Characterization of PmfR, the Transcriptional Activator of the pAO1-Borne *purU-mabO-fold* Operon of *Arthrobacter nicotinovorans*

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Nicotine catabolism by *Arthrobacter nicotinovorans* is linked to the presence of the megaplasmid pAO1. Genes involved in this catabolic pathway are arranged on the plasmid into gene modules according to function. During nicotine degradation γ -*N*-methylaminobutyrate is formed from the pyrrolidine ring of nicotine. Analysis of the pAO1 open reading frames (ORF) resulted in identification of the gene encoding a demethylating γ -*N*-methylaminobutyrate oxidase (*mabO*). This gene was shown to form an operon with *purU*- and *fold*-like genes. Only in bacteria grown in the presence of nicotine could transcripts of the *purU-mabO-fold* operon be detected, demonstrating that this operon constitutes part of the pAO1 nicotine regulon. Its transcriptional start site was determined by primer extension analysis. Transcription of the operon was shown to be controlled by a new transcriptional regulator, PmfR, the product of a gene that is transcribed divergently from the *purU*, *mabO*, and *fold* genes. PmfR was purified, and electromobility shift assays and DNase I-nuclease digestion experiments were used to determine that its DNA binding site is located between –48 and –88 nucleotides upstream of the transcriptional start site of the operon. Disruption of *pmfR* by homologous recombination with a chloramphenicol resistance cassette demonstrated that PmfR acts in vivo as a transcriptional activator. Mutagenesis of the PmfR target DNA suggested that the sequence GTTT-14 bp-AAAC is the core binding site of the regulator upstream of the –35 promoter region of the *purU-mabO-fold* operon.

Nicotine catabolism by *Arthrobacter nicotinovorans* is dependent on the presence of the megaplasmid pAO1 (1, 5). Genes that have previously been shown to be implicated in this metabolic pathway (1, 20, 24) are clustered on pAO1 in modules (22). Expression of these genes is induced by the presence in the growth medium of nicotine or of nicotine breakdown products (13). The metabolic fate of γ -*N*-methylaminobutyrate generated from the pyrrolidine ring of nicotine (13, 19, 21) and the location of the pertinent genes on the chromosome or on pAO1 are not known. The *nic* gene cluster on pAO1 (1, 22) is flanked by a group of open reading frames (ORFs) consisting of ORF63 (Fig. 1A) encoding a γ -*N*-methylaminobutyrate oxidase (MABO) (11), ORF64 encoding a protein with a high level of similarity to formyltetrahydrofolate deformylase (the gene product of *purU*) (37), and ORF62 encoding a protein with a high level of similarity to the bifunctional enzyme methylenetetrahydrofolate dehydrogenase/cyclohydrolase (the gene product of *fold*) (23). In bacteria the combination of genes encoding demethylating oxidases and enzymes of tetrahydrofolate C₁ metabolism into one functional unit has been observed previously (12, 25).

Here we show that these genes, whose expression is nicotine dependent, form an operon on pAO1 and thus constitute part of the pAO1 nicotine regulon. Upstream of *purU* are ORF66, which encodes a protein with similarity to amino acid permeases, and the divergently oriented ORF67, which encodes a

protein with similarity to transcriptional regulators having unknown functions (22) (Fig. 1A). We cloned and expressed the gene encoding the potential transcriptional regulator PmfR, purified and characterized the recombinant protein, and defined its binding site upstream of the *purU-mabO-fold* operon promoter. We demonstrated by disrupting the *pmfR* gene with a chloramphenicol resistance cassette that PmfR is the transcriptional activator of the *purU-mabO-fold* operon. The transcriptional regulator described in this paper is the first identified transcriptional activator of the nicotine regulon.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *A. nicotinovorans* pAO1 was grown at 30°C on citrate medium supplemented with vitamins and trace elements (6) in the presence of 5 mM L-nicotine, as required. Growth of the culture was monitored by determining the increase in absorbance at 600 nm.

Escherichia coli XL-1 Blue was employed as the host for plasmids and was grown on lytic broth (LB) supplemented with the appropriate antibiotics at 37°C.

