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Molecular characterization of the CmbR activator-binding site in the *metC–cysK* promoter region in *Lactococcus lactis*

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The *metC–cysK* operon involved in sulphur metabolism in *Lactococcus lactis* is positively regulated by the LysR-type protein CmbR. Transcription from the *metC* promoter is activated when concentrations of methionine and cysteine in the growth medium are low. The *metC* promoter region contains two direct and three inverted repeats. Deletion analysis indicated that direct repeat 2 (DR2) is required for activation of the *metC* promoter by CmbR. Gel mobility shift assays confirmed that CmbR binds to a 407 bp DNA fragment containing the *metC* promoter. This binding was stimulated by *O*-acetyl-L-serine. Competition experiments with deletion variants of the *metC* promoter showed that CmbR binding only occurred with fragments containing an intact DR2, confirming that DR2 is the CmbR binding site within the *metC* promoter.

INTRODUCTION

Cysteine biosynthesis in bacteria represents the way in which inorganic sulphur is reduced and incorporated into organic compounds. The assimilatory reduction of sulphate and cysteine formation has been extensively studied in the Gram-negative bacteria *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. In contrast, only little information is available for Gram-positive bacteria. For *Lactococcus lactis*, the biosynthesis of methionine and cysteine has been poorly characterized. This micro-organism has been described as prototrophic for cysteine and auxotrophic for methionine (Chopin, 1993). Homologues for all the genes involved in methionine biosynthesis in *E. coli* (*metA*, *metB*, *metC*, *metF* and *glyA*) are present together with *cysE* and two *cysK* homologues from the cysteine biosynthesis pathway (Fernández *et al.*, 2002). In contrast, no homologues of the *E. coli* genes responsible for sulphate uptake and reduction seem to be present, although there is a putative sulphate transporter (*yafB*) of the SulP family (Bolotin *et al.*, 2001).

Genes of the cysteine regulon in *E. coli* and *S. enterica* serovar Typhimurium require a positive regulator protein CysB, a member of the LysR family of bacterial transcriptional regulators, as well as sulphur limitation and the presence of the inducer *N*-acetyl-L-serine, often provided as *O*-acetyl-L-serine (OAS) (Kredich, 1996; Ostrowski & Kredich, 1989). OAS is a substrate for the cysteine synthase (CysK). The pathway involves transport and reduction of inorganic sulphate to sulphide in one branch and the synthesis of OAS from serine in another. The subsequent reaction of sulphide with OAS results in cysteine synthesis.

The genes involved in methionine biosynthesis in *E. coli* and *S. enterica* serovar Typhimurium are regulated by two regulator proteins, MetJ and MetR. The *met* regulon genes, except *metH*, are under negative transcriptional control of the MetJ repressor, with *S*-adenosylmethionine as a co-repressor (Saint-Girons *et al.*, 1988). In addition to MetJ-mediated negative control the *metE*, *metA*, *metF*, *metH* and *glyA* genes are under positive control of the MetR activator (Cowan *et al.*, 1993; Lorenz & Stauffer, 1996; Mares *et al.*, 1992; Maxon *et al.*, 1989; Urbanowski & Stauffer, 1989; Weissbach & Brot, 1991).

Information on methionine and cysteine biosynthesis, and regulation of sulphur metabolism, in Gram-positive bacteria is limited. A number of *Bacillus subtilis* genes involved in cysteine and methionine biosynthesis contain a highly conserved sequence upstream of the coding region called the S-box (Grundy & Henkin, 1998). This motif contains a putative transcriptional terminator, suggesting that regulation is controlled via a transcription-termination mechanism. The S-box has a key role in the transcription control of

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Abbreviations: CFE, cell-free extract; DR, direct repeat; LTTR, LysR-type transcriptional regulator; OAS, *O*-acetyl-L-serine.

the *metIC* operon (Auger *et al.*, 2002). In contrast, Mansilla *et al.* (2000) showed that *cysH* operon transcription is independent of the S-box motif present upstream of this operon. CysL, a LysR-type transcriptional regulator, is the only regulator of sulphur genes identified in *B. subtilis* and acts as an activator of the *cysJI* operon (Guillouard *et al.*, 2002).

Previously, the *metC* gene and the encoded cystathionine β-lyase, which has both β- and γ-lyase activity, were characterized in *L. lactis* (Alting *et al.*, 1995; Fernández *et al.*, 2000). Cystathionine β-lyase is a tetrameric protein and its physiological function is the catalysis of a reaction in the methionine synthesis pathway, namely an α,β-elimination reaction from cystathionine to produce homocysteine, pyruvate and ammonia. Subsequently, the homocysteine is methylated to form methionine (Gottschalk, 1988). The *metC* gene is present in an operon with the *cysK* gene, encoding a cysteine synthase, which catalyses the formation of cysteine from OAS and sulphide (Fernández *et al.*, 2000). Expression of the *metC–cysK* operon is repressed by cysteine and, to a lesser extent, by methionine. Two genes, *cmbR* and *cmbT*, have been identified that are involved in regulation of *metC–cysK* transcription (Fernández *et al.*, 2000).

