Nucleotide sequence and over-expression of morphine dehydrogenase, a plasmid-encoded gene from *Pseudomonas putida* M10

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Pseudomonas putida M10 was originally isolated from factory waste liquors by selection for growth on morphine. The $NADP^+$-dependent morphine dehydrogenase that initiates morphine catabolism is encoded by a large plasmid of 165 kb. Treatment of *P. putida* M10 with ethidium bromide led to the isolation of a putative plasmid-free strain that was incapable of growth on morphine. The structural gene for morphine dehydrogenase, *morA*, has been located on the plasmid by oligonucleotide hybridization, by coupled transcription–translation of cloned restriction fragments and by nucleotide sequence analysis and is contained within a 1.7 kb *Sph*I fragment that has been cloned into *Escherichia coli*. The cloned dehydrogenase enzyme is expressed at high levels in *E. coli* resulting in a 65-fold increase in morphine dehydrogenase activity in cell-free extracts compared with *P. putida* M10. Morphine dehydrogenase was rapidly purified to homogeneity, as judged by SDS/PAGE, by a one-step affinity chromatography procedure on Mimetic Orange 3 A6XL. The properties of the purified enzyme were identical with those previously reported for *P. putida* M10 morphine dehydrogenase. The *morA* gene was sequenced and the deduced amino acid sequence confirmed by N-terminal amino acid sequencing of the over-expressed protein. The predicted amino acid sequence of *morA*, deduced from the nucleotide sequence, indicated that morphine dehydrogenase did not belong to the non-metal-requiring short-chain class of dehydrogenases, but was more closely related to the aldo-ketoreductases.

INTRODUCTION

Bacteria which degrade plant alkaloids are important in the synthesis of compounds with pharmaceutical properties. The initial steps in the metabolism of the morphine alkaloids in *P. putida* M10 have potential applications in the biological synthesis of analgesic compounds and narcotic antagonists (Bruce et al., 1990), since synthetic routes for many of these compounds are not entirely satisfactory and precursors are often in limited supply. An understanding of the structural and regulatory genes involved in the metabolism of morphine is necessary before genetic manipulation to engineer pathways for the synthesis of analgesics and narcotic antagonists in bacteria can be attempted. The $NADP^+$-dependent morphine dehydrogenase (MDH) has been shown to initiate morphine catabolism in *P. putida* M10 and catalyses the oxidation of morphine and codeine to morphinone and codeinone, respectively (Bruce et al., 1990). MDH has been purified to homogeneity and extensively characterized previously (Bruce et al., 1991). The enzyme is highly specific for its alkaloid substrate, oxidizing only the C-6 hydroxy group of morphine and closely related analogues. It consists of a single polypeptide unit which has an M_r of 32000. In this paper we show that MDH is encoded on a large plasmid in *P. putida* M10 and we report on the cloning and over-expression of MDH. The complete nucleotide sequence of *morA*, the structural gene for MDH, permits a first primary structure comparison with the aldo-ketoreductases.

MATERIALS AND METHODS

Chemicals, enzyme and isotopes

Mimetic Orange 3 A6XL was obtained from Affinity Chromatography Ltd. (Freeport, Ballasalla, Isle of Man, U.K.). Morphine and codeine were purchased from Macfarlan Smith Ltd. (Edinburgh, Scotland, U.K.). $NADP^+$ and protein A-Sepharose were obtained from Sigma (Poole, Dorset, U.K.). Restriction enzymes *Bam*HI, *Sac*I and *Xho*I were from Pharmacia (Milton Keynes, Bucks, U.K.), *Bgl*II, *Eco*RI, *Hin*dIII and *Sal*I from Boehringer Mannheim (Lewes, U.K.), and *Sph*I from New England Biolabs (Bishops Stortford, U.K.). Isopropyl β-thiogalactoside, 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) and T4 DNA ligase were purchased from Boehringer Mannheim. The prokaryotic DNA-directed translation system, Hybond-N^+ membrane and L-[^{35}S]methionine were from Amersham International p.l.c. (Amersham, U.K.); [α-^{35}S]dATP and [γ-^{32}P]ATP were from Du Pont (U.K.) Ltd. (Stevenage, U.K.). T4 polynucleotide kinase was from New England Biolabs. All reagents were of the highest grade that could be obtained commercially.