Cloning of the *pmfR* gene. pH6EX3 (3) was used as the expression vector for cloning of the *pmfR* gene. The *pmfR* gene was amplified by PCR with primers 1 and 2 (see the supplemental material at <http://www.biochemie.uni-freiburg.de/brandsch/supplement.pdf>) carrying EcoRI and XhoI restriction enzyme recognition sites, respectively. pAO1 DNA isolated as described previously (5) was employed as the template in PCRs performed as follows: 95°C for 1 min, 62°C for 40 s, and 72°C for 2 min for 30 cycles, followed by one amplification cycle of 95°C for 1 min, 62°C for 40 s, and 72°C for 10 min. Pfu-Turbo high-fidelity polymerase (Stratagene, Heidelberg, Germany) was used in the PCR. The amplified DNA fragments were ligated, after restriction, into EcoRI-XhoI-digested pH6EX3, resulting in pH6EX3*pmfR*. *E. coli* XL-1 Blue cells, made transformation competent with a Roti-Transform kit (Roth, Karlsruhe, Germany), were transformed with pH6EX3*pmfR* DNA. The bacteria were plated on LB plates supplemented with 50 µg of ampicillin per ml. Recombinant clones were verified by sequencing.

Purification of His-tagged PmfR. The recombinant plasmid carrying the *pmfR* gene was transformed into *E. coli* BL21 (Novagen, Schwalbach, Germany) and was selected on LB plates supplemented with 50 µg of ampicillin per ml. One hundred milliliters of LB was inoculated with a single colony, which was grown overnight at 30°C and used to inoculate 1 liter of LB. Overexpression of PmfR

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FIG. 1. Arrangement of the ORFs in pAO1 investigated and RT-PCR analysis of transcripts generated from this region. (A) Schematic diagram of ORFs and RT-PCR strategy. The numbers below the diagram indicate primers (see the supplemental material at <http://www.biochemie.uni-freiburg.de/brandsch/supplement.pdf>). (B) Lanes 1 to 3, PCR with primers 3 and 4 and with pAO1 DNA as the template (lane 1), with cDNA as the template (lane 2), and with RNA as the template (lane 3) (expected size, 350 bp); lanes 4 to 6, PCR with primers 5 and 6 and with pAO1 DNA as the template (lane 4), with cDNA as the template (lane 5), and with RNA as the template (lane 6) (expected size, 310 bp); lanes 7 to 9, PCR with primers 7 and 8 and with pAO1 DNA as the template (lane 7), with cDNA as the template (lane 8), and with RNA as the template (lane 9) (expected size, 775 bp). (C) Primer extension analysis was performed as described in Materials and Methods. Lanes G, A, T, and C, sequencing reactions; lane 1, control without RNA template; lane 2, product of primer extension reactions (indicated by an arrow). In the sequence on the left the transcriptional start site is indicated by an asterisk.

was induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 5 h at 30°C. Bacteria were harvested by centrifugation at $5,000 \times g$, resuspended in 40 mM HEPES buffer containing 0.5 M NaCl (pH 7.4), and disrupted with a Branson Sonifier. The supernatant (30 ml, with a protein concentration of 5 mg/ml) obtained by centrifugation of the bacterial lysate at $13,000 \times g$ was used to purify the protein on Ni-chelating Sepharose as described by the supplier (Amersham Biosciences, Freiburg, Germany). The yield of the PmfR protein was 1 to 1.5 mg per liter of culture, and samples were analyzed on Coomassie blue-stained sodium dodecyl sulfate (SDS)–10% polyacrylamide gels. Gel permeation chromatography for determination of the native sizes of the proteins was performed on Superdex-200 gels with the aid of an ÄKTA Basic chromatography device (Amersham Biosciences) and by blue native polyacrylamide gel electrophoresis (BN-PAGE) on 6 to 15% polyacrylamide gradient gels as described by Schagger and von Jagow (34).

Western blotting of *A. nicotinovorans* pAO1 extracts. His-tagged MABO protein purified as described previously (11) was used to raise an antiserum in rabbits by using standard protocols. Bacterial pellets from 1-liter cultures of *A. nicotinovorans* pAO1 grown as described above were suspended in 5 ml of 0.1 M phosphate buffer (pH 7.4) containing 58 mM Na_2HPO_4 , 17 mM NaH_2PO_4 , 68 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 5 mg of lysozyme per ml. After 1 h of incubation on ice, the bacterial suspensions were passed through a French pressure cell at 132 MPa, and the lysates were centrifuged for 30 min at $12,000 \times g$. The extracts were analyzed by SDS-PAGE on 10% polyacrylamide gels and blotted onto Optitrans BA-S 85 nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were decorated with the antiserum and were visualized by using alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Sigma, Steinheim, Germany) and nitroblue tetrazolium chloride as an indicator.

