Expression of the mau gene cluster of *Paracoccus denitrificans* is controlled by MauR and a second transcription regulator

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The *mau* gene cluster of *Paracoccus denitrificans* constitutes 11 genes (10 are located in the transcriptional order *mau*FBEDACJGMN; the 11th, *mauR*, is located upstream and divergently transcribed from these genes) that encode a functional methylamine-oxidizing electron transport branch. The *mauR* gene encodes a LysR-type transcriptional activator essential for induction of the *mau* operon. In this study, the characteristics of that process were established. By using *lacZ* transcriptional fusions integrated into the genome of *P. denitrificans*, it was found that the expression of the *mauR* gene during growth on methylamine and/or succinate was not autoregulated, but proceeded at a low and constant level. The *mauF* promoter activity was shown to be controlled by MauR and a second transcriptional regulator. This activity was very high during growth on methylamine, low on succinate plus methylamine, and absent on succinate alone. MauR was overexpressed in *Escherichia coli* by using a T7 RNA polymerase expression system. Gel shift assays indicated that MauR binds to a 403 bp DNA fragment spanning the *mauR–mauF* promoter region. It is concluded from these results that the expression of the structural *mau* genes is dependent on MauR and its inducer, methylamine, as well as on another transcription factor. Both activators are required for high-level transcription from the *mauF* promoter. It is hypothesized that the two activators act synergistically to activate transcription: the effects of the two activators are not additive and either one alone activates the *mauF* promoter rather weakly.

Keywords: LysR, *Paracoccus denitrificans*, *mau*, methylamine dehydrogenase, regulation

INTRODUCTION

*Paracoccus denitrificans* is a Gram-negative soil bacterium that is able to grow under a variety of conditions. In the presence of oxygen, it can grow on the C₁ substrate methylamine as sole source of carbon and energy (Harms & van Spanning, 1991). For growth on this substrate, methylamine dehydrogenase (MADH) and its obligate electron acceptor amicyanin are indispensable (Van der Palen *et al.*, 1995; van Spanning *et al.*, 1990b). Both proteins are specifically induced during growth on methylamine (Husain & Davidson, 1985, 1987) while they are absent when succinate is also present in the growth medium (Page & Ferguson, 1993; van Spanning *et al.*, 1994). MADH is a periplasmic protein which catalyses the oxidation of methylamine to formaldehyde and ammonium. MADH has an α₂β₂ composition, with molecular masses of 467 and 15.5 kDa for the α and β subunits, respectively (Husain & Davidson, 1985, 1987). The small subunit contains the tryptophan tryptophylquinone cofactor, which is constructed after linkage and modification of two specific tryptophan residues in the protein chain (McIntire *et al.*, 1991).

The genes involved in methylamine metabolism are clustered in the *mau* locus. The *mau* locus of *P.
denitrificans constitutes 11 genes organized in the transcription order mauRFEBDACJGMN (Chistoserdov et al., 1992; Van der Palen et al., 1995; van Spanning et al., 1990b, 1994) (Fig. 1a). The mauB and mauA genes encode the large and small subunits of MADH, respectively, while mauC encodes amicyanin. MauG is a peroxidase-like cytochrome c suggested to be involved in the processing of the tryptophan tryptophylquinone cofactor (Chistoserdov et al., 1994; Van der Palen et al., 1995). MauFEDJMN are accessory proteins involved in maturation and/or transport of MADH to the periplasm. Similar mau loci or parts of them have been found in other methylamine-utilizing organisms, including Methylobacterium extorquens AM1, Methylophilus methylotrophus W3A1-NS and the closely related strain Thiobacillus versutus (Huittema et al., 1993; Lidstrom & Chistoserdov, 1993; Ubink et al., 1991). Induction of the mau gene cluster has been suggested to be under control of the regulatory gene mauR, which is located upstream of mauF and divergently transcribed from the structural mau genes (van Spanning et al., 1994).

