Characterization of HdonOR, the Transcriptional Repressor of the 6-Hydroxy-D-nicotine Oxidase Gene of *Arthrobacter nicotinovorans* pAO1, and its DNA-binding Activity in Response to L- and d-Nicotine Derivatives

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Utilization of L-nicotine as growth substrate by *Arthrobacter nicotinovorans* pAO1 starts with hydroxylation of the pyridine ring at C6. Next, the pyrrolidine ring is oxidized by 6-hydroxy-L-nicotine oxidase, which acts strictly stereo-specific on the L-enantiomer. Surprisingly, L-nicotine also induces the synthesis of a 6-hydroxy-D-nicotine-specific oxidase in the bacteria. Genes of nicotine-degrading enzymes are located on the catabolic plasmid pAO1. The pAO1 sequence revealed that the 6-hydroxy-D-nicotine oxidase gene is flanked by two open reading frames with a similarity to amino acid permeases and a divergently transcribed open reading frame with a similarity to proteins of the tetracycline repressor TetR family. Reverse transcription PCR and primer extension analysis of RNA transcripts isolated from *A. nicotinovorans* pAO1 indicated that the 6-hydroxy-D-nicotine oxidase gene represents a transcriptional unit. DNA electromobility shift assays established that the purified TetR-similar protein represents the 6-hydroxy-D-nicotine oxidase gene repressor HdnoR and binds to the 6-hydroxy-D-nicotine oxidase gene operator with a *Kd* of 21 nM. The enantiomers 6-hydroxy-D- and 6-hydroxy-L-nicotine acted *in vitro* as inducers. *In vivo* analysis of 6-hydroxy-D-nicotine oxidase gene transcripts from bacteria grown with L- and D-nicotine confirmed this conclusion. The poor discrimination by HdnoR between the 6-hydroxy-D- and 6-hydroxy-L-nicotine enantiomers explains the presence of the 6-hydroxy-D-nicotine-specific enzyme in bacteria grown on L-nicotine.

The Gram-positive soil bacterium *Arthrobacter nicotinovorans* pAO1, formerly known as *Arthrobacter oxidans* (1), has the metabolic ability to grow on the tobacco alkaloid nicotine (2). The main alkaloid produced by the tobacco plant is L-nicotine and, in the presence of this compound, the bacteria produce a nicotine dehydrogenase that hydroxylates C6 of the pyridine ring of nicotine (3, 4). A stereo-specific 6-hydroxy-L-nicotine oxidase, (6HLDNO), leads to the formation of N-methylaminopropyl-(6-hydroxyprpyridyl-3)-ketone (5). Surprisingly, L-nicotine also induces the synthesis of a 6-hydroxy-D-nicotine-specific oxidase (6HNO) (Refs. 6 and 7 and Fig. 1). When chemically synthesized D-nicotine was added to *A. nicotinovorans* pAO1 cultures, the same enzyme activities were induced. The induction of both stereo-specific enzymes, 6HLDNO and 6HNO, by either L- or D-nicotine was a long standing puzzle (3). It could be explained by the presence of an L-nicotine racemase, which produces the d-enantiomer. However, there was no evidence found for the presence of an L-nicotine racemase (3, 5, 8).

Genes of nicotine-degrading enzymes are situated on the catabolic plasmid pAO1 (9). It has been shown before that a protein present in *A. nicotinovorans* pAO1 extracts binds to an operator site consisting of two inverted repeats, namely IR1, covering the 6hdno promoter region, and IR2, situated upstream from the 6hdno promoter (10). However, the protein of the transcriptional regulator remained elusive and had not been identified. Recently, the position on pAO1 of genes of enzymes involved in nicotine catabolism by *A. nicotinovorans* has been determined (11). The gene of 6HNO was not part of this gene cluster. The sequence of pAO1 (12) revealed that 6hdno is positioned in close proximity to two open reading frames, ORF111 and ORF113, with high similarity to amino acid permeases, and to ORF114, with similarity to transcriptional regulators of the TetR family (PROSITE accession code PS01081; Refs. 13 and 14).