RNA extraction and reverse transcription PCR (RT-PCR). Total RNA was extracted from *A. nicotinovorans* pAO1 cultures and transcribed into cDNA as described previously (33). One microliter (1:10) of the cDNA was used as a template in PCRs with primers specific for *purU-mabO* and *mabO-folD* transcripts (primers 3, 4, 5, and 6 [see the supplemental material at <http://www.biochemie.uni-freiburg.de/brandsch/supplement.pdf>]). In addition, PCRs were performed to amplify the region between the permease gene-like ORF and *pmfR* (primers 7 and 8 [see the supplemental material]). The pAO1 DNA template was employed as a positive PCR control, and untranscribed RNA was used as a

negative control, in order to confirm the absence of contaminating DNA in the RNA preparation.

EMSA. DNA fragments were generated by PCR by using primers described in the supplemental material and pAO1 DNA as the template. The fragments were 5' end labeled with [γ - ^{32}P]ATP by using a Ready-to-Go polynucleotide kinase kit according to the instructions of the supplier (Amersham Biosciences). Electrophoretic mobility shift assays (EMSA) of the labeled DNA fragments in the presence of various amounts of purified PmfR or *A. nicotinovorans* extracts were performed as described by Foster-Hartnett and Kranz (15). The effects of potential inducers on PmfR DNA binding were tested by preincubation of the purified protein or *A. nicotinovorans* extracts with nicotine or nicotine derivatives (6-hydroxynicotine, 2,6-dihydroxypyridine, methylmyosmine) and with γ -N-methylaminobutyrate or γ -N-methylaminobutyrate breakdown products (γ -aminobutyrate, formaldehyde, succinic semialdehyde) for 5 min on ice before the radiolabeled DNA was added. Radioactive gels were analyzed by digital autoradiography by using the PhosphorImager technology (Amersham Biosciences).

DNase I-nuclease digestion experiments. The DNA binding site of PmfR was determined by performing DNase I-nuclease digestion experiments as described by Galas and Schmitz (16). An EcoRI restriction site was introduced by PCR with a degenerate primer (primer 17 [see the supplemental material]) into a 308-bp DNA fragment carrying the PmfR binding site. The fragment was ^{32}P end labeled as described above and digested overnight with EcoRI. The DNase I-generated fragments from the assay were electrophoresed on a 6% polyacrylamide–7 M urea denaturing gel along with a sequencing reaction mixture containing the undigested fragment and were visualized by autoradiography.

Determination of the transcriptional start site of the *purU-mabO-folD* operon. Primer extension analysis with total *A. nicotinovorans* pAO1 RNA was performed with a 5' [γ - ^{32}P]ATP-labeled oligonucleotide (primer 10 [see the supplemental material]) and a primer extension system-avian myeloblastosis virus reverse transcriptase kit from Promega (Madison, Wis.). A DNA sequencing reaction with the same primer and with fragment I (see the supplemental material and Fig. 4A) as the DNA template was performed with a Thermo Sequenase cycle sequencing kit used according to the instructions of the supplier (Amersham Biosciences), and the product was analyzed on a 6% polyacrylamide–7 M urea denaturing gel along with the product of the primer extension reaction.

Disruption of the *pmfR* gene of *A. nicotinovorans* pAO1. The cloning and expression vector pH6EX3 is unable to replicate in *A. nicotinovorans* and thus may be used as a DNA carrier in gene disruption experiments. pH6EX3*pmfR* was digested with restriction enzyme BglII, and the resulting overhangs were made blunt by treatment with the Klenow fragment of *E. coli* DNA polymerase I (New England BioLabs, Frankfurt, Germany), followed by digestion with SalI. This treatment excised a 316-bp fragment from *pmfR*, leaving 737 bp of *pmfR* to the 5' end and 390 bp to the 3' end. The DNA of the chloramphenicol resistance (*cmx*) gene (36) of pOKU9-cmB alpha (kindly provided by K.-H. Gartemann, Bielefeld, Germany) was excised with KpnI (blunt ended with the Klenow fragment) and SalI and was inserted into pH6EX3*pmfR* prepared as described above. Two micrograms of pH6EX3 carrying the *pmfR* gene disrupted by *cmx* was used to transform *A. nicotinovorans* pAO1 competent cells by electroporation (18). Chloramphenicol-resistant colonies were selected on NBYE plates (18) supplemented with 15 µg of chloramphenicol per ml following growth at 30°C for 48 h. The colonies were tested for homologous recombination by PCR amplification of DNA fragments by using primers 21 and 22 (see the supplemental material) specific for *cmx* and primers 23 and 24 (see the supplemental material), which flank the insertion sites of *cmx*, or by using primer 20, whose sequence is present only on the pAO1 plasmid flanking *pmfR*, and primer 24.