MauR is a member of the LysR family of transcriptional regulatory proteins (for a review see Schell, 1993). In general, genes encoding LysR-type regulators are divergently transcribed from the operon they regulate and their expression is often subject to negative autoregulation (Malakooti & Ely, 1994; Parke, 1996; Wang et al., 1992). Most LysR-type proteins activate transcription of target genes after binding of an inducer. They also share a sequence homology in the N-terminal region including a helix-turn-helix DNA-binding motif (Henikoff et al., 1988). In the absence of inducer, the LysR-type activators bind to promoter regions via a partially symmetrical sequence containing the T-N11-A motif, generally located 65 bp upstream from the transcription start of the regulated gene (Coco et al., 1994; Goethals et al., 1992). The presence of inducer may cause additional interactions of the activator with DNA sequences near the -35 RNA polymerase binding site and/or relaxed DNA bending that results in transcription activation (Huang & Schell, 1991; Parsek et al., 1995; Wang et al., 1992).

The aim of this study was to investigate further the characteristics of expression of the mau operon. The results indicate that MauR can bind to the mau promoter DNA region and fully activate the mau promoter, but only in the presence of an additional transcription regulator.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The strains and plasmids used are listed in Table 1. Escherichia coli strains were grown aerobically at 37 °C in YT medium (5 g yeast extract l−1, 8 g tryptone l−1 and 5 g NaCl l−1). P. denitrificans was grown aerobically at 30 °C in batch culture or on plates, either on rich medium [Brain Heart Infusion (BHI); Gibco] or on mineral salts medium supplemented with 100 mM methyamine, 10 mM choline or 25 mM succinate as carbon and energy source. The mineral salts medium was as described by Chang & Morris (1962) but supplemented with 1 ml trace elements solution l−1 (van Spanning et al., 1990a). When necessary, antibiotics were added to final concentrations of 100 μg ampicillin ml−1, 25 μg kanamycin ml−1, 40 μg rifampicin ml−1, 50 μg streptomycin ml−1, 20 μg chloramphenicol ml−1 or 12.5 μg tetracycline ml−1.

**β-Galactosidase assay.** Cells were grown on mineral salts medium supplemented with various carbon sources as described above and harvested during the exponential phase of growth. When both methylamine and succinate were present, cells were harvested in the early-exponential phase of growth (van Spanning et al., 1994). After the cells had been permeabilized with toluene, β-galactosidase activity was measured with ONPG as a chromogenic substrate as described by Miller (1972).

**Expression of mauR in E. coli.** E. coli BL21(DE3)(pLysS) transformed with plasmid pET9a or its derivative harbouring the mauR gene, plasmid pET.MauR (see Results), was grown in YT medium to an OD600 of 0.4–0.5. Expression of the T7 RNA polymerase was induced by adding 0.4 mM IPTG to the cultures, which were then grown for an additional 30 min (Studier et al., 1990). After induction, 200 μg rifampicin (ml culture)−1 was added and the culture was grown for another 90 min. Cells were harvested by centrifugation, and re-suspended in binding buffer containing 40 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 mM DTT and 60 mM KCl. The cell suspensions were sonicated (4 × 30 s) and centrifuged at 11,000 g for 10 min to remove cell debris. The resulting cell-free extracts were stored at −80 °C.

For the purification of the MauR protein, the cell-free extracts of the overproducing strain were dissolved in 10 mM potassium phosphate buffer (pH 7–7) containing 0.25 mM EDTA and 15 mM β-mercaptoethanol, and were directly applied to an hydroxyapatite column (Bio-Rad), equilibrated with the same buffer. Proteins were eluted by using a linear gradient of 10–400 mM potassium phosphate buffer. By using SDS-PAGE analysis, the collected fractions were tested for the presence of a 39 kDa protein band corresponding to the size of MauR. MauR-containing fractions were pooled and subsequently dialysed against 10 mM Tris/HCl (pH 7.6) containing 0.25 mM EDTA and 15 mM β-mercaptoethanol (TEM buffer) for 2 h. The dialysed sample was loaded onto a DEAE column (DE52), which was equilibrated with TEM buffer. The MauR protein was eluted from this column by using a linear salt gradient of 45–750 mM KCl in TEM buffer. MauR-containing fractions were then dialysed overnight against TEM buffer and stored at −80 °C.