Bacterial strains, plasmids and growth conditions

P. putida M10 was originally isolated from industrial waste liquors (Bruce et al., 1990). *E. coli* DH5α (Hanahan, 1983) was used as the host for all recombinant plasmids. The plasmid pTZ19R (United States Biochemical Corporation, Cambridge Bioscience, Cambridge, U.K.) was used to construct clones and subclones. Cultures were grown on Luria broth with 100 μg of ampicillin/ml or on minimal salts medium supplemented with glucose and morphine, as described previously (Bruce et al., 1990).

Plasmid construction

Plasmid DNA was extracted from *P. putida* M10 and purified by alkaline lysis (Morelle, 1989). Restriction fragments of *P. putida*

Abbreviations used: MDH, morphine dehydrogenase, X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; SSPE, 0.9 M NaCl, 50 mM NaH_2PO_4 and 5 mM EDTA (pH 7.7).

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The nucleotide sequence data reported have been submitted to the GenBank and EMBL databases under accession number M94775.

M10 plasmid DNA were separated by agarose gel electrophoresis and the DNA was recovered by binding and elution from Whatman DE81 paper (Dretzen et al., 1981). The purified restriction fragments were inserted into pTZ19R which had been cut with the appropriate restriction enzyme and used to transform *E. coli* strain DH5α by electroporation. Transformants were selected by antibiotic resistance and by screening on X-gal plates. Ten transformants were picked, plasmid DNA was extracted and analysed by restriction enzyme digestion and Southern hybridization (Maniatis et al., 1982).

Oligonucleotide hybridization

An oligonucleotide mixture ($AA^C/_TAA^C/_TGG^C/_TGT^G/_CAA^G/_AATGCC$) was synthesized based on the sequence Asn-Asn-Gly-Val-Lys-Met-Pro from the N-terminal amino acid sequence of MDH from *P. putida* M10 (Bruce et al., 1991). The oligonucleotide mixture was end-labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP.

Restriction fragments of *P. putida* M10 plasmid DNA were electrophoresed in 0.75% (w/v) agarose gels and transferred to a Hybond-N$^+$ membrane using the alkaline method, exactly as described by the suppliers (Amersham International). The filters were prehybridized at 65 °C for 16 h in 5 × SSPE (0.9 M NaCl, 50 mM NaH_2PO_4 and 5 mM EDTA; pH 7.7), 5 × Denhardt's solution, 50 μg/ml salmon sperm DNA and 0.5% (w/v) SDS. Hybridization with labelled oligonucleotides was carried out at 35 °C for 16 h in prehybridization buffer. The filters were washed with four changes of 5 × SSPE and 0.1% (w/v) SDS at 45 °C for 40 min and exposed to Fuji RX film with intensifying screens.

In vitro coupled transcription–translation of DNA

Coupled transcription–translation of DNA was carried out using a prokaryotic DNA-directed translation kit (Amersham International), exactly as described by the manufacturer. [^{35}S]Methionine-labelled products were identified by immunoprecipitation with antibodies to MDH and to protein A-Sepharose (Howe et al., 1982). Polypeptides were analysed by SDS/PAGE and autoradiography using β-max film (Amersham International).

Sequencing techniques

The N-terminal sequence of MDH was determined by automated Edman degradation with an Applied Biosystems 470A sequencer, by the Protein and Nucleic Acid Chemistry Facility, Cambridge Centre for Molecular Recognition, Department of Biochemistry, University of Cambridge, U.K. Plasmid DNA was sequenced by the dideoxy chain-termination method (Sanger et al., 1977) using modified T7 polymerase (Sequenase version 2.0 DNA polymerase; Cambridge Bioscience, Cambridge, U.K.) and [α-^{35}S]dATP, as described in the Sequenase protocol, using synthetic oligonucleotides based on the previously determined nucleotide sequence as primers. Electrophoresis was on 6% (w/v) acrylamide/8 M urea sequencing gels (0.2 mm × 20 cm × 40 cm), at 1500 V. After electrophoresis the gels were fixed in 10% (v/v) acetic acid and 10% (v/v) methanol for 20 min, dried at 70 °C on Whatman 3MM paper using a BioRad 583 gel dryer and exposed to Fuji RX film. Sequence data were compiled and analysed with the GCG computer programs (Devereux et al., 1984). Sequence comparisons were made using FASTA and ALIGN (Pearson and Lipman, 1988).

Morphine dehydrogenase assay

Morphine dehydrogenase activity was determined as described by Bruce et al. (1991).