CmbR is a LysR-type regulator protein essential for expression of the *metC–cysK* operon in *L. lactis* (Fernández *et al.*, 2002). LysR-type transcriptional regulators (LTTRs) constitute a large family of prokaryotic regulator proteins that includes the well-characterized CysB and MetR regulators (Henikoff *et al.*, 1988). The CysB regulator is a coinducer-responsive transcription regulator, which positively regulates the transcription of the *cys* regulon and negatively regulates its own transcription and transcription of the *hslJ* gene. CysB binds to cognate promoters via a 15 bp dyad repeat with a common structure and position independently of the presence of a coinducer. The coinducer causes additional interactions of the regulator with sequences near the −35 RNA polymerase binding site that result in bending and transcription activation (Schell, 1993; Jovanovic *et al.*, 2003).

By analogy with CysB, CmbR is expected to require a coinducer (*N*-acetylserine) to allow binding to the activation site. However, the activator-binding sequences described for the CysB-responsive promoters in *E. coli* and *S. enterica* serovar Typhimurium (Hryniewicz & Kredich, 1995; Wu *et al.*, 1995) were not found in the lactococcal *metC* promoter (Fernández *et al.*, 2002). For a number of LTTRs such as AmpR (Lindquist *et al.*, 1989), IlvY (Wek & Hatfield, 1988), NahR (Huang & Schell, 1991), OccR (Cho & Winans, 1993), TrpI (Chang & Crawford, 1991) and MetR (Byerly *et al.*, 1991; Urbanowski & Stauffer, 1989), dyad symmetry elements have been described in the binding sites, while both inverted and direct repeats have been found in NodD (Fisher & Long, 1993; Goethals *et al.*, 1992; Wang & Stacey, 1991) and CysB (Hryniewicz & Kredich, 1995) binding sites.

In this paper we analyse the *metC* promoter in *L. lactis*, which contains three inverted repeat sequences and two direct repeats located upstream of the −35 region. We demonstrate the role of the second direct repeat sequence (DR2) in the regulation by CmbR.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria–Bertani broth at 37 °C (Sambrook *et al.*, 1989). *L. lactis* strain NZ9000*cmbR* is a single-crossover *cmbR* disruption derivative of NZ9000 (Kuipers *et al.*, 1998), obtained by site-directed integration of plasmid pNZ9348 (Fernández *et al.*, 2002). *L. lactis* strains were grown in M17 broth (Difco) supplemented with 0·5 % glucose (GM17) or in modified chemically defined medium (CDM) (Looijesteijn & Hugenholtz, 1999; Fernández *et al.*, 2002) at 30 °C. The sulphate salts of the CDM were replaced by equimolar amounts of chloride or nitrate salts, and the medium contained 0·82 mM cysteine (1 × cysteine) and 0·84 mM methionine (1 × methionine). For activation of P_{metC}, cysteine was omitted and the methionine concentration was reduced to 0·1 × methionine (0·084 mM), while for repression both methionine and cysteine were increased to 10 × methionine and 10 × cysteine. When appropriate, the media contained ampicillin (100 μg ml^{-1}), chloramphenicol (5 μg ml^{-1}) or erythromycin (2·5 μg ml^{-1}). Agar plates were prepared by adding agar (1·5 %, w/v; Difco) to the medium.

DNA isolation and manipulation. Isolation and transformation of plasmid DNA from lactococci were performed as described previously (de Vos *et al.*, 1989). Isolation of plasmid DNA from *E. coli* and all other recombinant DNA techniques were performed as described by Sambrook *et al.* (1989). Large-scale isolation of *E. coli* plasmids for nucleotide sequence analysis was performed using the Jet Star system (Genomed), according to the manufacturer's instructions.

Plasmid constructions. To study the regulation of the *metC–cysK* operon, deletion derivatives were made of pNZ9340 (Fernández *et al.*, 2002) that contain the *gusA* gene under control of P_{metC}. A schematic representation of the deletion derivatives is shown in Fig. 1. Smaller promoter regions were amplified by PCR using the primers P_{metC}F1 (5′-GCGCCGGGATCCCGAAAGATTTAGAAAATATTAAA-3′), P_{metC}F2 (5′-GCGCCGGGATCCGATGTCCTCGTTTTTTTAT-3′), P_{metC}F3 (5′-GCGCCGGGATCCATAAAAAAAGTTAATTCTGCT-3′), P_{metC}F4 (5′-GCGCCGGGATCCGTTAATTCTGCTATAAAAAAATCT-3′) or P_{metC}F5 (5′-GCGCCGGGATCCCTTATAGCACTGGGCACAC-3′) in combination with P_{metC}R (5′-GGCCGCGAATTCCAGCAAGTCCTGAACTAAATG-3′). All PCR amplifications were performed by using the Advantage Genomic Polymerase Mix (Clontech) in 25 successive cycles according to the instructions of the manufacturer. Resulting DNA fragments were digested with *Bam*HI and *Eco*RI (sites introduced into the primers; underlined) and cloned into similarly digested pUC18 (Yanish-Perron *et al.*, 1985). The integrity of the inserts was confirmed by sequencing and, subsequently, the *Bam*HI–*Eco*RI fragments were subcloned upstream of the promoterless *gusA* gene in pNZ273 (Platteeuw *et al.*, 1994). The resulting P_{metC}–*gusA* fusions were subcloned into the low-copy-number plasmid pIL252 (Simon & Chopin, 1988) as *Eco*RI–*Hin*dIII DNA fragments, generating the plasmids pNZ7211, pNZ7212, pNZ7213, pNZ7214 and pNZ7215, respectively.