**Preparation of the DNA probe used in the binding studies.** Plasmid pPr471 was digested with BamHI and HindIII and the 0.4 kb fragment (60 ng) containing the mauR–mauF intergenic region (Fig. 1b) was extracted from the agarose gel and radio-labelled with [α-32P]dCTP by filling the recessed ends with Klenow DNA polymerase.

**Gel shift DNA-binding assays.** Each binding mixture (20 μl) contained 30000 c.p.m. of the 32P-labelled target DNA, 10 μg of sonicated herring sperm DNA and various amounts of cell-free extract containing MauR in binding buffer. The reaction mixture was incubated for 15 min at 30 °C and then loaded onto a low-ionic-strength acrylamide gel as previously described by Ausubel et al. (1989). The gels were subsequently dried and autoradiographed with intensifying screens.

**Gel electrophoresis, electroblotting and protein sequencing.** Proteins were analysed by SDS-PAGE according to the method of Laemmli (1970). Proteins were then transferred to a PVDF-type membrane (ProBlott; Applied Biosystems) as previously
**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
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<th>Source/reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
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<td></td>
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<td>E. coli BL21(DE3)pLysS</td>
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<td>Novagen</td>
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<td>TG1</td>
<td>supE hisD55 thi Δ(lac–proAB) F' (traD36 proAB lacC lacZ ΔM15)</td>
<td>Sambrook et al. (1989)</td>
</tr>
<tr>
<td>HB101</td>
<td>recA hisD R hsD M tra leu thi</td>
<td>Boyer &amp; Roulland-Dussoix (1969)</td>
</tr>
<tr>
<td><strong>P. denitrificans</strong></td>
<td></td>
<td></td>
</tr>
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<td>DSM413, Rif', Spec', enhanced conjuration frequencies</td>
<td>De Vries et al. (1989)</td>
</tr>
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<td>Pd1222::Pr541a</td>
<td>Pd1222, pPr541 integrated once</td>
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<td>Pd1222::Pr541b</td>
<td>Pd1222, pPr541 integrated twice</td>
<td>This work</td>
</tr>
<tr>
<td>Pd1222::Pr471a</td>
<td>Pd1222, pPr471 integrated once</td>
<td>This work</td>
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<td>This work</td>
</tr>
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<td>Pd4721</td>
<td>Pd1222, mauR::Km'</td>
<td>van Spanning et al. (1994)</td>
</tr>
<tr>
<td>Pd4721::Pr541a</td>
<td>Pd4721, pPr541 integrated once</td>
<td>This work</td>
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<td>Pd4721::Pr541b</td>
<td>Pd4721, pPr541 integrated twice</td>
<td>This work</td>
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<td>Pd4721::Pr471b</td>
<td>Pd4721, pPr471 integrated twice</td>
<td>This work</td>
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<td><strong>Plasmids</strong></td>
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<td>pET9a</td>
<td>Km', φ10 promoter</td>
<td>Novagen</td>
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<td>pET.MauR</td>
<td>pET9a, mauR</td>
<td>This work</td>
</tr>
<tr>
<td>pBS(II)KS</td>
<td>oriV (colE1), Amp, lacZ'</td>
<td>Stratagene</td>
</tr>
<tr>
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<td>pBS(II)KS, mauR, mauR–mauF intergenic region and S' end mauF</td>
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<td>pBK11</td>
<td>Sm', Spec', lacZ promoter–probe vector</td>
<td>Kessler et al. (1992)</td>
</tr>
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<td>pBK11, mauF promoter–lacZ fusion</td>
<td>This work</td>
</tr>
<tr>
<td>pPr471</td>
<td>pBK11, mauR promoter–lacZ fusion</td>
<td>This work</td>
</tr>
<tr>
<td>pRK2020</td>
<td>Tc', pRK2013::Tn10</td>
<td>Ditta et al. (1980)</td>
</tr>
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</table>

Described by Towbin et al. (1979). The ProBlott membrane was stained with Coomassie Brilliant Blue and then the protein band with the size corresponding to that of MAUR was excised, after which its N-terminal sequence was determined. Protein concentrations were determined using a BCA protein assay (Pierce) with bovine serum albumin as a standard.