RESULTS

***mabO* gene and *purU*- and *folD*-like genes of pAO1 form a transcriptional unit.** Figure 1A shows a schematic diagram of the arrangement of ORF62 to ORF67 in the pAO1 DNA discussed here (22). The stop codon of ORF64 is separated from the translation start codon of ORF63 by 56 bp, and the stop codon of ORF63 is separated from the translation start codon of ORF62 by 60 bp. The genes carrying these ORFs may form a transcriptional unit. To examine this possibility, RT-PCR was performed with RNA extracted from *A. nicotinovorans* pAO1 grown in the presence of nicotine. The resulting cDNA was employed in PCR with primers 3 and 4, primers 5 and 6, and primers 7 and 8, as shown in Fig. 1A (see the supplemental material at <http://www.biochemie.uni-freiburg.de/brandsch/supplement.pdf>). Figure 1B shows the results. In the RT-PCR, primer pairs derived from ORF62 and ORF63 and from ORF63 and ORF64 resulted in amplification of a DNA fragment, which is consistent with the idea that the genes carrying these ORFs are transcribed as one mRNA molecule. As expected, primer pairs derived from the DNA carrying the divergently transcribed permease-like gene and the gene encoding the hypothetical transcriptional regulator failed to amplify a DNA fragment. Thus, we concluded that the pAO1 *purU*-, *mabO*-, and *folD*-like genes form a transcriptional unit.

The analysis of cDNA prepared from RNA extracted from *A. nicotinovorans* pAO1 grown in the absence of nicotine and amplified with primers 3 and 4 and with primers 5 and 6 (see the supplemental material), covering the *purU*-*mabO* and *mabO*-*folD* boundaries (Fig. 1A), showed that transcripts of the operon were not present in bacteria grown in the absence of nicotine (data not shown).

Determination of the transcriptional start site of the *purU*-*mabO*-*folD* operon. In order to define the transcriptional start site of the operon, we performed primer extension assays (Fig. 1C) with RNA extracted from nicotine-grown *A. nicotinovorans* pAO1. Primer 10 (see the supplemental material) starting 30 nucleotides downstream from the ATG of the *purU* gene was used. The ³²P-labeled product of the runoff reaction was analyzed along with the product of a sequencing reaction with the DNA fragment performed with the same primer used for the runoff assay (Fig. 1C). In this analysis, a strong signal

corresponding to an A residue 107 nucleotides upstream from the translation start codon ATG was identified as transcriptional start site 1. A putative σ⁷⁰ RNA polymerase -10 region with the sequence TATGGT (*E. coli* consensus sequence, TA TAAT) was proposed starting 10 nucleotides upstream from the A nucleotide of the transcriptional start. The sequence ATGGAA (*E. coli* consensus sequence, TTGACA), 18 nucleotides further upstream, may represent the -35 region of the promoter.

Cloning, expression, and purification of the putative regulatory protein of ORF67. The arrangement on pAO1 of ORFs analyzed in this study (Fig. 1A) suggests that ORF67 may represent the transcriptional regulator of the hypothetical permease gene and of the genes of the *purU*-*mabO*-*folD* gene cluster. Database searches revealed that the amino acid sequence encoded by ORF67 shows similarity to the sequences of several putative regulatory proteins present in recently sequenced bacterial genomes (2, 27, 28, 35), none of which has yet been experimentally associated with a function. The DNA fragment carrying ORF67 was cloned in the expression vector pH6EX3, and the protein was purified as a His₆-tagged protein by Ni-chelating chromatography. The N-terminal sequence of the fusion protein was MSPIHHHHHHLVPRGSEASG SRM (the underlined M was the translational start methionine of ORF67). As determined by SDS-PAGE, the protein had an apparent molecular mass of 50 kDa, which was close to the calculated molecular mass (50.5 kDa) (data not shown). BN-PAGE and gel permeation chromatography on Superdex-200 indicated that the protein formed oligomers in solution, probably tetramers, corresponding to a molecular mass of about 190 kDa (data not shown).