In this work we performed a transcriptional analysis of the 6hdno gene cluster and present evidence that 6hdno represents a transcriptional unit. We cloned and expressed the DNA carrying ORF114, purified the TetR-similar transcriptional regulator, and show that it represents the 6hdno repressor. Evidence is presented that 6-hydroxy-D-nicotine and 6-hydroxy-L-nicotine act as inducers of 6hdno expression. Induction of 6hdno expression by both the L- and the D-nicotine enantiomer can be explained by the poor discrimination by HdnoR between 6-hydroxy-L- and 6-hydroxy-D-nicotine.

**EXPERIMENTAL PROCEDURES**

Growth of *A. nicotinovorans* pAO1 and Preparation of Bacterial Extracts—*A. nicotinovorans* pAO1 was grown at 30 °C on citrate medium supplemented with trace elements and a vitamin solution (2). For enzyme assays and Western blots, the cultures were induced with 0.05% of different nicotine derivatives for 3 h at 30 °C. Bacterial pellets harvested from 100-ml cultures were re-suspended in 3 ml of 0.1 M phosphate buffer, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, and 5 mg/ml lysozyme. After 1 h of incubation on ice, the suspensions were passed three times through a French pressure cell at 132 megapascals, tetracycline repressor; EMSA, electromobility shift assay; IR1 or IR2, inverted repeat 1 or 2; RT, reverse transcription.
and the lysate was centrifuged for 30 min at 12,000 × g.

For total RNA isolation, a 5-ml *A. nicotinovorans* pAO1 culture, grown overnight in citrate medium at 30 °C, was induced with 0.05% of L-nicotine, D-nicotine, 6-hydroxy-L-nicotine, or 6-hydroxy-D-nicotine, and growth was continued for 3 h. The cultures were then frozen in liquid nitrogen to stabilize the RNA, melted again, and the bacteria were harvested by centrifugation at 4,000 × g for 10 min, re-suspended in 100 μl of 14 mg/ml lysozyme, and incubated at 28 °C for 3 h. The suspension was used for RNA isolation following the protocol of the supplier of the RNA isolation kit.

**Total RNA Isolation**—RNA was isolated from bacteria pre-treated as described above with the RNeasy kit (Qiagen, Hilden, Germany). Contaminating DNA was digested by “on column” DNase I treatment as described by the supplier. A second round of DNase I digestion was performed to remove traces of DNA as follows. 3 μg of RNA was incubated in a 15-μl assay with three units of RNase-free DNase I in
buffer supplied with the kit for 1 h at 30 °C. The DNase I was inactivated by the addition of 1 mM EDTA and incubation at 65 °C for 15 min.

**Reverse Transcription (RT)-PCR**—cDNA was prepared from 1 μg of total RNA with avian myeloblastoma virus reverse transcriptase (20 units/μg RNA; Amersham Biosciences) and a mixture of random hexamers in the presence of 1 unit of RNasin (Amersham Biosciences) and a mixture of random hexamers in the presence of 1 unit of RNasin (Amersham Biosciences) and 0.5 mM dithiothreitol, 0.1 mM dithiothreitol, and 0.1 M NaCl, and lysed by sonication at 4 °C.

### Primer Extension Analysis

**A. nicotinovorans** HDnoR was cloned from the genome of *A. nicotinovorans* strain CAM001 by PCR using the primers listed in Table I and labeled with [γ-32P]ATP using the Ready-to-Go T4 polynucleotide kinase kit (Amersham Biosciences). Binding of HDnoR to DNA was carried out in TBE buffer at a constant current of 30 mA for 1.5 h.