**DNA manipulations.** General cloning techniques were carried out essentially as described by Ausubel et al. (1993). Plasmid DNA was isolated from E. coli by the alkaline lysis method (Birnboim, 1983) and purified by using the Qiagen plasmid kit. DNA restriction fragments were purified from agarose gels with the Qiaex gel extraction kit (Qiagen). Using the acetyltrimethylammonium bromide procedure, genomic DNA from P. denitrificans was isolated from cultures harvested during the stationary phase of growth (van Spanning et al., 1990a).

Digested chromosomal DNA was subjected to agarose gel electrophoresis on 0.8% gels; the fragments were then denatured and transferred to positively charged nylon membranes (Boehringer Mannheim) according to the method of Southern (1975). Probes were made by labelling DNA with DIG-DUTP (DIG Nucleic Acid Detection kit; Boehringer Mannheim). After hybridization, visualization of the positively reacting fragments was performed with the chemiluminescent substrate CSPD (Lumigen-PPD; Boehringer Mannheim) essentially as described by the manufacturer.

**Conjugations.** Conjugations were carried out by streaking cells of donor and recipient strains together with the helper strain HB101(pRK2020) on BHI plates. After 1 d incubation at 30 °C, cells were collected and plated on selective media, after which the exconjugants were isolated.

**RESULTS**

**Construction of mau promoter–lacZ fusion vectors**

Two promoter–lacZ fusions were constructed; one to the mauF promoter (pPr541) and the second to the mauR promoter (pPr471). The starting materials were the lacZ promoter-probe vector pBK11 (Kessler et al., 1992), and plasmid pMAU32, which was constructed by insertion of a 1.3 kb XmnI–HindIII fragment (with mauR and the mau promoter region) into the pBluescript vector. For the construction of pPr541, a 0.4 kb HinII–BamHI fragment from pMAU32 (the HinII site is located in mauR; the BamHI site is located in the multiple cloning site of the vector), containing the mauR and mauF promoter regions and flanking regions, was
cloned into the Smal–BamHI sites of pBK11 upstream of the lacZ gene (Fig. 1b). The plasmid pPr471 was constructed by insertion of the same fragment in the opposite orientation into the polylinker of pBK11 (using HindIII–EcoRI and Smal–EcoRI sites for the insert and the vector, respectively). The pPr471 and pPr541 vectors were transferred by conjugation to P. denitrificans, the wild-type strain Pd1222 and the mutant strain Pd4721 (mauR::Km). The exconjugants that had integrated the vector with the Pmau–lacZ fusion into the chromosome by homologous recombination were selected for their resistance to streptomycin. A scheme showing the genomic organization expected after integration of these fusions by homologous recombination is shown in Results. The 0.4 kb fragment shown in (b) was also used as probe in the Southern blot analysis and in the gel shift assays. (c) Genomic organization expected after integration of these fusions by homologous recombination. The different strains are shown to the right. The shaded boxes are elements of pBK11 (7.9 kb; not to scale), and the broken double-headed arrows at the top indicate the distances between relevant restriction sites located upstream and downstream. The mau promoter region (P P) sandwiched by the hatched boxes is the genomic part that was used for construction of pPr541 and pPr471. The direction of transcription is indicated by the direction of the thin arrows under the genes. The direction of the thick arrows indicates the position of the fragment relative to the transcription direction of lacZ. Restriction sites in bold belong to the multiple cloning site of the vector.

Southern analysis of these strains with the 0.4 kb fragment as a probe (Fig. 1b) revealed that both constructs were properly integrated within the chromosomal copy of the mau promoter region. In some cases the vectors were integrated twice at the same location, yielding an organization of two Pmau–lacZ fusions in tandem. A typical example of these analyses is shown in Fig. 2. When the chromosomal DNA was digested with EcoRI (Fig. 2a), the probe hybridized with an approximately 12 kb DNA fragment of the wild-type strain Pd1222 (lane 4), with 4 and 7.4 kb fragments of the insertion strains Pd1222::Pr541b and a (lanes 1 and 2), and with 13 and 7.4 kb fragments of the insertion strains Pd1222::Pr471b and a (lanes 3 and 5), as expected. The presence of faint 7-0 kb bands in lanes 1 and 2 are most likely due to incomplete digestion of the chromosomal DNA. Digestion with SalI (Fig. 2b) gave rise to a band of 4-3 kb which hybridized with the probe for the wild-type strain (lane 1), as expected. Two distinct bands of either 12.6 kb (lanes 3 and 4) or 20.9 kb (lanes 2 and 5) were obtained for the insertion strains, depending on whether one (Pd1222::Pr541a and Pr471a) or two (Pd1222:: Pr541b and Pr471b) copies of the lacZ fusion vector had been integrated into the genome. As expected, similar band patterns were obtained with the insertion strains derived from the mutant strain Pd4721 (results not shown). All plasmid integrated derivatives of strains Pd1222 and Pd4721 were used for further analyses.