Purification of morphine dehydrogenase

All procedures were performed at 4 °C. A crude extract was prepared from 0.1 g (wet weight) of *E. coli* DH5α transformed with pMdh1.7. The cells were resuspended in buffer A (50 mM KH_2PO_4/NaOH buffer, pH 7.0, containing 1 mM dithiothreitol) at a concentration of 0.1 g (wet weight)/1.0 ml of buffer and disrupted by sonication in a Soniprep MSE Ultrasonic Disintegrator at an amplitude of 12 μm. The sonicated cell suspension was centrifuged at 13000 *g* for 10 min to remove the cell debris. The cell-free extract was then applied to a Mimetic Orange 3 A6XL column (2 cm × 1 cm) that had been previously equilibrated with buffer A. After adsorption, the column was washed extensively with buffer A containing 0.25 M KCl until no further absorbance at 280 nm was evident in the eluate. The enzyme was eluted batch-wise, in the reverse direction, with buffer A containing 0.8 M KCl. Fractions of 0.8 ml were collected at a flow rate of 4 ml cm^{-2} h^{-1}.

The protein concentration was measured in solution by the method of Bradford (1976), using bovine serum albumin as the standard.

SDS/PAGE

SDS/PAGE analyses were carried out by the method of Laemmli (1970). Vertical slab gels (0.75 mm × 75 mm × 80 mm) containing 12.5% (w/v) acrylamide were run at a constant voltage of 200 V. Protein was detected by staining the gels with 0.1% (w/v) Coomassie Blue R-250, dissolved in methanol/water/acetic acid (4:5:1, by vol.). Gels were destained by repeatedly washing in the above solvent mixture. The M_r of the purified enzyme was determined by using BioRad (Watford, Herts., U.K.) SDS/PAGE low molecular mass standards.

Immunological techniques

Antibodies against purified MDH were prepared by injecting a rabbit subcutaneously at multiple sites with 150 μg of enzyme in complete Freund's adjuvant. A second intramuscular injection of 50 μg of enzyme was given 6 weeks later in incomplete Freund's adjuvant. Serum taken 14 days after the second injection was positive for anti-MDH activity in an e.l.i.s.a.

Plasmid curing experiments

Cured strains of *P. putida* M10 were obtained by growing cultures in 3 ml of Luria broth containing 100 μg of ethidium bromide/ml at 30 °C. After 24 h of growth, 50 μl of the cell suspension was subcultured under the same conditions. After repeating this process three times 0.5 ml of cell suspension was serially diluted and plated onto minimal media agar containing 5 mM morphine and 5 mM glucose. Morphine-grown cells of *P. putida* M10 generally turned brown, thus normal coloured (non-brown) colonies were picked and assayed for MDH activity. This procedure led to the isolation of putative cured strains.

RESULTS

Evidence for the existence of a catabolic plasmid

Agarose gel electrophoresis of purified plasmid preparations from *P. putida* M10 revealed the presence of a natural plasmid of high molecular mass. The first indication that MDH was encoded on this plasmid was the apparent loss of the plasmid from *P. putida* M10 after curing with ethidium bromide, which resulted in the organism being unable to use morphine as its sole carbon

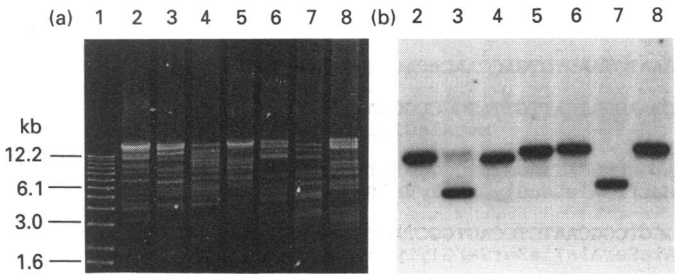

Figure 1 Localization of the *morA* gene on the *P. putida* M10 (pMor) plasmid

(**a**) Agarose gel electrophoresis of restriction enzyme digests of plasmid DNA from *P. putida* M10. (**b**) Autoradiograph of the Hybond-N$^+$ membrane after blotting and hybridization with a radiolabelled mixed 20mer oligonucleotide based upon the amino acid sequence of the N-terminus of purified MDH. In (**a**) and (**b**), lane 1, markers; lane 2, *Bam*HI; lane 3, *Bgl*II; lane 4, *Eco*RI; lane 5, *Hin*dIII; lane 6, *Sac*I; lane 7, *Sal*I; lane 8, *Xho*I.