| Primer | Sequence | Length of DNA (bp) | Fragment |
|--------|----------|--------------------|----------|
| 1      | Forward 5'-ctttgctgacccctctccc-3' | 228 | Fragment I |
| 2      | Reverse 5'-cctgcttggttgcctacctac-3' | 535 | Fragment II |
| 3      | Forward 5'-cctgctgctgagcagagcagc-3' | 194 | Fragment III |
| 4      | Reverse 5'-gctttgctggttgcctacctac-3' | 1193 | Fragment IV |
| 5      | Forward 5'-ctctgctgacccctctccc-3' | 197 | Fragment V |
| 6      | Reverse 5'-gctttgctgacccctctccc-3' | 208 | Fragment VI |
| 7      | Forward 5'-ccctgctgacccctctccc-3' | 205 | Fragment 5' to ORF 111 |
| 8      | Reverse 5'-gctttgctgacccctctccc-3' | 105 | Fragment 5' to 6hdno carrying IR1 |
| 9      | Forward 5'-ccctgctgacccctctccc-3' | 191 | Fragment 5' to 6hdno carrying IR1 + IR2 |
| 10     | Reverse 5'-gctttgctgacccctctccc-3' | 107 | Fragment carrying IR2 |
| 11     | Forward 5'-gctttgctgacccctctccc-3' | 531 | Fragment 5' to ORF 113 |
| 12     | Reverse 5'-gctttgctgacccctctccc-3' | 662 | hdnoR (ORF114) |
| 13     | Forward 5'-gctttgctgacccctctccc-3' | 41 | Double stranded oligo carrying IR1 |
| 14     | Reverse 5'-gctttgctgacccctctccc-3' | 25 | Primer extension analysis |
| 15     | Forward 5'-gctttgctgacccctctccc-3' | 307 | Fragment VII |
| 16     | Reverse 5'-gctttgctgacccctctccc-3' | 112 | Fragment VIII |
| 17     | Forward 5'-gctttgctgacccctctccc-3' | 137 | Fragment IX |
| 18     | Reverse 5'-gctttgctgacccctctccc-3' | 189 | Fragment X |

**Overexpression and Purification of HDnoR**—An overnight culture of *E. coli* XL-1 blue harboring the pH6EX3-HdnoR was diluted 10 times in LB medium, supplemented with 50 μg/ml ampicillin, and induced for 3 h at 37 °C with 1 mM isopropyl-1-thio-β-d-galactopyranoside. The bacteria were harvested by centrifugation, re-suspended in 40 mM Hepes, pH 7.4, 0.5 mM NaCl, and lysed by sonication at 4 °C in a Branson sonifier J17V (scale adjustment 1). The lysate was centrifuged for 15 min at 12,000 × g, and the supernatant was used to purify the His6-HDnoR protein on Talon-Sepharose (Clontech). The protein was eluted at 0.5 M imidazole in 40 mM Hepes, pH 7.4, 0.5 M NaCl and revealed a single band in SDS-PAGE. The HDnoR protein fractions were concentrated by 50% ammonium sulfate precipitation and centrifugation at 4,000 × g for 5 min. The protein pellet was re-suspended in 10 mM Tris, 1 mM EDTA, 10 mM dithiothreitol, and 10% glycerol, and the concentration of the protein was adjusted to 0.1 μg/μl in the same buffer. Aliquots of the protein were frozen in liquid nitrogen and kept until use at −70 °C.

For electromobility shift assays (EMSAs), the protein was diluted at the required concentration as indicated in the legends to Figs. 3, 4, 6, and 7.

**Protein Cross-linking**—2 μg of HDnoR protein in a final volume of 25 μl of TBE buffer (89 mM Tris borate, pH 8.0, and 2 mM EDTA) were cross-linked with 1% formaldehyde (16) for 10 min at room temperature. The reaction was stopped by acetone precipitation, and the samples were analyzed by SDS-PAGE.

**Electromobility Shift Assay**—Protein DNA-binding assays were performed according to Ref. 17. DNA fragments employed in the EMSA were amplified by PCR using the primers listed in Table I and labeled with [γ-32P]ATP using the Ready-to-Go T4 polynucleotide kinase kit (Amersham Biosciences). Binding of HDnoR to DNA was carried out in 25 μl of a reaction mixture containing 0.3 ng DNA and various amounts of HDnoR in 10 mM Tris, pH 8, 50 mM KCN, 0.1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, and 5% glycerol. After incubation at room temperature for 20 min, loading buffer was added to a final concentration of 10% glycerol and 0.05% bromphenol blue, and the mixture was immediately applied to a 6% native polyacrylamide gel. Electrophoresis was carried out in TBE buffer at a constant current of 30 mA for 1.5 h. After drying, the gel was developed by incubation with a phosphorim-
aging plate. The effect of potential inducers on HdnoR DNA binding was tested by pre-incubation of the protein for 2 min with nicotine derivatives dissolved in H2O prior to adding the radiolabeled DNA. Incubation continued for 20 min at room temperature before the sample was loaded onto a polyacrylamide gel as described above.