For the construction of CmbR containing a C-terminal extension of six His residues, the *cmbR* gene from *L. lactis* MG1363 was placed under control of the *nisA* promoter in plasmid pNZ8113. Plasmid pNZ8113 is a derivative of pNZ8048 (Kuipers *et al.*, 1998), containing

Table 1. Strains and plasmids used in this study

Strains or plasmids	Relevant characteristic(s)*	Reference
Escherichia coli		
DH5α	*supE44 ΔlacU169 (ϕ80 lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1983)
Lactococcus lactis		
NZ9000	Plasmid-free, Lac$^-$ Prt$^-$ *nisR nisK* derivative of *L. lactis* MG1363	Kuipers *et al.* (1998)
NZ9000*cmbR*	Ery^r derivative of NZ9000 with a *cmbR* disruption by single crossover	This study
Plasmids		
pUC18	Amp^r, 2·8 kb multiple cloning vector	Yanisch-Perron *et al.* (1985)
pNZ273	Cm^r, promoter screening vector with the promoterless *gusA* reporter gene	Platteeuw *et al.* (1994)
pIL252	Ery^r, 4·7 kb low-copy vector with theta replicon	Simon & Chopin (1988)
pNZ9340	Cm^r, 5·2 kb pNZ273 derivative carrying the *gusA* gene fused to $P_{metCcysK}$	Fernández *et al.* (2002)
pNZ9341	Ery^r, 7·1 kb pIL252 derivative carrying the *gusA* gene fused to $P_{metCcysK}$	Fernández *et al.* (2002)
pNZ9347	Amp^r, 9·5 kb pUC18 derivative carrying a 6·8 kb *SstI–SalI* fragment with *cmbR*	Fernández *et al.* (2002)
pNZ9348	Ery^r, 4·0 kb pUC18Ery derivative carrying $P_{metCcysK}$	Fernández *et al.* (2002)
pNZ8048	Cm^r, 3·3 kb, expression vector for translational fusions to P_{nisA}	Kuipers *et al.* (1998)
pNZ8113	Cm^r, pNZ8048 derivative containing P_{nisA} and Histag	This study
pNZ7200	Cm^r, 3·4 kb pNZ8113 derivative containing *cmbR* gene placed under the P_{nisA} and with Histag at 3′ terminus	This study
pNZ7201	Amp^r, 3·0 kb pUC18 derivative with F1 derivative of $P_{metCcysK}$	This study
pNZ7202	Amp^r, 3·0 kb pUC18 derivative with F2 derivative of $P_{metCcysK}$	This study
pNZ7203	Amp^r, 3·0 kb pUC18 derivative with F3 derivative of $P_{metCcysK}$	This study
pNZ7204	Amp^r, 3·0 kb pUC18 derivative with F4 derivative of $P_{metCcysK}$	This study
pNZ7205	Amp^r, 3·0 kb pUC18 derivative with F5 derivative of $P_{metCcysK}$	This study
pNZ7206	Cm^r, 5·2 kb pNZ273 derivative with F1 derivative of $P_{metCcysK}$	This study
pNZ7207	Cm^r, 5·2 kb pNZ273 derivative with F2 derivative of $P_{metCcysK}$	This study
pNZ7208	Cm^r, 5·2 kb pNZ273 derivative with F3 derivative of $P_{metCcysK}$	This study
pNZ7209	Cm^r, 5·2 kb pNZ273 derivative with F4 derivative of $P_{metCcysK}$	This study
pNZ7210	Cm^r, 5·2 kb pNZ273 derivative with F5 derivative of $P_{metCcysK}$	This study
pNZ7211	Ery^r, 7·1 kb pIL252 derivative with F1 derivative of $P_{metCcysK}$	This study
pNZ7212	Ery^r, 7·1 kb pIL252 derivative with F2 derivative of $P_{metCcysK}$	This study
pNZ7213	Ery^r, 7·1 kb pIL252 derivative with F3 derivative of $P_{metCcysK}$	This study
pNZ7214	Ery^r, 7·1 kb pIL252 derivative with F4 derivative of $P_{metCcysK}$	This study
pNZ7215	Ery^r, 7·1 kb pIL252 derivative with F5 derivative of $P_{metCcysK}$	This study

*Amp^r, ampicillin resistant; Ery^r, erythromycin resistant; Cm^r, chloramphenicol resistant.

six histidine codons next to the multiple cloning sites to enable a His-tag fusion. To create pNZ8113, pNZ8048 was digested with *Xba*I and *Hin*dIII and ligated with a synthetic linker encoding six His residues, a stop codon (bold), a *Nru*I restriction site (underlined) and extensions for ligation into plasmid digested with *Xba*I and *Hin*dIII restriction enzymes (underlined) using the oligonucleotides 5′-CTAGATCGCGACATCACCATCACCATCAC**TA**-3′ and 5′-AGCT-**TA**GTGATGGTGATGGTGATGTCGCGAT-3′.