Interaction of the ORF67 protein with DNA. The ability of the purified protein to bind DNA was tested with PCR-amplified fragments derived from the regions 5' of *purU* (fragment I, 308 bp) and 5' of the permease gene (fragment VII, 304 bp) (Fig. 2A). Fragment I was subdivided into two overlapping fragments (fragment II [154 bp] and fragment III [178 bp]) by PCR amplification (for primers see the supplemental material). Fragments IV (78 bp) and V (100 bp) were generated from fragment III by digestion with RsaI. Fragment VI was generated by the annealing of two complementary 39-nucleotide primers (see the supplemental material) located at the middle of fragment III (39 bp). Fragments I, III, and VI showed an electrophoretic mobility shift (EMS) in the presence of the protein (Fig. 2B), whereas fragments II, IV, V and VII did not (data not shown). The absence of an EMS with fragment VII suggests that expression of the permease-like gene is not regulated by the ORF67 protein and does not belong to the operon formed by ORF62, ORF63, and ORF64. The protein-DNA interaction was specific, since in the presence of unlabeled DNA fragment I the shift was greatly reduced (Fig. 2D), but nonspecific DNA [salmon sperm or poly(dI-dC)] had no influence on the electrophoretic mobility shift (Fig. 2E). Titration of fragment I with increasing amounts of protein (Fig. 2C) allowed calculation of an apparent *K_d* (50% binding of PmfR to its recognition sequence) of 65 nM. From these results we concluded that the protein encoded by ORF67 is the transcriptional regulator (designated PmfR) of the *purU*-*mabO*-*folD* operon. Attempts to identify potential low-molecular-weight modulators of the DNA binding activity

FIG. 2. EMSA with PmfR. (A) Schematic diagram of DNA fragments used in EMSA. (B) EMSA with fragments I, IV, and VI in the absence of PmfR (lanes 1, 3, and 5) and in the presence of PmfR (lanes 2, 4, and 5). (C) Titration of DNA fragment I with PmfR protein. Lane 1, titration in the absence of protein; lanes 2 to 9, titration in the presence of increasing PmfR concentrations (0.15, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1 µg, respectively). (D) EMSA performed in the presence of unlabeled fragment I. Lane 1, no PmfR; lane 2, EMSA with 0.6 µg of PmfR and no competitor DNA; lanes 3 to 7, EMSA with PmfR and increasing concentrations of unlabeled fragment I (0.1, 0.25, 0.5, 0.75, and 1 µg, respectively). (E) EMSA performed in the presence of nonspecific competitor DNA. Lane 1, EMSA without PmfR; lane 2, EMSA with 0.6 µg of PmfR; lanes 3 to 6, EMSA with PmfR and increasing amounts of poly(dI-dC) (0.5, 1, 1.5, and 2.5 µg, respectively); lanes 7 to 10, EMSA with PmfR and increasing amounts of salmon sperm DNA (0.5, 1, 1.5, and 2.5 µg, respectively).

of PmfR derived from the degradation of nicotine and γ -N-methylaminobutyrate were unsuccessful (data not shown).

Determination of the PmfR-DNA contact sites by DNase I-nuclease digestion experiments. To determine the site of PmfR-DNA interaction, nuclease digestion experiments were performed with fragment I (5' *purU*) (Fig. 2A). The assay was analyzed in parallel with a sequencing reaction performed with the same DNA fragment (Fig. 3A). Several areas protected from DNase I-nuclease digestion in the presence of PmfR were detected in the DNA. The sequence is as follows: 5'-ATCAA AGTCGCAGTTTAGACCTTAGTACCAAAACCATGGTC TTTTTC-3' (the protected nucleotides are underlined, and the *RsaI* endonuclease recognition sequence is in boldface type). The double-stranded oligonucleotide VI (Fig. 2A) that was generated by annealing of complementary primers 15 and 16 (see the supplemental material) and was positive as determined by the EMSA (Fig. 2B, lane 6) contained the first four stretches of protected base pairs, confirming the protection pattern observed in the DNase I-nuclease digestion experiments. The PmfR protected DNA sequence determined by the DNase I-nuclease digestion spanned nucleotides -48 to -88 (Fig. 3B).