\[\textbf{\textit{\beta}-Galactosidase assays}\]

The eight different insertion strains were grown on succinate, on methylamine, or on a combination of the two carbon sources. After harvesting the cells, the transcriptional activities of the mau promoters, as reflected by the \(\beta\)-galactosidase activities, were assessed (Table 2). For supporting the growth of the mauR mutant strains, which are unable to grow on methylamine, a second substrate had to be supplied (choline or succinate) as source of carbon and energy in addition to the first substrate as inducer of the mau gene cluster (van Spanning et al., 1994). The \(\beta\)-galactosidase activities of the parent strains Pd1222 and Pd4721 were below 1 Miller unit.

The \(\beta\)-galactosidase activity of succinate-grown Pd1222::Pr541a was at background level, suggesting that the mauF promoter is inactive under those growth conditions.
P-galactosidase activity was assayed with ONPG as substrate. All strains carry a genomic visualized with the labelled 0.4 kb SO-fold lower than when they were grown in the presence grown cells (4 versus 8755 Miller units). galactosidase activity as compared with succinate-grown cells (4 versus 8755 Miller units).

The mean of three independent determinations, each performed in triplicate. All standard deviations are within 15 % of the mean.

Fig. 2. Southern blot analysis of P. denitrificans plasmid integrant strains. (a) Southern blot of P. denitrificans chromosomal DNA digested with EcoRI. Lanes: 1, Pdl222::Pr541b; 2, Pdl222::Pr541a; 3, Pdl222::Pr471b; 4, Pdl222 wild-type strain; 5, Pdl222::Pr471a. (b) Southern blot of P. denitrificans chromosomal DNA digested with Sall. Lanes: 1, Pdl222; 2, Pdl222::Pr471b; 3, Pdl222::Pr471a; 4, Pdl222::Pr541a; 5, Pdl222::Pr541b. DNA fragments were visualized with the labelled 04 kb EcoRI–BamHI fragment of pPrAT1. The size of DIG-labelled DNA molecular size markers is indicated to the right (in kb).

conditions. In contrast, methylamine-grown Pdl222::Pr541a cells displayed a 2000-fold increase in β-galactosidase activity as compared with succinate-grown cells (4 versus 8755 Miller units).

The mauF promoter activity in Pdl222::Pr541a cells grown on both succinate and methylamine was about 50-fold lower than when they were grown in the presence of methylamine alone (148 versus 8755 Miller units), but higher than when they were grown in the presence of succinate alone. The mauF promoter activity in mutant strain Pd4721::Pr541a grown under the same conditions was reduced to background level, indicating that this activity is strictly dependent on MauR (2 versus 148 Miller units).

The β-galactosidase activity of the strain Pdl222::Pr541a was much lower when the cells were grown on choline than when they were grown on methylamine (35 versus 8755 Miller units). The activity of the former cells, however, was significantly higher than that of the background level (35 versus 4 Miller units). Furthermore, this low level of activity of the mauF promoter was independent of the activator MauR, since it was also observed in the Pd4721::Pr541a strain.

When Pdl222::Pr541a cells were grown in the simultaneous presence of methylamine and choline, the transcriptional activity of the mauF promoter was very strong and only twofold lower than in the presence of methylamine alone (4007 versus 8755 Miller units). In Pd4721::Pr541a grown under the same conditions, this activity was strongly reduced (12 versus 4007 Miller units), but it was significantly higher than the background level.