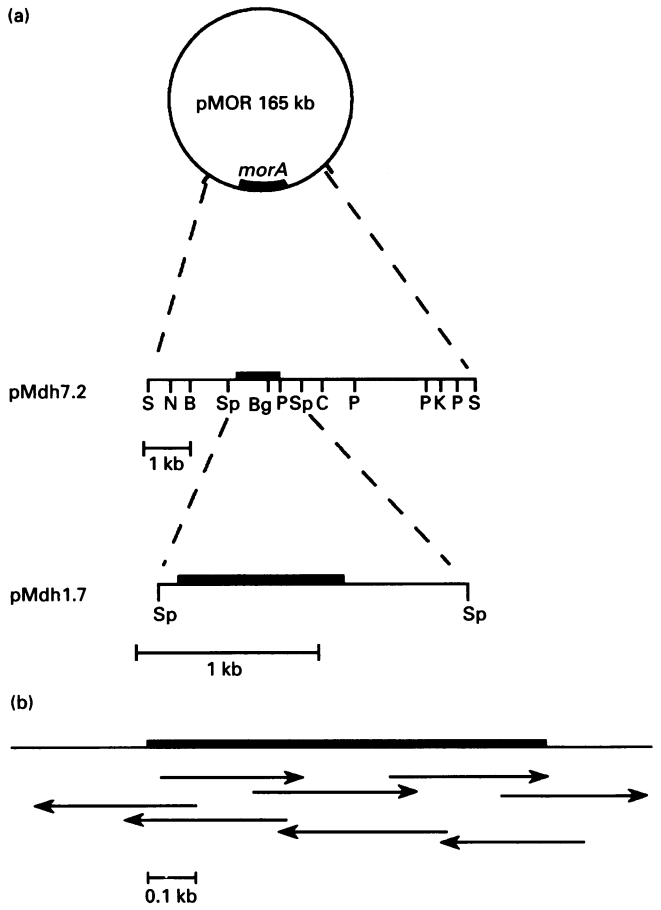

Figure 2 Cloning and sequencing strategy of the *morA* constructs

(**a**) Schematic representation of the inserts in pMdh7.2 and pMdh1.7 and the restriction map of the 7.2 kb fragment encoding the *morA* gene (closed box). Restriction sites are abbreviated as follows: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; K, *Kpn*I; N, *Nco*I; P, *Pst*I; S, *Sal*I; Sp, *Sph*I. (**b**) Sequencing strategy followed. The horizontal arrows indicate the direction and extent of sequence determinations.

and energy source and, furthermore, resulted in the loss of the constitutive morphine dehydrogenase activity. Agarose gel electrophoresis indicated that the plasmid was no longer present in the cured strain of *P. putida* M10 (data not shown). Analysis of the plasmid by restriction endonuclease digestion with the enzyme

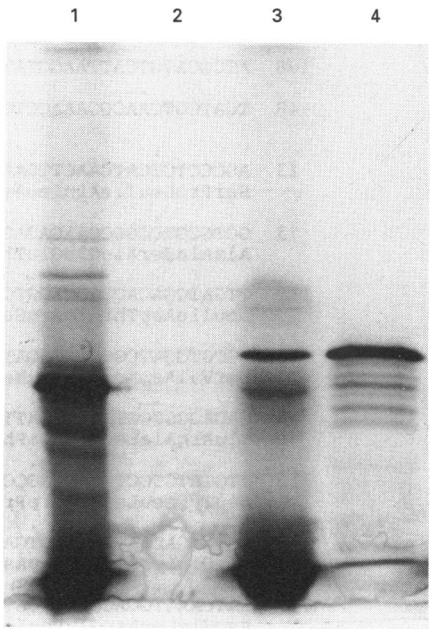

Figure 3 *In vitro* coupled transcription–translation of the *morA* gene

Analysis of the products of *in vitro* coupled transcription–translation of pMdh7.2 by immunoprecipitation, SDS/PAGE and autoradiography. Lane 1, total protein translated from pTZ19R (control DNA); lane 2, immunoprecipitate from total protein translated from pTZ19R with anti-MDH antibodies; lane 3, total protein translated from pMdh7.2; lane 4, immunoprecipitate from total protein translated from pMdh7.2 with anti-MDH antibodies.

*Sal*I revealed the presence of many bands, making it difficult to determine the precise size of the plasmid; however, summation of fragment sizes from *Sal*I digests gave an approximate size of 165 kb (Figure 1).