Western Blotting—Cell extracts of A. nicotinovorans pAO1 induced with L- and D-nicotine enantiomers were separated by SDS-PAGE on a 12% polyacrylamide gel and blotted onto nitrocellulose membrane (Millipore, Bedford, Germany). Polyclonal antibodies raised in rabbit against A. nicotinovorans pAO1 6HDNO were used to detect the presence of the protein in a second antibody-bound, peroxidase-mediated color reaction.

Enzyme Assays—Extracts of noninduced, or L- or D-nicotine-induced A. nicotinovorans pAO1 grown on a citrate medium were prepared as described above, and 6HDNO activity was determined in the cleared lysates as outlined in Ref. 18.

RESULTS

Organization and Transcriptional Analysis of the 6HDNO Gene Region of pAO1—The 6HDNO gene of pAO1 is flanked by two ORFs, ORF111 and ORF113 (GenBank™ accession number AJ507836, 12), both with similarity to amino acid permeases. ORF111 is positioned 550 bp upstream of and divergently oriented to 6hdno, and ORF113 is positioned 505 bp downstream of 6hdno and oriented into the same direction (Fig. 2A). 81 bp downstream of ORF113 and in the opposite orientation is ORF114, with a similarity to transcriptional repressors of the TetR family. An analysis of transcripts generated from this DNA region was performed to establish whether 6hdno and the hypothetical gene of ORF113 form a transcriptional unit. RNA was isolated from A. nicotinovorans pAO1 grown in the presence of L-nicotine, DNase I-treated, and reverse transcribed into cDNA with the aid of a random hexanucleotide. The cDNA was then employed in PCR with specific primers (Table I) derived from regions I to VI, as indicated in Fig. 2A. Fig. 2B shows the results. Lanes marked M show a 1-kb DNA ladder as the molecular weight marker. Lanes 1, 4, 7, 10, 13, and 16 show the PCR products obtained with pAO1 DNA as template and primers amplifying fragments I, II, III, IV, V, and VI, respectively. This PCR control was positive with all primer pairs. Lanes 2, 5, 8, 11, 14, and 17 show the results of the negative control PCR with RNA as template, which were all negative and are proof that the RNA samples did not contain DNA. Lanes 3, 6, 9, 12, 15, and 18 show the PCR results with cDNA as template. Only primer pairs derived from coding regions of the ORFs amplified the expected DNA fragments in the PCR. No amplification product was obtained with primers devised to amplify region II of Fig. 2A, as was expected for an intergenic region. The absence of a PCR product with cDNA as template and primers devised to amplify region IV of Fig. 2A supports the conclusion that 6hdno and the gene carrying ORF113 do not form a transcriptional unit.

The transcriptional analysis also indicated that the genes of this pAO1 region are expressed in the presence of L-nicotine. PCR assays performed with cDNA derived from RNA prepared from A. nicotinovorans pAO1 grown without nicotine gave no amplification products (data not shown). Transcripts corresponding to ORF114 could be detected in the absence of nicotine at a level corresponding to that shown in lane 18 of Fig. 2B.

Fig. 3. Purification and DNA-binding activity of the ORF114 protein. Panel A, alignment of the N-terminal amino acid sequence of ORF114 with the amino acid sequence of TetR family repressors. Underlined is the amino acid sequence predicted to form a helix-turn-helix domain of the protein. Panel B, lane 1, protein molecular weight (Mr) standard; lane 2, purified ORF114 protein; and lane 3, dimers of the protein following formaldehyde cross-linking. Panel C, EMS assays with 32P-labeled DNA-fragments a, b, and c (see Fig. 2A and Table I); minus sign (−), no protein; plus sign (+), with protein added to the assays. Panel D, the specificity of interaction of HdnoR (0.5 μM) with 32P-labeled DNA fragment b (30 ng) was tested by competition with 1 μg (lane 2), 500 ng (lane 3), and 100 ng (lane 4) of unlabeled DNA fragment b and with 2.5 μg (lane 7), 1.2 μg (lane 8), and 0.25 μg (lane 9) of salmon sperm DNA. Lanes 1 and 6, 30 ng of 32P-labeled fragment only; lanes 5 and 10, EMSA in the presence of HdnoR only.
Expression and Purification of the ORF114 Protein

