The Maltose/Maltodextrin Regulon of *Streptococcus pneumoniae*

DIFFERENTIAL PROMOTER REGULATION BY THE TRANSCRIPTIONAL REPRESSOR MalR

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The *Streptococcus pneumoniae* MalR protein regulates the transcription of two divergent operons, **malXCD** and **malMP**, involved in maltosaccharide uptake and utilization, respectively. MalR belongs to the LacI-GalR family of transcription repressors. The protein binds specifically to two operator sequences in the intergenic region between these operons. The affinity of MalR for the **malMP** binding sequence is higher than for the **malXCD** site. Results obtained in *vivo* using transcriptional fusions with reporter genes indicate low repression level of **malXCD** by MalR when compared with **malMP**. This behavior may be correlated with the existence of separate induction pathways for maltose, maltotriose, and maltotetraose. The similarities found at the operator sequences and binding domains for MalR and enterococcal repressor proteins suggest that the pneumococcal maltosaccharide regulation system is closely related to several Gram-negative metabolic pathways, but not to the structurally similar *Escherichia coli* maltose regulon.

Bacterial regulons are often arranged as a network of genes or operons coordinately triggered by a DNA-binding protein, which interacts with the inducer molecule. Transcriptional control of gene expression would be expected to have evolved to optimize the performance of specific functions in response to nutritional and environmental changes. However several findings suggest that regulatory genes may have evolved relatively independently from the target genes (1, 2). The bacterial maltosaccharide regulon may represent a paradigm of independent evolution of the regulatory and metabolic genes. This regulon contains several genetic regions encoding peptides needed for the uptake and metabolism of maltosaccharides (3). Coordinated expression of the **mal** operones requires the synthesis and induction of a transcriptional activator, MalT, which is directly controlled by maltosaccharides and by the cAMP-CAP system (4, 5). Maltose/maltodextrin regulons have also been found in, at least, two Gram-positive bacteria, namely *Streptococcus pneumoniae* (6) and *Streptomyces coelicolor* (7). Although all three systems display structural and genetic similarities suggesting a common evolutionary origin, transcriptional regulation for these regulons in the Gram-positive bacteria appears to be attained by a repressor protein unrelated to MalT. This work is focused on the transcriptional control of the streptococcal **mal** regulon.

The maltosaccharide regulon of *S. pneumoniae* is organized in three operons (see Fig. 1A). Two operons are transcribed in opposite orientation: (i) **malXCD**, whose products were proposed to be involved in the uptake of maltotetraose (6), and (ii) **malMP**, which encodes enzymes involved in the metabolism of maltosaccharides: amyloglucosidase (MalM), essential for growth on maltose, and the maltodextrin phosphorylase MalP (8, 9). The gene products of these two operons share structural similarities with their enterococcal counterparts, which are located in the **malEFG** and the **malPQ** operons, respectively (6, 10). The third operon, **malAR**, is located 345 bp downstream from gene **malD**, and its products are involved in the global regulation of the pneumococcal **mal** operons. MalR has been shown to be the maltosaccharide pathway negative effector. Pioneer genetic work in the MAL locus (8), led to the isolation of several mutants yielding constitutive and noninducible expression of **malM**. These mutations were located in a region proximal to **malD**. In addition, integrative mutations in the **malR** gene resulted in constitutive expression of **malM**, denoting that the product of **malR** is involved at least in **malM** repression (11). The 36.9-kDa product of **malR** belongs to the LacI-GalR family of transcriptional repressors, and it was suggested to bind to an intergenic region located between the **malXCD** and the **malMP** operons repressing both operons (11, 12). Gene **malA** encodes a protein of 25 kDa, which could be identified using *E. coli* cell extracts for *in vitro* transcription-translation (11). Mutants in **malA** are unable to grow in maltodextrin as the only carbon source; however, the precise function of the protein is not known yet. Data collected on the streptococcal system suggest a more sophisticated regulation of maltosaccharide utilization. Short chain molecules like maltose and maltotriose may use an uptake pathway independent of **malXCD** (6, 8). However, **malM** and **malP** may be required for metabolization of all maltosaccharides. If this is the case, regulation of **malXCD** and **malMP** may not be necessarily coupled.