Identification of nucleotides in the PmfR recognition sequence that are important for PmfR binding. The DNase

I-nuclease digestion experiments revealed several PmfR protected regions on the DNA sequence between nucleotides -48 and -88 (see above). A DNA fragment containing this sequence shortened by the 3'-terminal sequence TCTTTT was still bound by PmfR in EMSA (fragment VI) (Fig. 2A). However, digestion of DNA fragments I, III, and VI (Fig. 2A) with *RsaI* abolished the EMS (data not shown), indicating that the partially palindromic sequences CAGTTTA and AAAACCA (Fig. 4A) protected by PmfR were important for PmfR binding. In order to define more precisely the importance of these sequences for the interaction of PmfR with its target DNA, we exchanged nucleotides in these sequences (Fig. 4A). The mutant oligonucleotides were then tested in an EMSA for PmfR binding at two protein concentrations (Fig. 4B). Replacement of two T residues with two G residues in the sequence of the first protected region reduced the amount of radiolabeled mutant oligonucleotide in the EMS complex to about 10% of the amount observed with the wild-type oligonucleotide, as judged from a densitometric evaluation of the intensity of the EMS bands on the exposed PhosphorImager plate (Fig. 4B, compare lanes *wt* and M1). Replacement of two A residues from the second protected sequence with C residues decreased the amount of mutant oligonucleotide bound in the EMS complex to about 84% of the amount observed with the wild-type oli-

FIG. 3. DNase I-nuclease digestion assay with PmfR and a DNA fragment 5' of *purU*. (A) DNase I-nuclease digestion assay performed with PmfR and fragment I as described in Materials and Methods. Lanes G, A, T, and C, sequencing reactions; lane 1, control without protein; lanes 2 to 8, reactions with increasing amounts of PmfR protein (0.05, 0.125, 0.25, 0.5, 1.25, 2.5, and 3.75 μ g, respectively). (B) Sequence of protected sites (in boldface type), the proposed promoter (–35 and –10 regions), the transcriptional start site (+1), and translation start codon ATG of the first gene (*purU*) of the *purU-mabO-fold* operon. The *RsaI* restriction enzyme recognition site is in italics.

gonucleotide, which was defined as 100% (Fig. 4B, lanes M2). The intensity of the EMS for the oligonucleotide with the nucleotide replacements at both sites decreased to 8% of the wild-type value (Fig. 4B, lanes M3). Transformation of the sequence GTTTAGA into the G-rich sequence AGGGGTC (Fig. 4B, lanes M4) reduced the amount of DNA bound in the EMS complex to almost background levels, and when 22 nucleotides were replaced with a random sequence (lanes M5), no EMS could be detected. PmfR apparently requires the TA-rich sequence GTTT-14 bp-AAAC for binding to its target DNA, as suggested by the results obtained with the mutated oligonucleotides.

Disruption of the *pmfR* gene revealed that PmfR acts as a transcriptional activator of the *purU-mabO-fold* operon. In order to determine the *in vivo* function of PmfR, we attempted

to inactivate its gene by disruption with a chloramphenicol resistance gene (*cmx*). Insertion of the chloramphenicol resistance cassette into the *pmfR* gene of pAO1 was verified by PCR. The use of primers specific for *cmx* (primers 21 and 22 [see the supplemental material]) resulted in amplification of a 1.15-kb *cmx* gene fragment only in the chloramphenicol-resistant *A. nicotinovorans* pAO1 transformants and not when bacteria carrying wild-type pAO1 were used as the template. With primers flanking the insertion sites of *cmx* (primers 23 and 24 [see the supplemental material]), a 0.57-kb fragment, corresponding to pAO1 DNA, was amplified in the wild type, and a 1.7-kb fragment, corresponding to pAO1 DNA plus the *cmx* gene DNA, was amplified in the mutant strain. Finally, PCR amplification with primer 20, whose sequence was present only on the pAO1 plasmid flanking *pmfR*, and primer 24 (see the

FIG. 4. Identification of nucleotides important for PmfR binding to its DNA target sequence. (A) PmfR DNA target sequence for wild-type *purU* oligonucleotide (see the supplemental material). wt, wild-type sequence. The PmfR protected regions are enclosed in boxes. M1 to M5 are mutant oligonucleotides with the nucleotide replacements indicated. (B) Lanes wt, EMSA performed with wild-type oligonucleotide in the absence of PmfR and in the presence of 0.3 and 0.6 μ g of PmfR; lanes M1 to M5, same as lanes wt but in the presence of the mutated oligonucleotides indicated in panel A.

supplemental material) resulted, as expected, in amplification of a 1.43-kb fragment for the wild-type strain and in amplification of a 2.6-kb fragment for the chloramphenicol-resistant *A. nicotinovorans* pAO1 transformants. From these results we concluded that *cmx* was successfully inserted into the *pmfR* gene of pAO1 by homologous recombination.