The hypothetical protein of ORF114, with a predicted molecular weight of 21,934, typical of TetR family repressors (PROSITE accession code PS01081), contains a predicted helix-turn-helix (HTH) motif at its N-terminal amino acid sequence (Fig. 3A) and shows the highest similarity to hypothetical repressor proteins from *Streptomyces coelicolor* (21.1 kDa protein EbrA, 26% identity in 167 amino acids, AN Q9X9V5), *Actinosynnema pretiosum* (transcriptional regulator Asm29, 24% identity in 197 amino acids, AN Q8KUH9), and the regulatory protein AcrR from *Proteus mirabilis* (21% identity in 195 amino acids, AN Q8VPBO0).

The DNA carrying ORF114 was inserted into pH6EX3 and expressed in *E. coli* as a His<sub>6</sub>-tagged protein. Thus, a fusion protein was generated with the N-terminal amino acid sequence MSPIHIIIIIIIIHLVPRGSKL<sub>L</sub>, with L corresponding to the UUG translation start of ORF114. The protein was purified, and the formation of dimers was determined by cross-linking with formaldehyde. Fig. 3B shows the purified protein (lane 2) and the cross-link product migrating at the size of a homodimer (lane 3). The purified protein was tested for DNA binding activity in EMSA with DNA fragments a, b, and c shown in Fig. 2A. Only fragment b, which carries the 6hdno promoter, gave a band shift in the presence of the protein (Fig. 3C). The specificity of the protein-DNA complex formed was evaluated in EMSA in the presence of competing unlabeled fragment b and salmon sperm DNA, respectively (Fig. 3D). Only the unlabeled fragment b competed with the labeled fragment for DNA binding, but not the unspecific salmon sperm DNA. Thus, ORF114 represents the gene of the transcriptional regulator of the 6HDNO gene, and the protein was named the 6hdno repressor (HdnoR) accordingly.

Interaction of HdnoR with IR1 and IR2 of the 6hdno Operator DNA

A schematic representation of the 5′-DNA region of 6hdno is presented in Fig. 4A. The transcriptional start site (+1), situated 51 nucleotides upstream of the translation start codon UUG, and the −10 and −35 elements of the 6hdno
The two inverted repeats, IR1 and IR2, were shown by DNase I protection assays to be the recognition sites of a DNA-binding protein present in crude extracts and (NH₄)₂SO₄ fractions of *A. nicotinovorans* (10). This DNA-binding protein was present in nicotine-noninduced and -induced bacterial extracts but did not react to the presence of L-nicotine when tested in vitro in an EMSA. When a DNA-fragment of 191 bp (Table I) carrying the two inverted repeats was employed in EMSA with purified HdnoR, the protein did bind concentration-dependently, first to one site (middle band in Fig. 4B, 20 nM) and then to both sites (Fig. 4B, 20 nM), an indication that binding of HdnoR to one site may stimulate binding of the protein to the second site and that binding of HdnoR to both sites was co-operative (10). Titration of the 105-bp DNA encompassing IR1 and the 107 bp DNA fragment encompassing IR2, respectively, with HdnoR (Fig. 4, C and D) allowed the determination of a *Kₐ* (50% binding of HdnoR to its recognition sequence) of −20 nM for the protein-DNA interaction at IR1 and IR2, respectively. When a 41-bp double-stranded oligonucleotide (Table I, number 13) with the 37-bp sequence of IR1 at its center was tested in EMSA for HdnoR-binding, the same results as with the 105-bp fragment (Fig. 4C) were obtained (not shown).