To gain knowledge on the relationship between MalR and other bacterial repressor proteins, we have analyzed its DNA binding activity and role in the maltosaccharide pathway regulation. In the present work, we have purified MalR and determined the MalR binding capacity to its target DNA. We have defined the DNA sequences to which MalR binds (the **malAR** operators) within the maltose/maltodextrin operons of *S. pneumoniae*. The operators overlap the expression signals for both the **malXCD** and the **malMP** operons. Maltose inhibited the binding of MalR protein to its target, indicating that this sugar acts as the positive effector of the **mal** operons. The MalR binding activity matched with its repression function, as shown

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1 The abbreviations used are: bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside.
through transcriptional fusions with β-galactosidase and luciferase in E. coli cells, showing different binding properties for malXCD and malMP.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—Overexpression of the malR gene was performed in E. coli BL21(DE3) using the pET5-derived plasmid pAPM60, which contains an KpnI-EcoRI fragment from pAPM34 (11). Plasmid pLS70 harbors a PstI 3.5-kilobase fragment of the S. pneumoniae maltose region cloned into the streptococcal plasmid pMV158 (13). Plasmid pQFXM (14) harbors two promoterless divergent reporter genes for β-galactosidase and luciferase. Plasmid pREP4 carries the lacI gene in a plasmid p15A-derived replicon (Quiggin). The recombinant plasmid pQFXM derives from pQFX2 by cloning the 674-bp NheI-Sau3AI fragment from pLS70 in the BglII-XhoI of the vector. pVTLMR was constructed by the subcloning of the BglII-ResAI fragment from plasmid pAM60 (see Fig. 1) containing a promoterless malR gene, into the expression vector pVLT31 (15). This construction places malR under the control of the IPTG-inducible tac promoter.

Growth and Transformation of Bacteria—E. coli was grown on LB medium and transformed by electroporation, as described (16), using 25 microfarads, 2.5 kV, and 200 Ω. Transformants were selected on agar medium with ampicillin (100 μg/ml), kanamycin (25 μg/ml), or tetracycline (15 μg/ml). The entire nucleotide sequence of the NheI-Sau3AI fragment, contained in the recombinants pQFXM, was determined by automated sequencer equipment (Applied Biosystems 373) and the dye-deoxyterminator procedure.

Protein Overexpression and Purification of MalR Protein—The method was performed as described previously (11) except that the buffer B was supplemented with 1 μM NaCl. After selective precipitation of MalR at low ionic strength (salting out) and solubilization in buffer B, 400 μl of the soluble fraction was passed through an agarose column (Bio-Gel A-0.5m, Bio-Rad) with a bed volume of 39 ml, and the flow rate was set at 6 ml/h. Fractions (500 μl) were collected and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). The selected fractions were pooled and stored at −80 °C. MalR protein retained its activity for at least 1 year.

Oligonucleotides and PCR Amplification—The following four oligonucleotides were synthesized and used in the PCR amplification to obtain DNA template for the binding assays: oligonucleotide 1, 5′-ggttaacctgtttcagcagc-3′; oligonucleotide 2, 5′-tccgtgaggctctcggcgtgg-3′; oligonucleotide 3, 5′-ggggatcatcagggggaggtta-3′; oligonucleotide 4, 5′-taacctgcaggtcctatc-3′. The fragments XM, X, and M were obtained after PCR amplification for 20 cycles using as template the NheI-Sau3AI 674-bp fragment of pLS70. For hydroxyl radical interference analysis the PCR was carried out after 5′ labeling of one of the primers with 32P-γATP and polynucleotide kinase (41). For gel retardation experiments PCR products were uniformly labeled with a mixture of 70 μl dNTP and 30 μl of 1α-32PdCTP.

DNA Binding Reactions and Electrophoretic Mobility Shift Assay—DNA binding reactions were performed in buffer containing 10 mM Tris-HCl, pH 8, 1 mM EDTA, 400 mM NaCl, and 10% glycerol. Purified MalR protein was mixed (100–200 ng) with 32P-labeled DNA (1 ng) and 0.5–1.9 μg of poly(dI-dC). Incubated 30 min on ice. Free and bound DNAs were separated on 5% polyacrylamide native gels (30:0.8 bisacrylamide in 0.5× TBE, TBE 89 mM Tris borate and 2 mM EDTA). Hydroxyl-Radical Interference Assay—The hydroxyl-radical interference assay was performed as described (17, 18). Single strand terminally labeled DNA fragments were treated with the hydroxyl radical reagents (19), except that the concentration was increased 5 or 10 times. Chemicals were purchased to Sigma or Merck. Treated DNA (100 ng) were incubated with MalR (20 μg), in a final volume of 100 μl. Bound DNA was separated from free DNA by nondenaturing PAGE, eluted in a solution 0.5 M ammonium acetate, 0.1 mM EDTA, and 0.1% SDS, and analyzed by electrophoresis in denaturing 5% polyacrylamide gels. Maxam and Gilbert (20) sequencing reactions were run in parallel. β-Galactosidase and Luciferase Assays—Bacterial cultures were grown until an absorbance (A600) of 0.3, and 0.7 ml of IPTG was added, to induce MalR expression. Cultures were incubated for 2 h at 37 °C. After this time, β-galactosidase and luciferase activities were determined. β-Galactosidase activity was assayed as described (21). Three independent transformants were used to determine each value. Luciferase activity was assayed as reported (22).