The *cmbR* gene was amplified using primers 5′-GCCGCGTCA-TGAATATTAAACAATTACGGTACG-3′ and 5′-GCCGCGCTCGA-GTTATCGCGAGAATTCATAGTTTTCAAAATAAACTTCA-3′, and using plasmid pNZ9347 (Fernández *et al.*, 2002) as a template. The PCR amplifications were performed using *Pwo* DNA Polymerase (Roche) in 25 successive cycles following the instructions of the manufacturer. The resulting DNA fragment was digested with *Rca*I and *Nru*I (sites introduced in the primers; underlined) and cloned into similarly digested pNZ8113. The resulting plasmid, pNZ7200, was introduced into NZ9000*cmbR*, which was subsequently used for overexpression of CmbR-Histag protein under control of the NICE system (Kuipers *et al.*, 1998).

DNA sequencing. Automatic double-stranded DNA sequence analysis was performed on both strands with an ALF*red* DNA sequencer (Amersham Biosciences). Sequencing reactions were accomplished by using the AutoRead sequencing kit, using Cy5-labelled universal and reverse primers according to the instructions of the manufacturer (Amersham Biosciences).

Enzyme assays. For these assays the cells were grown in CDM with different methionine and cysteine concentrations to an OD_{600} of 0·7. For the preparation of extracts, cells were disrupted with zirconium beads in a Bio101 Fast Prep (two treatments of 30 s with intervals of 1 min on ice between treatments) and cellular debris was removed by centrifugation. The extracts were kept on ice, and enzyme assays were performed within 4 h. Activity of the β-glucuronidase (GusA) enzyme was measured as described previously (Kuipers *et al.*, 1995); 40 µl of extract was added to 950 µl buffer (50 mM sodium phosphate, pH 7·0, 10 mM β-mercaptoethanol, 1 mM EDTA, 0·1 % Triton X-100) and 10 µl 100 mM *p*-nitrophenyl-β-D-glucuronic acid (Clontech). The mixture was incubated, and the increase in A_{405} was measured at 37 °C in a Cary 1E UV–visible spectrophotometer (Varian) with a thermostatically controlled

compartment. Specific activity was calculated as increase in A_{405} min^{-1} (mg protein)$^{-1}$. Protein concentrations were determined with a protein assay based on the method of Bradford (1976), using bovine serum albumin as a standard.

Preparation of extracts enriched in CmbR protein. Cell-free extract (CFE) enriched in CmbR-Histag protein was isolated from NZ9000*cmbR* harbouring plasmid pNZ7200. As a negative control CFE was isolated from strain NZ9000*cmbR* without plasmid pNZ7200. Cells were grown to an OD_{600} of 0·5 and were subsequently induced with nisin (1 ng ml^{-1}). Following induction, growth was continued for 2 h, then cells were harvested and resuspended in 1 ml milliQ water. The cell-free extracts were prepared with 0·8 g zirconium beads (0·1 mm, Biospec Products) using a Bio101 FastPrep (two 30 s treatments, with cooling on ice between the runs). Samples were centrifuged for 1 min and the supernatants were used as CFEs. Samples were divided into small portions, frozen in liquid nitrogen, and stored at −70 °C until further use. Protein concentrations of the CFEs were determined as described above.

Gel mobility shift assay. A DNA fragment containing the F1*metC* promoter region was generated by PCR using primers P_{metC}F1 and P_{metC}R. The PCR product obtained was 5′-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Gibco-BRL), and purified by JETquick PCR Purification Spin Kit (Genomed). Binding reactions and gel mobility shift assay were performed as described previously (Lochowska *et al.*, 2001). Briefly, the reaction mixtures (40 µl) contained approximately 10 ng labelled DNA fragment and 1 µg poly(dIdC) (to reduce non-specific binding), in the CmbR buffer containing 40 mM Tris/HCl (pH 8·0), 10 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol and 0·2% Tween 20. Incubation (15 min at 37 °C) was initiated by adding a variable amount of CFE and OAS where indicated. OAS was always prepared fresh as a 10 mM stock solution. Samples were separated on 5% acrylamide/bisacrylamide (30:0·8) vertical gels in 0·5 M Tris/borate/EDTA buffer (pH 8·3) for 1·5 h at 10 V cm^{-1} and the radiolabelled bands were visualized by autoradiography.