Cloning and overexpression of *morA*

Plasmid DNA from *P. putida* M10 was digested with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Sac*I, *Sal*I and *Xho*I and the fragments separated by electrophoresis on agarose gels. The fragments were blotted onto a Hybond-N$^+$ membrane and hybridized with a labelled oligonucleotide corresponding to part of the N-terminal amino acid sequence of MDH (Bruce et al., 1991). The MDH-specific oligonucleotide hybridized to a single restriction fragment in each of the digests (Figure 1). The 7.2 kb *Sal*I fragments were removed from the gel by electrophoresis onto Whatman DE81 paper and inserted into the plasmid pTZ19R, generating the plasmid pMdh7.2 (Figure 2). The recombinant plasmids were transformed into *E. coli* DH5α and transformant colonies were analysed by restriction enzyme digestion and oligonucleotide hybridization. A restriction map of pMdh7.2 was constructed for use in further subcloning experiments (Figure 2). No MDH activity was detected in crude extracts of *E. coli* containing pMdh7.2; however, expression of pMdh7.2 in the coupled transcription–translation system revealed a [^{35}S]methionine-labelled polypeptide with an M_r approximately the same as MDH. This polypeptide was immunoprecipitated with anti-MDH antibodies (Figure 3), indicating that plasmid pMdh7.2 contained the complete coding sequence for MDH.

The gene for MDH, *morA*, was localized within a 1.7 kb *Sph*I fragment by restriction mapping, oligonucleotide hybridization and nucleotide sequencing (Figure 2). The 1.7 kb *Sph*I subfragment of pMdh7.2 was inserted into pTZ19R to generate

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-168  CTACGGATTCTGGAAACCATGGCTCTCTTACATACGAGTCGTATTGCAGTTGACATACGT  -109

-108  ATCGCATGTCATTAAGGTATTTTGGATACTCACGAATGTAAGTGTGACCAACACAGCGAG   -49

 -48  TGATCGTCAACGCAAACCTGCCGTCCCGTATATCGAAGGAGCATTGTTATGGCCGGAAAA    12
                                          ----       MetAlaGlyLys

  13  AGCCCTCTCATCAACTTGAATAATGGCGTGAAAATGCCGGCACTAGGTCTCGGAGTGTTC    72
      SerProLeuIleAsnLeuAsnAsnGlyValLysMetProAlaLeuGlyLeuGlyValPhe

  73  GCTGCGTCCGCCGAAGAGACCGCCTCCGCCATCGCGTCGGCAATCTCCAGTGGCTACCGT   132
      AlaAlaSerAlaGluGluThrAlaSerAlaIleAlaSerAlaIleSerSerGlyTyrArg

 133  CTGATCGACACCGCCAGGTCGTATAACAATGAGGCTCAGGTCGGTGAAGGCATCCGCAAC   192
      LeuIleAspThrAlaArgSerTyrAsnAsnGluAlaGlnValGlyGluGlyIleArgAsn

 193  AGCGTGGATCGCGCCGAGATGTTCGTCACCACAAAGCTGTTCAACTGCGACTATGGTTAC   252
      SerValAspArgAlaGluMetPheValThrThrLysLeuPheAsnCysAspTyrGlyTyr

 253  GAGAGGGCGCTCAGGGCATTCGACGAAAGCCTCGGCAGGTTGGGGCTCGACTATGTCGAT   312
      GluArgAlaLeuArgAlaPheAspGluSerLeuGlyArgLeuGlyLeuAspTyrValAsp

 313  TTGTATCTGCTGCACTGGCCGACCAAAGATTGGAACGCCACGATCCAGTCCTGGAAGGCG   372
      LeuTyrLeuLeuHisTrpProThrLysAspTrpAsnAlaThrIleGlnSerTrpLysAla

 373  GCGGAGAAAATTCTTGGTGACGGGCGTGCGCGCGCGATCGGTGTATGCAACTTCCTGGAG   432
      AlaGluLysIleLeuGlyAspGlyArgAlaArgAlaIleGlyValCysAsnPheLeuGlu

 433  GATCAACTGGACGAGCTGATCGCGGCAAGTGACGTCGTGCCGGCGGTCAACCAGATCGAG   492
      AspGlnLeuAspGluLeuIleAlaAlaSerAspValValProAlaValAsnGlnIleGlu