RESULTS

Purification of the MalR Protein—To purify the malR gene product, the plasmid pAPM60 (schematized in Fig. 1A) was transferred to E. coli BL21(DE3), and the malR gene was overexpressed by addition of IPTG. Previous efforts for the purification of MalR (11) indicated that a large fraction of the protein was insoluble. Attempts to purify the protein following the method reported for RhaS (23) were unsuccessful. For these reasons, the purification procedure was modified to include a step in which the ionic strength was increased to 1 M NaCl. The MalR-soluble fraction was passed through a gel filtration column as an additional purification step, after which the protein was estimated to be about 95% pure, as judged from polyacrylamide-SDS stained gels (Fig. 1B). Preliminary results of the elution profile suggested that, under the conditions used, MalR protein eluted as a mixture of 40% monomers and 60% dimers (not shown).

MalR Binds Specifically to the Pm and Px Promoters—To define the region to which MalR binds, we first performed specific DNA fragment mapping experiments. To this end, we made use of plasmid pLS70, in which a 3.5-kilobase PstI chromosomal fragment containing part of the S. pneumoniae mal operons is cloned (Fig. 1A) (13). The chromosomal region cloned in pLS70 includes a 674-bp NheI-Sau3AI fragment (Fig. 1A and 2), which contains the divergent promoter regions for the malXCD and the malMP operons. The 9-kilobase pair plasmid pLS70 DNA was digested with NheI and Sau3AI, yielding 14 fragments ranging from 2,583 bp to 12 bp, and the digestion products were incubated with increasing concentrations of MalR. Protein/DNA mixtures were loaded on agarose native gels, and the DNA fragments were stained with ethidium bro-
mide and visualized under UV light. The results showed that upon addition of increasing amounts of MalR, only the 674-bp fragment was specifically retarded by the protein, whereas the electrophoretic mobility of the other fragments was unaffected (Fig. 3). The retarded fragment appears to migrate as a faint smear near a position corresponding to 1.8 kilobases in the polyacrylamide gel. We conclude that purified MalR protein exhibits specific binding on the DNA fragment that contains its putative target sites.

The MalR binding sites within the 674-bp \textit{NheI-Sau3AI} fragment were defined by band-shift assays. To this end, four oligonucleotides were designed (see Fig. 2) and used as primers to amplify different regions within the \textit{NheI-Sau3AI} fragment. The DNA obtained was uniformly labeled with \([\alpha-32P]dCTP\), incubated with MalR, and the protein-DNA complexes were visualized after electrophoresis on native polyacrylamide gels and autoradiography. The results (Fig. 4) showed that the addition of MalR protein retarded the large XM fragment containing both divergent promoters \(P_m\) and \(P_x\), with their \(-35\) and \(-10\) regions, putative ribosome binding sites (RBS), and the initiation codons for \textit{malX} and \textit{malM} genes are underlined. The binding consensus sequence denoted as MalR operator (determined by footprint experiments; see Fig. 5) is shown as shadowed boxes. Numbered arrows represent the different oligonucleotides used in the PCR reactions. Nucleotide coordinates correspond to the sequence of plasmid pLS70 (12).

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**Fig. 2.** Promoter control region of \textit{malXCD} and \textit{malMP}, including the nucleotide sequence of the \textit{NheI-Sau3AI} fragment that contains the control signals for the operons. Promoters \(P_m\) and \(P_x\), with their \(-35\) and \(-10\) regions, putative ribosome binding sites (RBS), and the initiation codons for \textit{malX} and \textit{malM} genes are underlined. The binding consensus sequence denoted as MalR operator (determined by footprint experiments; see Fig. 5) is shown as shadowed boxes. Numbered arrows represent the different oligonucleotides used in the PCR reactions. Nucleotide coordinates correspond to the sequence of plasmid pLS70 (12).