In all cases, the strains carrying two copies of the lacZ fusion, Pd1222::Pr541b or Pd4721::Pr541b, exhibited β-galactosidase activities approximately twofold higher than those carrying a single copy of the vector, Pd1222::Pr541a or Pd4721::Pr541a.

In contrast to the mauF promoter, the transcriptional activity of the mauR promoter was low and constant, irrespective of whether the strains were grown on methylamine and/or succinate. However, when the growth medium contained choline alone or choline with methylamine, the activity was always slightly higher.

<table>
<thead>
<tr>
<th>Strain</th>
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<th>β-Galactosidase activity (Miller units) after growth on:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>MA</td>
</tr>
<tr>
<td>Pdl1222</td>
<td>Parent strain</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Pdl4721</td>
<td>mauR::Km'</td>
<td></td>
</tr>
<tr>
<td>Pdl1222::Pr541a</td>
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<td>8755</td>
</tr>
<tr>
<td>Pdl4721::Pr541a</td>
<td>mauR::Km', Pr mauF–lacZ</td>
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<tr>
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<td>16712</td>
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<td>Pdl4721::Pr541b</td>
<td>mauR::Km' (Pr mauF–lacZ) × 2</td>
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<td>Pdl1222::Pr471a</td>
<td>Pr mauR–lacZ</td>
<td>19</td>
</tr>
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<td>mauR::Km' Pr mauR–lacZ</td>
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<tr>
<td>Pdl4721::Pr471b</td>
<td>mauR::Km' (Pr mauR–lacZ) × 2</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Expression studies of the mauR and mauF promoters

Cells were grown in mineral salts medium supplemented with a carbon source as indicated. After disruption of the cells with toluene, β-galactosidase activity was assayed with ONPG as substrate. All strains carry a genomic lacZ fusion except the parent strains Pdl1222 and Pd4721 added as control. MA, Methylamine; Succ, succinate; Chol, choline; Pr, promoter; −, no growth. Data are the mean of three independent determinations, each performed in triplicate. All standard deviations are within 15% of the mean.
Fig. 3. Purification of the MauR protein from *E. coli* cell-free extracts overproducing MauR. Electrophoresis was performed on a 12.5% polyacrylamide gel containing 0.1% SDS and proteins were visualized by Coomassie Blue staining. Lanes: 1, cell extract of *E. coli* BL21(DE3)(pET.MauR) minus IPTG (20 µg); 2, cell extract of *E. coli* BL21(DE3)(pET.MauR) plus IPTG (20 µg); 3, cell extract of *E. coli* BL21(DE3)(pET9a) plus IPTG (20 µg); 4, purified MauR (2 µg). Molecular mass markers (in kDa) are indicated on the left. The arrow indicates the position of the 39 kDa mauR gene product.

Furthermore, the mauR mutants Pd4721::Pr471a and b displayed β-galactosidase activities similar to those of the corresponding wild-type strains Pd1222::Pr471a and b, respectively.

Expression of MauR in *E. coli*

Overexpression of mauR in *E. coli* was shown to be negatively affected when sequences upstream of the gene were cloned along with it. In order to avoid this negative effect, a translational fusion between the mauR gene and the promoter of the pET9a vector was generated. To achieve this, the mauR gene was amplified in a PCR with two primers that matched the upstream and downstream regions of the gene. The upstream primer 5'-AAGCTT-CATATGAACTGGGACGACCTTCG-3' was slightly modified in order to introduce an NdeI site at the position of the ATG translational start codon. The downstream primer 5'-GAATTCCGATCCCTACACCGCGTCGCGG-3' was equipped with a BamHI site. The resulting 0.9 kb PCR product was cut with NdeI and BamHI and cloned in pET9a, yielding pET.MauR. In this construct, the mauR coding region is now fused to the ATG start codon present on the T7 expression vector pET9a. Expression from the T7 promoter in *E. coli* BL21(DE3) harbouring either pET.MauR or pET9a was induced with IPTG. SDS-PAGE analysis of the cell-free extracts showed that a protein of approximately 39 kDa was expressed in *E. coli* BL21(DE3)(pET.MauR) upon induction with IPTG (Fig. 3, lane 2). The apparent molecular mass of this protein is in agreement with that predicted from the sequence translated from the mauR gene (32 kDa). This protein band was absent in *E. coli* BL21(DE3)(pET9a) (Fig. 3, lane 3). The cell-free extracts overproducing the 39 kDa protein were used for the purification of MauR. After two purification steps, first on a hydroxyapatite column and subsequently on a DEAE column (as described in Methods), a partially purified protein preparation containing some minor contaminants was obtained (Fig. 3, lane 4). Sequence analysis of the N-terminus of this 39 kDa protein band revealed the amino acid sequence MNWDDL, which was identical to that deduced from the sequence translated from the mauR gene, confirming that the overproduced protein was MauR.