**Fig. 5. Determination of the transcriptional start site of the permease gene carrying ORF111 by primer extension and RT-PCR.** Panel A, a schematic representation of the divergently transcribed permease and 6hdno genes, indicates fragment VII (see Table I) amplified for the sequencing reaction performed for the identification of the transcriptional start site of the permease gene, the +1 nucleotide of the transcript, the putative −10 and −35 regions of a proposed promoter of the permease gene, and a putative operator site. ORF111 starts with TTG. Also indicated is the transcriptional start of 6hdno. Panel B gives the result of the primer extension analysis. Lanes G, A, T, and C show the sequencing reaction of fragment VII; lanes 1, 2, and 3 are three independent primer extension reactions; lane 4, control reaction in the absence of RNA. The arrow indicates the fragment obtained by primer extension, and the asterisk indicates the first nucleotide of the transcript. Panel C, lanes 1, 3, and 5 show PCR reactions with primers 16, 17, and 18 and pAO1 DNA as template amplifying fragments VIII, IX, and X, respectively (see Table I); lanes 2, 4, and 6 show the results of RT-PCR with the primers 16, 17, and 18, respectively; lane 7 shows control RT-PCR in the absence of RNA; M, 100-bp DNA ladder. The amount of template and primers was the same in all reactions.

The promoter were established previously (20). The two inverted repeats, IR1 and IR2, were shown by DNase I protection assays to be the recognition sites of a DNA-binding protein present in crude extracts and (NH₄)₂SO₄ fractions of *A. nicotinovorans* (10). This DNA-binding protein was present in nicotine-noninduced and -induced bacterial extracts but did not react to the presence of L-nicotine when tested in vitro in an EMSA. When a DNA-fragment of 191 bp (Table I) carrying the two inverted repeats was employed in EMSA with purified HdnoR, the protein did bind concentration-dependently, first to one site (middle band in Fig. 4B, 20 nM) and then to both sites (Fig. 4B, 20 nM), an indication that binding of HdnoR to one site may stimulate binding of the protein to the second site and that binding of HdnoR to both sites was co-operative (10). Titration of the 105-bp DNA encompassing IR1 and the 107 bp DNA fragment encompassing IR2, respectively, with HdnoR (Fig. 4, C and D) allowed the determination of a *Kₐ* (50% binding of HdnoR to its recognition sequence) of −20 nM for the protein-DNA interaction at IR1 and IR2, respectively. When a 41-bp double-stranded oligonucleotide (Table I, number 13) with the 37-bp sequence of IR1 at its center was tested in EMSA for HdnoR-binding, the same results as with the 105-bp fragment (Fig. 4C) were obtained (not shown).
The first six nucleotides of IR2 (Fig. 4A) read on the complementary strand TTGTCA, which represents a reasonable −35 region, and are followed 17 nucleotides downstream by the sequence AATGAT, a possible −10 region. Therefore the transcriptional start site of the gene carrying ORF111 was determined by primer extension analysis. It revealed one strong signal as a potential transcriptional start site (Fig. 5) 85 bp upstream of the proposed translation start site of ORF111 (12). Weaker signals corresponding to shorter primer extension products may represent cDNAs generated from processed RNA molecules or premature termination products of the reverse transcriptase reaction. The strong termination signal of the primer extension reaction may represent a genuine transcriptional start site, because secondary structure predictions of a hypothetical RNA transcript upstream and downstream from the proposed transcriptional start site showed neither hairpin loop structures, which may act as potential stop sites for the reverse transcriptase, nor an unusually high GC content of the

![Diagram](image)
lanes 0, increasing inducer concentrations as indicated.

Thus, 6-hydroxy-nicotine may be regarded as the compound found in cultures grown in the presence of L-nicotine (Fig. 8, panel A) and with the 6H\textsubscript{D}NO protein levels revealed on Western blots (W-Blot) of A. nicotinovorans pAO1 extracts decorated with 6H\textsubscript{D}NO-specific antiserum. A. nicotinovorans pAO1 extracts were prepared from non-induced (n.i.), L-nicotine induced (L), 6-hydroxy-D-nicotine induced (D-OH), and \n\nsequence. The palindromic sequence 5’-CTCCCCGGGAG (Fig. 5, panel A), which may represent a potential binding site for a transcriptional regulator, is positioned 6 bp upstream of the proposed −35 promoter region.