**Fig. 3.** Specific DNA fragment missing assay. DNA from plasmid pLS70 (1 µg) was digested with \textit{NheI} and \textit{Sau3AI}, and the fragments were incubated with increasing amounts of purified MalR. Lane 1, MalR-untreated sample. Lanes 2–7, received 5, 10, 20, 40, 70, and 150 ng of MalR, respectively. The arrow points to the 674-bp fragment that progressively disappears as the MalR concentration increases. Molecular weight standards are those from bacteriophage T7 DNA digested with \textit{HpaII}.

ments carrying only one promoter. In addition, it appears that the affinity of MalR for promoter \(P_m\) is higher than for \(P_x\).

**Definition of the MalR Consensus Sequence on the DNA Binding Sites**—To determine the MalR recognition sequences in each of the above DNA fragments, hydroxyl radical interference analysis was performed. Hydroxyl radical-treated DNA was incubated with MalR, and free and bound DNA were separated by nondenaturing PAGE and eluted and analyzed in denaturing PAGE. Fig. 5 shows the regions protected by MalR on the short fragments carrying either the promoter/operator sequences of \textit{malX} or \textit{malM}. A protected region corresponding to a 15-bp imperfect palindromic was located 47 bp upstream of
the initiation codon for malM. A similar 14-bp sequence was also protected by MalR 44 bp upstream of the start codon for malX. In the hydroxyl radical interference experiments using the whole XM fragment, only the malM binding sequence was protected by MalR (not shown). No protection was detected at the malX binding site, probably due to the competition of the higher affinity malM site for MalR. The analysis of the consensus sequence for the binding of MalR on the operator/promoter region of malX and of malM revealed some differences, the major mismatch being located at the 3'-end (Fig. 5). The change observed could explain the differential affinity of MalR and may define the optimal consensus sequence for the repressor.

Maltose Inhibits the Binding of MalR to DNA—Physiological studies with S. pneumoniae cells grown in maltose as a carbon source showed that, in the presence of this sugar, the maltose/maltodextrin operon is derepressed (6, 8, 11). This suggests that maltose is one of the inducers of maltose/maltodextrin metabolism in this Gram-positive bacteria. Therefore we should expect that the affinity of MalR protein to DNA should be abolished in vitro when maltose is present in the assay. Indeed, the MalR band-shift assays shown in Fig. 6 indicate that the MalR DNA binding is dramatically reduced in the presence of maltose. It is worth pointing out that maltotriose or maltotetraose did not affect significantly the binding ability of MalR to its target DNA (data not shown).

MalR Represses Transcription from the malX-malM Promoters in E. coli—To test the differential affinity of MalR to the Pm or Px regions in vivo, we constructed the plasmid pQFXM which contains transcriptional fusions of malX and malM to the lacZ and lucAB reporter genes respectively. MalR was provided in trans by plasmid pVLTMR (Fig. 7). This plasmid could only be transferred to E. coli JM109 when this strain harbors pRep4, which overexpresses the lacI gene. This is probably due to a toxic effect of MalR expression in E. coli. To determine the effect of MalR on the expression of reporter genes, the strain JM109/pRep4, containing the pQFXM vector, was transformed with the plasmids pVLT31 or pVLTMR. To measure the basal levels of β-gal or Luciferase we used as a control the pQF120 plasmid. The results of the transcriptional repression assays are summarized in Fig. 7. The expression levels of the reporter genes obtained in the absence of IPTG indicate that MalR is expressed to some extent even under noninduced conditions, leading to a decrease of 30% in the malX-lacZ and 80% for the malM-luxAB fusion. Expression of MalR in IPTG-induced pVLTMR containing cultures leads to a reduction of 60% and 93% for Px and Pm, respectively, relative to the levels seen in the IPTG-treated pVLT31 containing control cells. These results suggest that, in vivo, MalR can repress the Pm promoter and thus the malMP operon to a greater extent that the Px promoter and its malXCD operon.