Bacterial extracts were prepared from *A. nicotinovorans* pAO1 and from the strain with the disrupted *pmfR* gene. As shown in Fig. 5A, a slightly more intense EMS signal was observed with 32 P-labeled DNA fragment I (upstream of *purU*) and an extract prepared from nicotine-grown bacteria than with extracts from bacteria grown in the absence of nicotine (compare lanes 2 and 3). The effect, although weak, may indicate that PmfR is a transcriptional activator. More importantly, no EMS was detected with extracts prepared from the *A. nicotinovorans* pAO1 strain carrying the disrupted *pmfR* gene (lanes 4 and 5), an indication that the translation of PmfR was blocked.

Disruption of the gene encoding a transcriptional activator should be reflected at the protein level by the lack of synthesis of the protein encoded by the gene controlled by the activator. We therefore performed Western blotting with extracts prepared from the wild type and from the *pmfR*-disrupted *A. nicotinovorans* strains and decorated them with MABO protein-specific antiserum. MABO protein was detected only in extracts of the nicotine-grown wild-type *A. nicotinovorans* pAO1 strain and not in bacterial extracts prepared from the strain carrying the inactivated *pmfR* gene (Fig. 5B).

Activation of transcription requires that the activator protein physically interacts with the RNA polymerase bound to

the promoter DNA (29, 31). A DNA fragment carrying the promoter and the recognition site of the activator should accommodate both proteins. The proposed promoter of the *purU-mabO-fold* operon resembles a sigma-70 promoter. Therefore, we tested the simultaneous binding of PmfR and *E. coli* sigma-70 RNA polymerase holoenzyme to DNA fragment I (which carried the PmfR binding site and the *purU-mabO-fold* promoter [Fig. 1A]) by EMSA. PmfR at the concentration used in the assay resulted in a significant electrophoretic mobility shift of the radioactive labeled DNA fragment (Fig. 5C, compare lanes 1 and 2). At a constant concentration of PmfR in the assay mixture and with increasing amounts of RNA polymerase, we observed an EMS at a higher molecular weight than the molecular weight when either PmfR or RNA polymerase was added individually to the assay mixture (Fig. 5C, lanes 4 to 7), which is consistent with simultaneous binding of the two proteins to the DNA fragment.

DISCUSSION

The results presented in this paper were used to identify a new operon in pAO1 that is functionally linked to the demethylation of γ -*N*-methylaminobutyrate generated during the catabolism of L-nicotine by *A. nicotinovorans* pAO1 (13, 19, 21). They indicate that the modular arrangement of operons in pAO1, with the *purU-mabO-fold* operon adjacent to the *nic* gene cluster, is not accidental but that in *A. nicotinovorans* pAO1 the gene products of the *purU-mabO-fold* operon are implicated in the nicotine catabolic pathway.

FIG. 5. Effects of *pmfR* disruption on expression of the *purU-mabO-fold* operon and the PmfR-RNA polymerase holoenzyme interaction. (A) EMSA performed with DNA fragment I (Fig. 1A) and 20 µg of extract of *A. nicotinovorans* pAO1 grown in the absence (lane 2) or in the presence (lane 3) of nicotine and with extract of the *pmfR*-disrupted *A. nicotinovorans* pAO1 strain grown in the absence (lane 4) or in the presence (lane 5) of nicotine. Lane 1 contained a control without extract. (B) Western blotting performed with 30 µg of extract of *A. nicotinovorans* pAO1 grown in the absence (lane 2) or in the presence (lane 3) of nicotine and with 30 µg of extract of the *pmfR*-disrupted *A. nicotinovorans* pAO1 strain grown in the absence (lane 4) or in the presence (lane 5) of nicotine, performed with MABO protein-specific antiserum. Lane 1 is a Western blot obtained with 10 ng of purified MABO protein. (C) EMSA with DNA fragment I (Fig. 1A) and PmfR in the presence of increasing amounts of *E. coli* sigma-70 RNA polymerase holoenzyme. Lane 1, no protein added; lane 2, EMS in the presence of 0.6 µg of PmfR; lane 3, EMS in the presence of 2 U of RNA polymerase; lanes 4 to 7, EMS in the presence of 0.6 µg of PmfR and 0.5, 0.75, 1, and 2 U of RNA polymerase, respectively. RNA Pol., RNA polymerase.