 493  CTGCACCCCTATTTCGCGCAGAAGCCGCTGCTGGCGAAGAACAGGGCGCTCGGCATCGTC   552
      LeuHisProTyrPheAlaGlnLysProLeuLeuAlaLysAsnArgAlaLeuGlyIleVal

 553  ACGGAGGCTTGGTCGCCGATCGGCTGCCATCAACGATGGCAGGATGGGGACAATCATGGC   612
      ThrGluAlaTrpSerProIleGlyCysHisGlnArgTrpGlnAspGlyAspAsnHisGly

 613  GGCAGGAAGCACCCGCTGACCGATCCGGTCATCACCACTATCGCCGAAGCCCATGGTAGA   672
      GlyArgLysHisProLeuThrAspProValIleThrThrIleAlaGluAlaHisGlyArg

 673  TCTGCCGCGCAGGTGATCTTGCGCTGGCACTTCCAGAACGATGTCGTTGCAATTCCGAAG   732
      SerAlaAlaGlnValIleLeuArgTrpHisPheGlnAsnAspValValAlaIleProLys

 733  TCAGTCAACCCCGAGCGCATTGCCAAGAATATTGACGTGTTCGATTTCGCGCTTAGCGAC   792
      SerValAsnProGluArgIleAlaLysAsnIleAspValPheAspPheAlaLeuSerAsp

 793  GCTGAGATGGCGCAGCTAGACGAGCTGGATACAGGGGTGCGCATCGGCCCTGATCCGCGC   852
      AlaGluMetAlaGlnLeuAspGluLeuAspThrGlyValArgIleGlyProAspProArg

 853  GACGTGGACACTAGCAGCTTTGCCGAATTTGTTTGATCTGAGATGATGCGATGAACCCCG   912
      AspValAspThrSerSerPheAlaGluPheValEnd                ~~~~~~~~

 913  CCTATGATCTCGGGGGTTCCTGCAGCAACATTTCGGCAAATCGGGAAACTGGTATCATCG   972
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 973  ATCGCATGAGATGACGTGCTCCCCTGAAATTCTGCCAGTCTGAGCTAGAGTCCTTCATGA  1032

1033  AGGAGGACGAAGATGAAGCGCCAGGATTTACCGAGGAGCAGATCATTGCGATCCTGCGAG  1092
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Figure 4 Complete nucleotide sequence of the *morA* gene and the corresponding amino acid sequence of morphine dehydrogenase

The top number in each line indicates the nucleotide number starting with the AUG initiation codon of *morA*. The Shine-Dalgarno sequene is underlined. The putative transcription termination sequence is indicated (~ ~ ~). Amino acid residues 2–25 have been confirmed by protein sequencing.

pMdh1.7. The activity of MDH in *E. coli* DH5α containing pMdh1.7 was measured and compared with that of the wild-type *P. putida* M10. Levels of MDH activity (4.08 units/mg of protein) were 65-fold higher than that observed in *P. putida* M10.

DNA sequence analysis

The strategy for determining the nucleotide sequence of *morA* is presented in Figure 2b. DNA sequencing was carried out by the dideoxy chain-termination procedure on pMdh7.2. The DNA sequence revealed a single open reading frame beginning with an initiation codon at nt 1, ending at a TGA triplet at nt 888 and encoding 295 amino acids, which includes the N-terminal sequence of MDH, as determined by sequencing the purified enzyme (Bruce et al., 1991) (Figure 4). A region 9–12 bp upstream from the proposed start codon shows a recognizable pseudomonad ribosome binding site, AGGA (Shine and Dalgarno, 1975). The polypeptide predicted by the nucleotide sequence has an M_r of 32378. Codon usage is typical of that found in *P. putida* in that there is a preference for G and C in the third base position in each codon.

Purification and properties of morphine dehydrogenase

Morphine dehydrogenase expressed in *E. coli* containing pMdh1.7 was purified 18.9-fold with an overall recovery of 87% (Table 1). Typically the one-step affinity chromatography protocol purified MDH to homogeneity, since SDS/PAGE revealed a distinct single protein band after staining with Coomassie Blue (Figure 5). The properties of the purified enzyme were compared

Table 1 Purification of morphine dehydrogenase from *E. coli* DH5α/pMdh1.7

The starting material was 0.1 g of cell paste. Full experimental details are given in the Materials and methods section.

