interaction of the CysR protein with its target DNA. Further research will be required to determine the reasons for such a discrepancy. In addition, as is shown in Fig. 4(h), the CmaR protein did not bind to the promoter region of *cmaR*. This implies that the *cmaR* gene does not control its own expression, excluding the possibility of autoregulation.

Interestingly, CmaR did not require any modulators for its binding to target DNAs (Fig. 4). In *E. coli*, the activity of the CysB protein is modulated by effectors, including *N*-acetylserine (NAS) and OAS (Ostrowski & Kredich, 1989), although its binding to DNA can be shown in the absence of such effectors *in vitro*. In addition, sulfide and thiosulfate function as negative effectors to inhibit the binding of CysB to DNA (Ostrowski & Kredich, 1990). Furthermore, the CysR of *C. glutamicum* requires OAH or OAS as a positive effector (Rückert *et al.*, 2008). DNA binding of the CysR protein was possible only in the presence of such effectors.

However, unlike CysR, CmaR required no effectors to bind to its target DNAs (Fig. 4). To monitor carefully the effects of possible effectors on the binding of CmaR to DNA, the DNA-binding assay was conducted with minimal protein concentrations, which resulted in a partial DNA shift of approximately 50 %. The incorporation of possible effectors, such as methionine, cysteine, OAS, OAH, sulfide, thiosulfate and SAM, in the assay mixture did not influence the pattern of band shifts (data not shown), thereby suggesting that the functional mechanism exerted by the CmaR protein of *C. ammoniagenes* differs from that of the CysR of *C. glutamicum*. This is in accordance with the data shown above which indicate that the expression of the *cmaR* gene is controlled at the level of transcription (Fig. 3), excluding the involvement of effectors. The data indicate that the mechanism by which the CmaR protein exerts its regulatory effect is via the control of the expression of the *cmaR* gene, rather than by modulating the activity of CmaR through effectors, as is the case for CysR. In *Burkholderia cenocepacia* and *P. putida*, the binding of the activator to the *cys* genes does not require effector molecules (Iwanicka-Nowicka *et al.*, 2007; Kouzuma *et al.*, 2008).

Knowing that CmaR binds to its target DNAs, we identified possible CmaR-binding sites on the regulatory regions of several genes with mapped transcriptional start sites. To accomplish this, we utilized the CysR-binding sequences of *C. glutamicum* (Rückert *et al.*, 2008). As is shown in Fig. 5(c), putative CysR-binding sequences could be located in the upstream region of the -35 promoter sequences of the *metB*, *metFE* and *cysIXY* genes. We speculate that the sequences function as CmaR-binding sites in *C. ammoniagenes*. In addition, McbR-binding sequences could be located between the -10 and -35 promoter sequences of the genes. These results suggest that the *cmaR* gene operates as a positive regulator for the subset of genes under the control of McbR.

Effect of *mcbR* and *cmaR*/*mcbR* deficiency

The TetR-type regulator McbR has been demonstrated to repress many of the genes involved in methionine and

cysteine metabolism in *C. glutamicum*. Under methionine-abundance conditions, the intracellular level of SAM is elevated. Subsequently, SAM binds to McbR, and the complex represses a range of methionine and cysteine biosynthetic genes (Suda *et al.*, 2008). Besides SAM, SAH also functions as a negative effector of McbR (Rey *et al.*, 2005). Recently, the regulatory model of sulfur metabolism in *C. glutamicum* has been proposed (Rückert *et al.*, 2008). According to this model, McbR functions as a master regulator of sulfur metabolism via the repression of genes that include *cysR* and *ssuR*. When released from the repression, CysR activates genes such as *cysIXHDNYZ*, *fpr2*, *cg3372–75*, *cg1514*, *cg4005* and *ssuR*. No *ssuR* homologue is present in the *C. ammoniagenes* genome (data not shown). Subsequently, we assessed the expression of genes involved in sulfur metabolism in the *mcbR*-deficient mutant strain ($\Delta mcbR$). As is shown in Fig. 5(b), the transcription of *cmaR* in the $\Delta mcbR$ strain was increased dramatically, as compared with that of the wild-type strain. This shows that McbR represses *cmaR* expression in *C. ammoniagenes*. In addition, as shown in Fig. 5(a), *metB* expression was also increased in the $\Delta mcbR$ strain, thereby indicating the repressive function of McbR in methionine biosynthesis in the organism. This phenomenon was also observed with *metYX*, *metFE*, *cysIXY*, *cysHDN* and *cysK* (Table 2). Derepressed transcription of the genes in the $\Delta mcbR$ strain was markedly diminished in the $\Delta cmaR\Delta mcbR$ double mutant strain, suggesting a critical role for the *cmaR* gene in the transcription of the genes (Table 2). The degree of influence on the transcription of the *aecD* and *cysK* genes of the mutations was relatively small. In addition to its role as an activator, CysR is also known to repress genes such as *cg2810* and *cg3138–39* in *C. glutamicum* (Rückert *et al.*, 2008). In order to determine whether CmaR functions in such a manner, we monitored the expression of the *C. ammoniagenes* gene which corresponds to *cg2810*. As compared with the wild-type strain, the expression level of the gene in the $\Delta cmaR$ mutant strain was unchanged (data not shown), thereby suggesting that there are differences in the mechanisms by which the *cmaR* and *cysR* genes exert their regulatory effects.

In conclusion, we present evidence in this study that CmaR positively controls not only the methionine biosynthetic and sulfur assimilatory pathways, but also conversion between cysteine and methionine. In addition, we have demonstrated that the presence of methionine or cysteine stimulates *cmaR* expression. Furthermore, the *cmaR* gene is negatively controlled by McbR, as is the case in *C. glutamicum*. In accordance with the physiological, genetic and biochemical data, we observed the direct binding of the purified CmaR protein to the promoter region of its target genes in the absence of any effector molecules. These results demonstrated that the *cmaR* gene of *C. ammoniagenes* plays a role similar to but distinct from that of the functional homologue *cysR* of *C. glutamicum*. Although not all genes and enzymes in sulfur metabolism have been identified in *C. ammoniagenes*, *cmaR* may prove an

attractive target for the development of methionine- or cysteine-producing strains.

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