The primer extension analysis was corroborated by the results of RT-PCR reactions performed with primer pairs covering the proposed 5’-end of the RNA transcript (Fig. 5, panel A) and primer pairs with one of the primes downstream of the +1 transcriptional start site (Fig. 5, panel A). Only primer 14, in combination with primer 16, gave an amplification product in RT-PCR (Fig. 5, panel C).

The Effect of Nicotine Derivatives on HdnOR Binding to IR1—The interaction of the protein with IR1, which covers the 6hdno promoter, was tested by EMSA in the presence of various nicotine-derived compounds (Fig. 6A). Only 6-hydroxy-D-nicotine and 6-hydroxy-L-nicotine prevented HdnOR from binding to the IR1 DNA (Fig. 6B).

The effect of 6-hydroxy-D-, 6-hydroxy-L-, and L-nicotine on HdnOR/DNA complex formation was analyzed in greater detail at 20 μM HdnOR, which gave half-maximal binding to IR1 (Fig. 7, panels A–C). Both 6-hydroxy-nicotine enantiomers prevented DNA-protein complex formation at μM concentrations, with complete inhibition at 50 μM 6-hydroxy-D-nicotine and 100 μM 6-hydroxy-L-nicotine, respectively. A thousand-fold higher L-nicotine concentration was required to elicit a similar effect.

Thus, 6-hydroxy-nicotine may be regarded as the compound active in 6hdno induction, with the L-enantiomer being twice as potent as the D-enantiomer.

The Effect of L-Nicotine, D-Nicotine, and 6-Hydroxy-L-nicotine in Vivo on 6HDNO Activity, Protein Level, and 6hdno Transcripts—A. nicotinovorans pAO1 cultures were grown in the presence of 0.05% L-nicotine, D-nicotine, or 6-hydroxy-D-nicotine, respectively, and 6HDNO activity in the bacterial extracts was determined. The highest specific activity was found in cultures grown with 6-hydroxy-D-nicotine, and the lowest was found in cultures grown in the presence of L-nicotine (Fig. 8A).

The enzyme activity levels correlated with the observed 6hdno transcript levels, which were lower in L-nicotine-grown bacteria and higher in 6-hydroxy-D-nicotine- and D-nicotine-grown bacteria (Fig. 8B), and with the 6HDNO protein levels on Western blots (Fig. 8C).

DISCUSSION

The experimental data presented in this paper demonstrate that the protein encoded by ORF114 of pAO1 represents the transcriptional repressor of 6hdno. The HdnOR protein shows all the characteristics of a repressor of the TetR family. Its predicted molecular mass of 21 kDa, the predicted helix-turn-helix motif at the initial third of the protein, and the formation of dimers are typical for these regulatory proteins. The DNA sequence of IR1 and IR2 protected by the protein was determined by S1mapping previously with a (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} fraction containing the transcriptional regulator, which has been reported previously (10), may be explained by the finding that, in EMSA solely, the 6-hydroxy-nicotine derivatives acted as inducers.

Expression of hdnOR seemed to be constitutive, because specific transcripts could be detected in both noninduced and nicotine-induced bacteria. This finding supports the conclusion that the genes code for a repressor. There are no HdnOR binding sites present on the DNA upstream of the repressor gene. Therefore, the repressor seems not to auto-regulate the transcription of its gene. Inspection of the 5’-regions of the ndh, kdh, and dhph (11) genes revealed no HdnOR binding site. A search for the core palindromic sequence of IR1 and IR2 revealed no additional consensus sequences on the pAO1 DNA.

Apparently, there is a complex regulation of genes involved in nicotine utilization, and one may assume that there are additional transcription factors in charge of regulating expression of the ndh-6hdno, kdh and dhph operons.

HdnOR is the first nicotine-responsive transcriptional regu-
labor of genes belonging to the pAO1-encoded nicotine regulon that has been characterized. The repressor discriminates poorly between 6-hydroxy-L-nicotine and 6-hydroxy-L-nicotine as inducers, which explains the surprising finding, made many years ago, that a strictly stereo-specific 6-hydroxy-D-nicotine oxidase is induced by L-nicotine (3, 5). From the effect of various nicotine derivatives on the formation of the HdnOR-DNA complex, one may conclude that the L- or D-position of the pyrrolidine ring of nicotine is not as important for binding to the repressor as is the presence of the hydroxyl group at C6 of the pyridine ring. L- and D-nicotine, N-methyl-myosmine, 6- and 2-amino-L-nicotine, or 6-hydroxy-pyridine did not prevent HdnOR DNA binding. Because 6-hydroxy-pyridine had no effect, the pyrrolidine ring of nicotine seems to be required for its interaction with HdnOR.