Purification step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Crude extract	0.8	41.4	10.2	4.06	100	–
Mimetic Orange 3	8.8	36	0.47	76.6	87	18.9

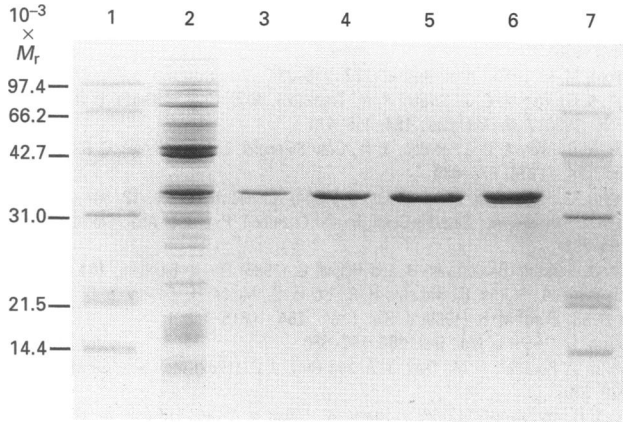

Figure 5 Electrophoresis of morphine dehydrogenase from the pMdh1.7 transformed strain of *E. coli*

The purification of MDH was monitored on a 12.5% (w/v) polyacrylamide gel containing SDS. Lanes 1 and 7; M_r markers; lane 2, cell-free extract; lane 3, 0.5 μg of purified MDH; lane 4, 1.0 μg of purified MDH; lane 5, 2.5 μg of purified MDH; lane 6, pure MDH from *P. putida* M10.

with MDH from *P. putida* M10. The enzymes were indistinguishable with respect to the pH optimum and K_m values for morphine and codeine (0.48 mM and 0.039 mM, respectively). The estimation of an M_r of 32000 for the gene product by SDS/PAGE agrees with the M_r of 32378 predicted from the open reading frame. The N-terminal amino acid sequence of MDH expressed in *E. coli* revealed that the N-terminal methionine is processed leaving Ala as the initial amino acid (see Flinta et al., 1986). The 25 N-terminal amino acid sequence of the protein expressed and purified from *E. coli*, Ala-Gly-Lys-Ser-Pro-Leu-Ile-Asn-Leu-Asn-Asn-Gly-Val-Lys-Met-Pro-Ala-Leu-Gly-Leu-Gly-Val-Phe-Ala-Ala, matches perfectly with the N-terminal 25 amino acids determined for MDH in *P. putida* M10 (Bruce et al., 1991) and is identical with that predicted by the nucleotide sequence.

DISCUSSION

The structural gene, designated *morA*, encodes the $NADP^+$-dependent MDH which initiates morphine catabolism in *P. putida* M10. We have shown that *morA* is located on a large plasmid of approximately 165 kb in *P. putida* M10 by curing and Southern hybridization experiments. This observation was further confirmed by subcloning a 7.2 kb *Sal*I fragment encompassing *morA* from the pseudomonad plasmid and analysing the polypeptide products in a coupled transcription–translation system. One of the products encoded by the *Sal*I fragment was a polypeptide with a similar relative molecular mass to MDH and which cross-reacted with anti-MDH antibodies. The lack of MDH activity in *E. coli* DH5α/pMdh7.2 was probably due to weak expression of *morA* from the promoter of plasmid pTZ19R located over 1.8 kb from the *morA* start codon. Crude extract from cells of *P. putida* M10 that had putatively lost the plasmid no longer possessed the ability to metabolize morphinone and codeinone further, implying that part, if not all, the morphine catabolic pathway is encoded on this plasmid (C. E. French and N. C. Bruce, unpublished results). DNA sequence analysis of *morA* and protein sequencing of MDH predict that MDH has 294 amino acid residues in the mature protein and a calculated M_r of 32247, which is similar to other short chain, non-zinc-dependent NAD(P) dehydrogenases, for example ribitol dehydrogenase from *Klebsiella aerogenes* (Morris et al., 1974) and glucose dehydrogenase from *Bacillus megaterium* (Jörnvall et al., 1984). The 5′ region flanking the MDH gene contains a Shine-Dalgarno sequence 9–12 bp upstream from the initiation codon, ATG (Figure 4). A putative rho-independent transcription termination sequence was found in the 3′ region 17 bp from the termination codon, TGA, of the MDH gene. The G+C content of the entire sequenced region is 59%. This elevated level is due in part to the codon usage preference, as 59% of codons have a guanine or cytosine in the wobble position. The end of a putative reading frame was detected 94 bp upstream of the *morA* start codon. The beginning of another open reading frame was observed 157 bp downstream from the *morA* termination codon, preceded by a putative ribosome-binding site. The identities of these genes are currently unknown, although they may encode other *mor* genes involved in morphine catabolism.