Magnetic DNA affinity purification. A biotin-labelled F1 P_{metC} fragment (F1b) was obtained by PCR by using the primers P_{metC}F1 and 5′-biotin-labelled P_{metC}R. One hundred microlitres of Magnasphere beads (Streptavidine Magne Sphere Paramagnetic Particles; Promega) were washed three times with 100 µl 0·5× SSC (1× SSC: 150 mM NaCl, 15 mM trisodium citrate) and three times in 100 µl TE buffer pH 8·0. Beads were resuspended in 100 µl TE buffer pH 8·0 containing the F1b fragment (4 µg), and incubated for 30 min at 25 °C with rotation until biotin-labelled DNA was bound to the beads. The beads were then washed three times with CmbR buffer to eliminate unbound DNA. Approximately 0·03 nmol of the F1b fragment was bound to the Magnasphere beads. A 1 ml sample of CFE enriched in CmbR-Histag protein (500 µg protein) in CmbR buffer was added and incubation was continued for 5 min at 30 °C. Unbound proteins were removed in five wash steps with 1 ml CmbR buffer. Proteins bound to the beads were eluted with 1 ml 1 M KCl. The solutions from the wash steps and elution were precipitated with TCA, and the precipitates were dissolved in 20 µl milliQ water. Residual TCA was neutralized by adding 4 µl 1 M Tris/HCl pH 8·0. Protein samples were run on 12·5% SDS-PAGE and analysed by silver staining (Wray *et al.*, 1981) or Western blotting. Western blotting was performed essentially as described by Towbin *et al.* (1979), using 1:5000 diluted anti-tetra-His antibody (Tetra-His Antibody, BSA-free; Qiagen) as a primary and 1:5000 diluted GAMPO [Goat-Anti-Mouse IgG (H+L) (HAS) Peroxidase Conjugate, Gibco-BRL] as a secondary antibody. Peroxidase activity was detected using 4-chloro-1-naphthol, according to the instructions of the manufacturer (Gibco-BRL).

RESULTS AND DISCUSSION

CmbR is a transcriptional activator of the *metC–cysK* operon and a member of a large family of bacterial activator proteins, termed LysR-type transcriptional regulators (LTTRs), that includes CysB and MetR (Henikoff *et al.*, 1988; Schell, 1993). In this work we studied the interaction of CmbR regulator with its responsive promoter P_{metC}. Positive regulation of prokaryotic gene expression involves binding of an activator protein upstream of the −35 promoter region. Binding sites of LTTRs, including those of the CysB and MetR regulators, are regions of dyad symmetry or direct repeats (Schell, 1993).

Deletion analysis of the *metC–cysK* promoter region

The region upstream of the transcription start of *metC* contains two direct (DR1 and DR2) and three inverted (IR1, IR2 and IR3) repeats (Fig. 1). These repeats were anticipated to play a role in the regulation of transcription of the *metC–cysK* operon, which is mediated by the transcriptional activator CmbR (Fernández *et al.*, 2002). To determine which of the repeats is involved in the regulation of *metC–cysK* transcription, we constructed fusions of *metC* promoter fragments of various lengths to the *β*-glucuronidase-encoding *gusA* gene in a low-copy plasmid (pIL252). All constructs contain the −35 and −10 sequences, the transcription start site, ribosome-binding site and the 5′-terminus of the *metC* gene (Fig. 1). Plasmid

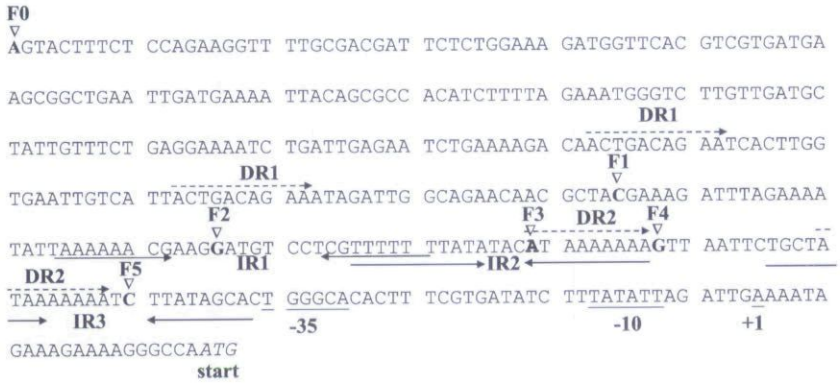

Fig. 1. Complete nucleotide sequence of P_{metC} (F0). F1, F2, F3, F4 and F5 represent the truncated fragments. The first nucleotide in each truncated fragment is boldface and indicated by a downward pointing arrowhead. Inverted repeats (IR1, IR2 and IR3) and direct repeats (DR1 and DR2) are represented by solid and dashed arrows, respectively. The −35 and −10 sequences, transcription start (+1) and start codon are indicated.

pNZ9340 contains the entire *metC* promoter (fragment F0, Fig. 1). Progressive 5′-truncations of the *metC* promoter fragment in pNZ9340 (Fig. 1) were constructed removing, successively, DR1 (fragment F1, pNZ7211); DR1 and the 5′-half of IR1 (fragment F2, pNZ7212); DR1, IR1 and the 5′-half of IR2 (fragment F3, pNZ7213); DR1, IR1, IR2 and the 5′-half of DR2 (fragment F4, pNZ7214); and DR1, IR1, IR2, DR2 and the 5′-half of IR3 (fragment F5, pNZ7215).