Expression of the *purU-mabO-fold* operon in bacteria grown in the presence of nicotine was found to be mediated by the transcriptional activator PmfR. Similar hypothetical transcriptional regulators have been revealed by the sequencing of bacterial genomes (2, 27, 28, 35). These regulators include the hypothetical protein PA1915 of *Pseudomonas aeruginosa* (accession number Q912I9; E-value, 6e-22), with 45% identity in a stretch of 111 N-terminal amino acids and 43% identity in a stretch of 88 C-terminal amino acids, and the putative transcriptional regulator SAV792 of *Streptomyces avermitilis* (accession number Q82PT0; E-value, 3e-16), with 45% identity in a stretch of 95 N-terminal amino acids and 37% identity in a stretch of 80 C-terminal amino acids (Fig. 6). PmfR is the first of these similar proteins that has been shown experimentally to exhibit DNA binding activity.

These transcriptional regulators appear to be organized into three functional domains. The very similar domains in the N-terminal and C-terminal parts of the proteins may be related to similar functions, whereas the dissimilar middle domains of the proteins may be connected to more specialized roles. Such a domain structure is reminiscent of the transcriptional regulators of the NtrC/XylR family (14, 17, 26, 30). Indeed, secondary structure prediction revealed a helix-turn-helix (HTH) motif in the C-terminal domain of these transcriptional regulators (Fig. 6C, PmfR sequence), which was identified by alignment with the amino acid sequence of the transcriptional activators Fis and XylR (4, 10) as a Fis-type HTH DNA-binding motif (Fig. 6C). PmfR forms oligomers in solution, as revealed by BN-PAGE. The N-terminal sequence of PmfR is leucine

rich (Fig. 6A), but it contains no typical leucine zipper motif (7). However, this part of the protein may be involved in oligomerization, since it was shown that leucine residues located in the N-terminal part of other transcriptional factors are important for dimerization (32).

In this work we defined the DNA recognition sequence of PmfR by performing DNase I-nuclease digestion experiments. The position of this sequence at nucleotides -48 to -88 from the transcriptional start site upstream from the promoter of the *purU-mabO-fold* operon is consistent with the assumption that PmfR acts as a transcriptional activator. Several lines of evidence support this conclusion. It has been shown previously that *A. nicotinovorans* pAO1 contains genes controlled by sigma-70 RNA polymerase-like promoters (22, 33). Promoters of genes regulated by transcriptional activators deviate considerably from the canonical -35 sequence (29), which also applies to the proposed promoter of the *purU-mabO-fold* operon.

The strongest support for the hypothesis that PmfR is a transcriptional activator comes from the gene disruption studies. To our knowledge, this is the first time that a system for gene-specific disruption by homologous recombination has been successfully established in an *Arthrobacter* strain. In the strain with *pmfR* disrupted by the *cmx* cassette, no synthesis of the MABO protein could be detected, as predicted for inactivation of the corresponding transcriptional activator. In addition, the EMSA performed simultaneously with both PmfR and sigma-70 RNA polymerase suggested that both proteins are accommodated on a DNA fragment carrying the recogni-

FIG. 6. Similarity of PmfR to putative transcriptional regulators and identification of an HTH motif in its C-terminal amino acid sequence. (A and B) N-terminal (A) and C-terminal (B) sequence alignments of PmfR, hypothetical protein PA1915 of *Pseudomonas aeruginosa* (database accession number Q91219), and hTF, a hypothetical transcription factor of *S. avermitilis* (database accession number Q82PTO). (C) Predicted HTH structure (underlined) in the C-terminal part of the PmfR amino acid sequence and alignment with the known Fis-type HTH motifs of XylR and Fis.

tion site for PmfR and the promoter of the *purU-mabO-fold* operon.

We recently characterized the first nicotine-responsive transcriptional regulator of *A. nicotinovorans* pAO1. This regulator acts as repressor of the 6-hydroxy-D-nicotine oxidase gene, and its DNA binding activity is modulated by the stereoisomers 6-hydroxy-L-nicotine and 6-hydroxy-D-nicotine (33). With PmfR there was no detectable in vitro effect of nicotine or γ -N-methylaminobutyrate or breakdown products derived from these compounds (6-hydroxy-L-nicotine, 6-hydroxy-pseudooxynicotine, dihydroxypyridine, γ -aminobutyrate, formaldehyde, succinic semialdehyde) on the DNA-binding ability of the protein in EMSA. Similarly, activators like MelR show no detectable in vitro effect of low-molecular-weight effectors on the interaction of the activator protein with its cognate nucleotide sequence (8, 9). The chemical nature of effector molecules which may modulate the PmfR activity is not known.

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