Binding of MauR to the mauR–mauF intergenic region

To determine whether MauR binds to the mau promoter region, gel mobility shift assays were performed. Increasing amounts of cell-free extracts of *E. coli* BL21(DE3) harbouring either pET.MauR or the vector pET9a were incubated with a 403 bp HindIII–BamHI DNA fragment containing the mauR–mauF intergenic region and flanking regions (Fig. 4). A clear shift of the complex was observed (Fig. 4, lane 5) and a saturating concentration of 8 µg cell-free extract of *E. coli* harbouring pET9a was used in all subsequent experiments. In the absence of inducer, MauR did not form a complex with the labelled DNA fragment (Fig. 4, lane 1), indicating that the expression of MauR was dependent on the presence of IPTG. In the presence of inducer, the complexes formed with 2 µg (lane 2), 4 µg (lane 3), and 8 µg (lane 4) cell-free extract of *E. coli* harbouring pET9a were indistinguishable, indicating that the complex formation was saturable. The complexes were shifted even further when 16 µg (lane 5) and 24 µg (lane 6) cell-free extract of *E. coli* harbouring pET9a were used. The arrow indicates the position of the single electrophoretically retarded complex.
labelled HindIII–BamHI fragment was observed when cell-free extracts of E. coli harbouring pET.MauR, in which mauR was expressed, were added to the assay mixture. In contrast, no retardation was observed when extracts harbouring the vector pET9a were used at similar concentrations. Increasing protein concentrations of cell-free extracts from E. coli harbouring pET.MauR (2–24 μg) caused an increasing amount of the labelled fragment to show retarded mobility (lanes 3–7). At protein levels higher than 30 μg, non-specific binding activity was observed (not shown). High concentrations of carrier DNA (10 μg herring sperm DNA) in the binding reaction were required in order to prevent non-specific binding. The presence of methylamine in this assay (Fig. 4b) did not alter the results.

DISCUSSION

Here we provide evidence that MauR, which is a LysR-type transcriptional activator, is able to bind to the promoter DNA region upstream of the mau gene cluster, and is essential for the transcription process during growth on methylamine. The concentration of MauR appeared to be relatively constant in the cells during growth on either succinate or methylamine. High levels of mauF gene expression, however, were only found when the cells were grown on methylamine. The latter molecule is thus very likely to be the chemical signal for activation of MauR. Indeed, many members of the LysR-type family are activated by the same molecule as that which is metabolized by the products of the genes that they control (Schell, 1993). When both succinate and methylamine were present in the growth medium, the expression of the mau genes decreased about 50-fold, as compared to during growth on methylamine alone, although the amount of MauR was the same in both cases. These results indicate that MauR-dependent expression of the mau gene cluster is either blocked due to the presence of a repressor protein or not induced due to the absence of a second activator protein during growth on succinate. We favour the concept of a second activator which acts in synergism with MauR during transcriptional control of the mau genes. This concept is in agreement with our earlier studies where we described the isolation of a mutant impaired in the expression of both the mox and mau genes (Harms et al., 1996). This mutant was unable to grow on methanol or methylamine, and was unable to synthesize methanol dehydrogenase and MADH under inducing conditions [i.e. when the cells were grown in media containing choline as the carbon and energy source plus either methanol or methylamine (inducers)].