L. lactis NZ9000 derivatives harbouring one of the *metC* promoter constructs were grown in CDM without cysteine and with 0·1× methionine (0·1×M), or with 10× methionine and 10× cysteine (10×M/10×C). Quantitative GusA analysis showed that the promoter fragments F0, F1, F2 and F3 could drive the high-level expression of β-glucuronidase in growth media with low methionine and without cysteine. In contrast, β-glucuronidase activity levels were barely detectable in cells harbouring the F4 and F5 promoter fusion constructs under the same conditions (Fig. 2). However, the apparent discrepancy between the β-glucuronidase activities in cells harbouring F0, F1, F2 and F3 promoter fusion constructs could be explained by different lengths of the DNA fragments. It is well known that upstream sequences can mediate the promoter activity (Watson *et al.*, 1988). For example, the nucleotide sequence in the deleted region could represent a target site for topoisomerases that change local DNA supercoiling. Alternatively, it is possible that differences in the upstream region might affect the bending of DNA in the DR2, resulting in different affinity of CmbR for its target sequence, considering the fact that LTTRs do not recognize consensus sequences, but DNA topography. Moreover, the bending of DNA could cause the difference in RNA polymerase promoter-binding affinity. Since the addition of methionine and cysteine (10×M/10×C) resulted in an almost complete loss of β-glucuronidase activity, the low concentration of methionine and lack of cysteine in the growth medium were essential for activation of the *metC* promoter fragments F0, F1, F2 and F3.

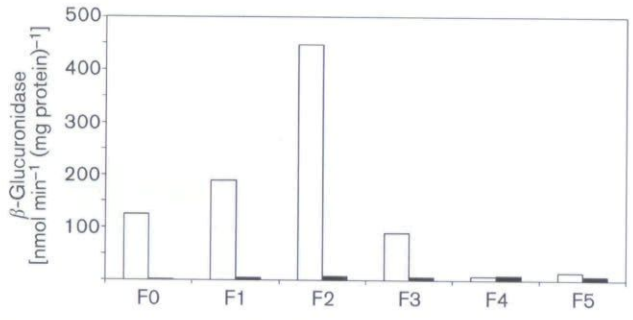

Fig. 2. β-Glucuronidase activities of promoter–*gusA* fusions with F0, F1, F2, F3, F4 and F5 promoter fragments of P_{metC}. White bars, CDM containing 0·1× Met; black bars, CDM containing 10× Met/10× Cys. Results are the means of three independent experiments.

The LysR type of transcriptional regulators may be the most common type of positive regulators in prokaryotes. DNA footprinting studies suggest that in the absence of coinducer many LTTRs bind to regulated promoters via a 15 bp dyadic sequence with a common structure and position (near −65) (Schell, 1993). The quantitative GusA analysis results demonstrate that the region upstream of the *metC* promoter from bp −80 to −67 of the transcription initiation site is required *in vivo* for expression of P_{metC}, which is in agreement with the data obtained for other target promoters for LysR-type regulators. The GusA assays with the progressive 5′ truncations of P_{metC} showed that deletion of this 13 bp region, containing half of the second direct repeat, results in a dramatic reduction of the P_{metC}-driven *gusA* expression, indicating that this region acts as a positive *cis* element.

Interaction of the CmbR protein with P_{metC}

Initially, interaction of CmbR with P_{metC} was evaluated by magnetic DNA affinity purification using a biotin-labelled 407 bp F1b PCR fragment, which contains the *metC* promoter region from positions −135 to +258 relative to the transcription start site. CFE of *L. lactis* NZ9000*cmbR* harbouring pNZ7200 in CmbR buffer was added to the beads. The magnasphere beads were washed with CmbR buffer to remove unbound proteins (lanes 1–5 in Fig. 3), after which bound proteins were eluted using a high-salt solution (EL in Fig. 3). Analysis of the protein fractions obtained by SDS-PAGE showed that the last wash step contained only minor amounts of protein, while the elution fraction contained a prominent protein band of approximately 36 kDa. This molecular mass is in good agreement with the predicted value for CmbR-Histag (35 691 Da) (Fig. 3a). Moreover, Western blot analysis using anti-tetra-His antibodies strongly suggested that the protein bound to the F1b fragment was CmbR-Histag (Fig. 3b).