6hdno is not co-transcribed with the permease-similar gene located downstream of it but appears to represent a transcriptional unit by itself. However, the genes of the two permeases appear to belong to a nicotine regulon, because transcripts of both of these genes can be detected only in nicotine-induced bacteria. 6hdno and the divergently transcribed permease gene may form a functional unit, with the permease responsible for uptake of the compound that serves as substrate for the enzyme HdnOR. However, as suggested by the primer extension analysis and the RT-PCR results, they seem not to form a transcriptional unit regulated by HdnOR. There are no hints yet available as to the function of these hypothetical permeases. It is tempting to assume a role in the transport of nicotine or nicotine derivatives in and/or out of the bacterial cell. Efforts to inactivate the permease genes in A. nicotinovorans pAO1 by homologous recombination with an antibiotic cassette have, to date, failed, as did the heterologous expression of the permease genes in E. coli.

A. nicotinovorans pAO1 contains promoters resembling, in principle, a35 E. coli promoters. The 6hdno promoter situated at IR1 exhibits a consensus – 35 TTGACA sequence separated by 16 bp from the sequence TATACT, which is very similar to the consensus – 10 sequence TATAAT, with one Cys residue inserted (Fig. 4, panel A). Whether the proposed promoter (Fig. 5, panel A), which resembles the 6hdno promoter, is indeed the promoter of the permease gene has to be proven in functional tests by fusion with an indicator gene. The accumulation of C residues at promoter sites, as suggested here for pAO1 promoters, has been observed in the case of other bacteria with a high GC content, like Mycobacterium tuberculosis (20). Consensus sequences typical for promoters regulated by alternative σ factors have not been detected by inspection of the intergenic regions of the 6hdno and permease genes.

The expression pattern of 6hdno in vivo in the presence of L-, D-, or 6-hydroxy-D-nicotine may be explained by the interaction of the HdnOR protein with these nicotine derivatives. L-nicotine induces the expression of both ndh and 6hdno, and, thus, L-nicotine is turned over into N-methylaminopropyl(6-hydroxypyridil-3)ketone, giving no time to 6-hydroxy-L-nicotine to accumulate and interact with HdnOR. This results in a low level of 6hdno expression. When turnover of L-nicotine slows down, possibly because of feedback inhibition of the pathway enzymes by end products, more 6-hydroxy-L-nicotine may accumulate, resulting in a delayed increase of 6hdno expression as has been observed in A. nicotinovorans pAO1 cultures grown with L-nicotine (3, 5).

In the presence of D-nicotine, D-nicotine is turned over into 6-hydroxy-D-nicotine by NDH, which does not discriminate between the L- and D-nicotine enantiomers (3, 5). The 6-hydroxy-D-nicotine formed, however, is no substrate for 6HlNO. Therefore, 6-hydroxy-D-nicotine accumulates, leading to a higher expression of 6hdno.

From the results presented in this study, one may assume that 6-hydroxy-D-nicotine represents the natural inducer of 6hdno expression. However, the question remains. Is D-nicotine found in nature? There are reports that L-nornicotine, a side product of nicotine biosynthesis by the tobacco plant, was racemized into the D-enantiomer in leaves of the plant (21). It has also been shown that, during cigarette smoking, L-nicotine is racemized into its enantiomer (22). Possibly, D-nicotine or D-nornicotine is formed during the decay of the tobacco plant in the soil. In this case, 6HDNO would be an essential enzyme, required for the biodegradation of these compounds, and regulation of the expression of its gene by HdnOR and 6-hydroxy-D-nicotine may reflect this fact. However, the possibility cannot be excluded that the natural inducer of 6hdno and the natural substrate of 6HDNO is a molecule that just resembles 6-hydroxy-D-nicotine.

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