In cases where MauR is activated (in the presence of methylamine), the expression level of the mau gene cluster increases almost 40-fold (4 Miller units in succinate-grown cells versus 148 in those grown on methylamine and succinate). When the second activator is also active in addition to MauR, the expression level of the mau gene cluster increases another 50-fold (148 Miller units in succinate- and methylamine-grown cells versus 8755 in methylamine-grown cells). Thus when both transcription factors are present an approximately 2000-fold increase of gene expression (4 Miller units in succinate-grown cells versus 8755 in methylamine-grown cells) is achieved. During growth on choline, MauR is inactive (no methylamine), but the expression level of the mau genes is almost 10-fold higher as compared to succinate-grown cells, which may reflect a higher activity of the second activator in the former cells. When the cells are grown on a mixture of choline and methylamine, both MauR and the second activator are active, and the expression levels increase almost 100-fold as compared to those of choline-grown cells. Apparently, the presence of choline still slightly suppresses the activity of the second activator, since the expression levels are half of those found during growth on methylamine alone. Exactly the same trends were observed when we analysed the data from the strains that had integrated the promoter–lacZ fusions twice (all activities were doubled as compared to those with a single integration). We may therefore conclude that the DNA is still limiting in the control of gene expression, and that regulatory proteins are not diluted due to the integration of more than one copy of the target DNA. During growth of the mauR mutants on succinate or choline, the mauF promoter activities were comparable to those of the wild-type. Furthermore, these promoter activities did not change significantly when methylamine was added to the growth medium, supporting the proposed model that MauR is essential for methylamine-induced expression.

The mauR promoter activity is relatively constant during growth on either succinate or methylamine, but increases two- to threefold when choline is added to the growth medium. The reason for this stimulation is not known. It is notable that the same promoter activities were found in the wild-type and mauR mutant strains, indicating that mauR gene expression is not auto-regulated. This feature makes the regulation of mauR unusual, since it has been described that the genes of many other LysR-type regulators are negatively auto-regulated (Schell, 1993). The only exceptions that have been described concern mleR and xapR (Renault et al., 1989; Seeger et al., 1995).

The DNA-binding site of all LysR regulatory proteins consists of an inverted repeat including the T-N_{11}-A motif (LysR motif) (Goethals et al., 1992), except in the case of CatR, where the G-N_{13}-A motif (CAGACC-N_{2}-GTTATG) replaces the common T-N_{11}-A motif within the inverted repeat (Parsek et al., 1994). There is no LysR consensus motif in the mau promoter region. Only an inverted repeat containing a G-N_{13}-A motif (GGCA-N_{2}-TCCAG) was found 26 bp upstream of the start codon of the mauR gene.

Although the exact site of binding is still unknown, gel mobility shift assays demonstrated that MauR binds specifically to the mau promoter region. This event occurred even in the absence of the inducer, methylamine. These in vitro binding experiments showed that the presence of the inducer does not alter the
apparent affinity of MauR for the mau promoter region, nor the mobility of the Maur-promoter–DNA complex as judged by the presence of one single retarded band exhibiting similar intensity and mobility, with or without methylamine. A similar lack of effect of the inducer on promoter-binding activity has also been reported for the LysR-type transcriptional activators NahR and CatM (Romero-Arroyo et al., 1995; Schell & Faris Poser, 1989). However, in vivo footprinting experiments in the case of NahR revealed that the presence of its inducer caused further interactions of NahR with sequences downstream of the primary binding site (−65), near position −40 (Huang & Schell, 1991). The reason why we did not observe a methylamine-mediated change in our gel shift assays might be due to the fact that the differences in the band-shift induced by methylamine are too small. Another possible explanation is that the methylamine-mediated change occurs only when the second activator is also present to enhance specific DNA-binding properties of MauR.

Our current model, which accounts for all our observations, predicts that MauR is present under all growth conditions, and is activated when methylamine is bound as a coinducer molecule. Both forms are able to bind to the DNA, but they bind weakly. Only when MauR is in the active configuration can it make contact with the second activator. The resulting complex may bind firmly to the DNA as a prerequisite for promoting mau gene expression. Another example of a LysR-type activator acting together with a second activator to activate transcription has been described for the dsdXA promoter (Narreggaard-Madsen et al., 1995). We are currently characterizing the second activator in order to confirm our model.

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