The interaction of the CmbR protein with P_{metC} was analysed in more detail by gel mobility shift assay (Lochowska *et al.*, 2001) using a radiolabelled 407 bp F1 PCR fragment (Fig. 1). Preincubation of 10 ng labelled *metC* promoter DNA fragment with rising concentrations of the CmbR-Histag-enriched CFE (from 7·5 μg to 30 μg) resulted in a reduced mobility of the F1 fragment when preincubations were performed in the presence of 10 mM OAS. In the absence of OAS the same CFE concentrations resulted in almost complete loss of mobility shift efficiency. Similar concentrations of CFE obtained from a strain that lacks CmbR (NZ9000*cmbR*) did not result in the mobility shift of the F1 promoter fragment, indicating that the change in F1 mobility was caused by binding of CmbR. These results confirm that CmbR binds to the *metC* promoter region and indicate that OAS strongly stimulates this binding (Fig. 4). Moreover, binding of CmbR appeared to occur in discrete dose-dependent stages, which could imply a multimeric nature of the regulator. Interestingly, all LTTRs are active *in vivo* as multimers. For example TrpI, CysB and NahR are tetramers, while MetR, CatR, IlvY, NodD3, Nac and IciA

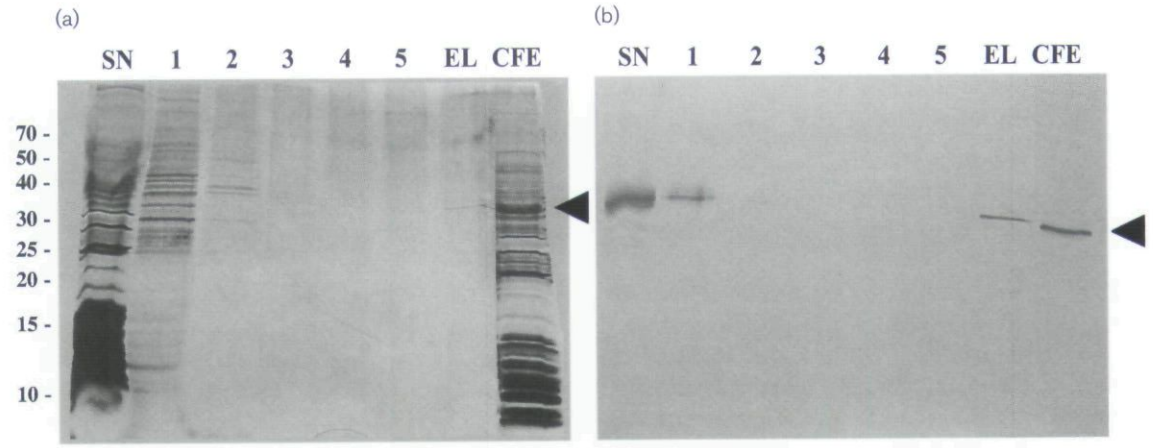

Fig. 3. Magnetic-bead-linked DNA affinity purification of CmbR-Histag. (a) Silver-stained SDS-PAGE. (b) Western blot analysis with anti-Histag antibody. SN, supernatant after incubation of CFE with magnetic-bead-linked P_{metC} fragment; 1–5: successive washing steps; EL, elution; CFE, cell-free extract. The positions of size markers (kDa) are shown on the left. The arrowheads indicate the position of CmbR-Histag.

are dimers (Schell, 1993). The CysB proteins of *E. coli* and *S. enterica* serovar Typhimurium are tetramers of identical 36 kDa subunits (Ostrowski *et al.*, 1987). By analogy, it seems likely that CmbR acts as a multimer, possibly as a tetramer. Two or more CmbR subunits might bind to the target site with different affinities and assemble to the proposed multimeric state of this regulator, which could explain the dose-dependent binding stages observed.

The exact region of the *metC* promoter involved in CmbR binding was localized by competition studies using the gel mobility shift assay in which unlabelled 5′-truncated variants of the *metC* promoter were added in excess of 500 ng (Fig. 5). This experiment showed that the unlabelled fragments F1, F2 and F3 could compete for the binding of CmbR with the labelled F1 fragment; the most efficient competition was obtained with the unlabelled F1 fragment. Truncation variants F4 and F5 could not compete with the binding of CmbR to the labelled F1 fragment (Fig. 5). These results are in agreement with the results obtained using the GusA assays and suggest that the most probable region for the CmbR binding in the *metC* promoter is the second direct repeat, which is present in the F1, F2 and F3 fragments (Fig. 1), but not in the F4 and F5 fragments, which were unable to compete for CmbR-Histag binding in our binding experiments. However, the F4 fragment still contains one of the two ATAAAAAAA-halves of the DR2 motif, but apparently could not compete for CmbR binding *in vitro*, nor activate *gusA* transcription in the corresponding fusion construct. Remarkably, many LysR-type regulators bind to a sequence of dyad symmetry, and a complete target sequence is required for recognition and binding (Schell, 1993). By analogy, the presence of both ATAAAAAAA regions of DR2 may be required to achieve the appropriate CmbR orientation or conformation to allow binding.