DISCUSSION

The results presented in this work indicate that the pneumococcal repressor MalR binds to two slightly different sequences. The consensus sequence shares homology with other consensus sites defined for some of the LacI-GalR proteins family. The consensus sequence is identical to the operator site found for PurR (24), while high homology is also found in the HTH motif for these two proteins (11, 25, 26) (Fig. 8). Such similarity might explain the toxicity of MalR found in E. coli as the binding of MalR to the PurR consensus sequences may cause interference with the de novo purine biosynthesis and, in part, with pyrimidine biosynthesis pathways, which are regul-
Transcriptional Repression by MalR

FIG. 7. Repression by MalR in E. coli. β-Galactosidase and luciferase activities were measured in extracts obtained from E. coli harboring either the promoterless pQF120 vector or pQFXM recombinant plasmid in which lacZ and LuxAB are under the control of Px and Pm, respectively. pVLT31 is a control non-MalR containing vector. Overexpression of malR cloned in pVLTMR is achieved by the addition of IPTG to the culture. Open and filled symbols represent malX and malM control signals respectively; dashed box, multicloning site. tsc, translational stop codons. The S.D. values for all assays did not exceed 20% from the values obtained in three independent experiments.

FIG. 8. Comparative sequence analysis. A, alignment of the binding consensus sequences for different proteins of the LacI-GalR family. The dashed box shows the homology between the MalR and PurR binding sequences, and the asterisk shows the axis of dyad symmetry in the sequences. B, alignment of HTH motifs in the LacI-GalR protein family. The identity in amino acid between MalR and PurR is indicated as dashed areas.

Published between positions 42 and 57 due to two sequencing errors found during the progress of this work (11). Amino acid residues involved in specific contacts such as Leu54 and Ala51 in the hinge helix of PurR are conserved in the MalR protein. The second residue of the recognition helix is serine, which is also found in MalI and RbtR (26, 30). The basic residue Lys55 in PurR discriminates against G-C. In MalR, this position is occupied by valine, which is not found in any other member of the family, and may not discriminate like the Ala residue found in GalR and LacI.

We have found a differential binding ability of MalR for the two target sites within the malXCD and the malMP promoter/operator regions. The results on MalR binding to both operators suggests that malMP should compete strongly with the binding to the malXCD operator. Apparently a small change in the adjacent bases to the G/C symmetry axis is enough to dramatically decrease the MalR binding affinity to its target sequence. The differential affinity observed in vitro provides a likely explanation for the differences seen in the in vivo expression from the transcriptional fusions. In addition, our finding agrees with previous results on MalM induction by maltose (11), and with the small differences found for the malX transcript accumulation under induced/uninduced conditions (6).

Several possibilities may explain the poor repression of promoter Px by MalR: (i) expression of the malXCD uptake operon is needed prior to the induction of the system, thus allowing the uptake of the inducer molecules; (ii) MalR may not be the only regulator of malXCD, and other proteins may play a role as co-repressors for this operon; and (iii) MalX might be shared with other carbohydrate uptake pathways (i.e. maltose and maltodextrins), making it likely that basal levels of the proteins should be expressed. These three possibilities are not self-exclusive. Expression of amylo maltase (codified by gene malM) is significantly affected by MalR as shown by transcriptional repression assays in E. coli (this work) and in experiments on MalM expression in S. pneumoniae (11). The failure of longer maltosacharide molecules, such as maltotriose and maltotetraose, to inactivate MalR, the requirement for malXCD for maltotetraose but not maltose uptake, and the differential expression of the two operons might be explained assuming the presence of additional genes for maltose uptake, possibly regulated by MalR, and additional effectors which could control malXCD in response to higher order maltodextrins.

The amino acid sequence homology at the binding consensus DNA binding domain among the members of LacI-GalR family (11, 24, 26, 30–36) suggests a common control model for all of
them. A dimeric configuration is required for DNA binding of the LacI-GalI repressor family. The generation of tetramers has been shown for the lactose, catabolite, and fructose repressors, and the ability of the tetramer to bind simultaneously to two different operators has been shown for LacI (29, 37–40). In the case of MalR, we could envisage that at low concentrations of the repressor, its preferred target would be the malM operator/promoter region. Although MalR appears as a mixture of monomers and dimers after purification we cannot exclude that binding of MalR to promoter Pm could favor the binding of the protein to the malX region through the generation of higher order oligomers. At high MalR concentrations, a DNA loop could be formed bringing close two distant DNA regions through protein-protein interactions, as it has been shown for other members of the LacI protein family (29). At present we have no indication of the participation of a host factor in the binding of MalR protein to these regions.

The similarities found between MalR and the other members of the family, even at the operator sequence among Gram-positive and Gram-negative bacteria, suggest a high conservation of the family, even at the operator sequence among Gram-negative bacteria, and enterococcal *MalT* seems to be the result of genetic shuffling rather than progressive mutation events. The selection of these positively regulated systems might be favored by the particular activity activated by nonrelated regulation proteins such as the *MalT* protein. A dimeric configuration is required for DNA binding of MalR protein to these regions.

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