Sequence homologies

MDH is a low M_r monomeric $NADP^+$-dependent oxidoreductase that could belong either to the non-metal-requiring short-chain class of dehydrogenases or to the aldo-ketoreductases (Bruce et al., 1991). However, comparison of the peptide sequence of the MDH gene using the FASTA and ALIGN programs (Pearson and Lipman, 1988) revealed significant sequence similarities with the aldose reductases [alditol:$NADP^+$ 1-oxidoreductase; EC 1.1.1.21] (Figure 6). Strong sequence similarities also exist with 2,5-diketo-D-gluconate reductase (Anderson et al., 1985), chlordecone reductase (Winters et al., 1990) and a yeast protein encoded by the GCY gene (Oeschsner et al., 1988). Aldose reductase is the enzyme that mediates polyol metabolism, catalysing the NADH-dependent reduction of D-glucose to D-sorbitol. From the amino acid comparisons it appears that aldose reductase and MDH are very similar, since MDH was found to have 38.2%, 39.9%, 38.3% and 38.7% identical amino acid residues with bovine (Schade et al., 1990), human (Bohren et al., 1989), rabbit (Garcia-Perez et al., 1989) and rat (Nishimura et al., 1989) aldose reductases, respectively. The homology is scattered throughout the polypeptide sequences, but there are several highly conserved regions. These homologous residues may be involved in forming the nicotinamide-binding sites of the

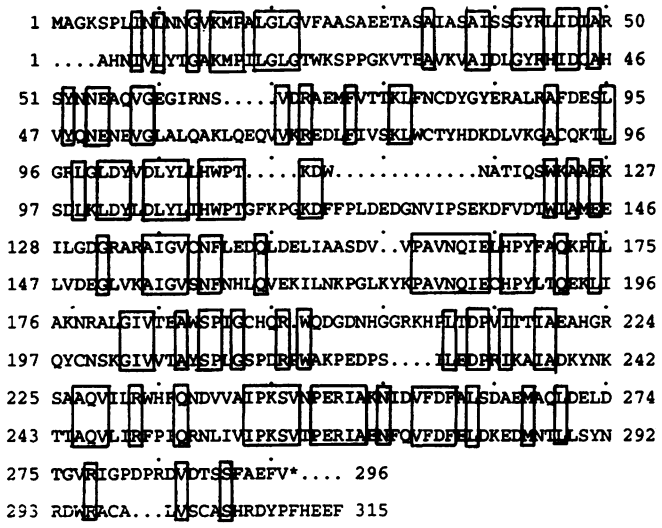

Figure 6 Comparison of the deduced amino acid sequence of *P. putida* M10 morphine dehydrogenase with the amino acid sequence of bovine aldose reductase

Residues which are the same in both sequences are boxed, gaps, indicated by dotted lines, are used to optimize the pairing of the two sequences.

two enzymes, which, in the case of pig lens aldose reductase, exhibits a single domain folded in an eight-stranded parallel α/β barrel, where an analogue of NADPH has been shown to bind to the C-terminal end of the barrel (Rondeau et al., 1992). This suggests that MDH may possess a similar nicotinamide binding site. The amino acid sequence of the N-terminal region of MDH, between residues 20 and 25 (Figure 6), seems to show the recognizable conserved sequence Gly-Xaa-Gly-Xaa-Xaa-Ala (where Xaa is any amino acid) that could indicate the 'fingerprint' motif identified by Wierenga et al. (1985) and common to many enzymes where the $NADP^+$-binding domain is a $\beta\alpha\beta$ fold (Rossmann et al., 1975). If this were the case, Ala-25 would be an essential residue for $NADP^+$-binding in MDH and an important target for site-directed mutagenesis. The striking degree of sequence identity between MDH and aldose reductase encourages speculation about the origins of the two polypeptides, suggesting either divergent evolution from a common ancestral gene or convergent evolution of dissimilar genes. Currently, this laboratory is trying to establish whether there is any structural similarity between the two proteins. The recombinant strain pMdh1.7 will prove a valuable source of protein for future work on crystallizing MDH for X-ray crystallography, which, combined with the complete amino acid sequence and site-directed mutagenesis of specific residues, will help confirm whether or not MDH belongs to the aldo-ketoreductases.

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