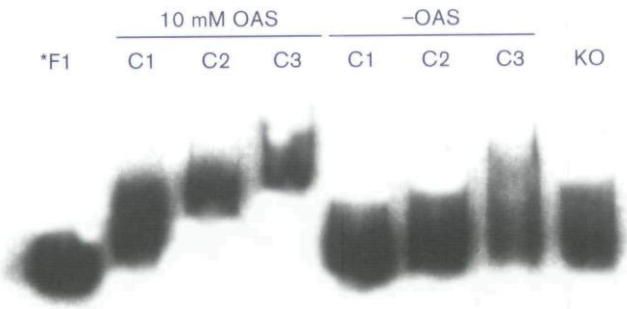

Fig. 4. Electrophoretic mobility shift assay: influence of OAS on CmbR binding at P_{metC}. *F1, radiolabelled fragment P_{metC}F1–P_{metC}R; C1, C2 and C3, 7·5, 15 and 30 μg CFE, respectively; KO, CFE from strain NZ9000*cmbR* with disrupted *cmbR* gene. Reactions were performed with 10 mM OAS or without (–OAS).

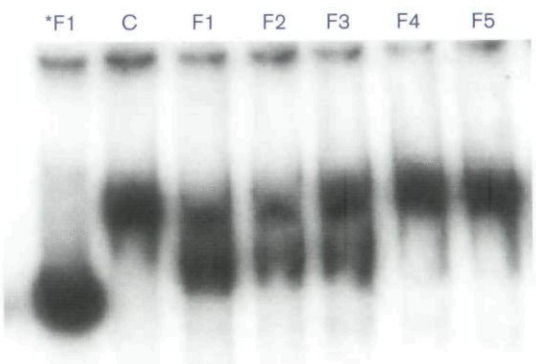

Fig. 5. Electrophoretic mobility shift assay: competition. *F1, radiolabelled fragment P_{metC}F1–P_{metC}R; C, P_{metC}–CmbR complex; F1, F2, F3, F4 and F5, unlabelled deletion variants.

Therefore, it is tempting to speculate that the complete DR2, which is present in fragment F3 but not in fragment F4, is a target sequence for the transcriptional activator CmbR.

In previous work the putative involvement of CmbR in the regulation of other genes involved in lactococcal sulphur metabolism has been described (Fernández *et al.*, 2002). Preliminary Northern blot analyses indicated that CmbR might be involved in regulation of the *metAB* operon. CmbR involvement in regulation of the genetically linked *fhu* genes was also indicated (Fernández *et al.*, 2002). Notably, in the *L. lactis* IL1403 genome sequence, CmbR is annotated as FhuR, which is the proposed regulator of the *fhu* operon (Bolotin *et al.*, 2001). The upstream regions of the *metAB* genes and the *fhu* operon were analysed, but no sequences identical to DR2 were detected. However, the upstream regions of both genes are AT-rich, which could obscure the presence of a DR2-like repeat. Our current studies are focusing on the role of the CmbR regulator in the regulation of other genes in *L. lactis*.

Many LTTRs bind to their target sequences without the presence of coinducer. The coinducer causes a conformational change of the LTTR, altering the bending of the DNA and activating transcription (Schell, 1993). This has been demonstrated for promoters that are positively regulated by MetR, with homocysteine as a co-inducer (Urbanowski & Stauffer, 1989; Martin *et al.*, 1986; Wek & Hatfield, 1988), and for promoters that are positively regulated by CysB (*cysK*, *cysP*), with *N*-acetylserine as a co-inducer (Hryniewicz & Kredich, 1995; Monroe *et al.*, 1990). In the binding experiments performed with CysB and its regulated promoters, 'slow' and 'fast' complexes were detected (Monroe *et al.*, 1990). A model was proposed in which a single molecule of CysB bends the *cysK* promoter by binding simultaneously to two cognate sites, in the absence of acetyl-L-serine. This DNA–protein interaction is unfavourable for transcriptional activation of the promoter. As the bending affects electrophoretic mobility, this complex migrates slowly during gel electrophoresis. Acetyl-L-serine is thought to induce a conformational change of CysB, allowing the protein to interact preferentially with one of the cognate sites. Bending is eliminated and fast complex is formed. In this study we did not observe slow and fast complexes of CmbR bound to P_{metC} in the presence and absence of OAS. CmbR binds to P_{metC} in the presence of OAS, while in the absence of OAS only a small amount of DNA fragment was bound. These results suggest that the presence of OAS is essential for the binding of CmbR to the *metC* promoter, and that in contrast to CysB, it is more likely that OAS does not induce the conformational change of already existing complex but facilitates the binding of CmbR to its binding site.

In conclusion, we have demonstrated that P_{metC} is regulated at the transcriptional level by interaction of the activator protein CmbR with P_{metC}, and shown that the nucleotide sequence positioned from −80 to −67 is involved in this interaction. The presence of OAS is essential for proper binding of CmbR to the promoter region. This mechanism of gene expression regulation by CmbR from *L. lactis* is different from that of CysB and MetR from *E. coli*, in which their respective coinducers cause a conformational change of the regulator that is already bound to the DNA.

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