Analysis of the nearly identical morpholine monooxygenase-encoding \textit{mor} genes from different \textit{Mycobacterium} strains and characterization of the specific NADH:ferredoxin oxidoreductase of this cytochrome P450 system

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Cloning and sequencing of the \textit{morABC} operon region revealed the genes encoding the three components of a cytochrome P450 monooxygenase, which is required for the degradation of the N-heterocycle morpholine by \textit{Mycobacterium} sp. strain HE5. The cytochrome P450 (P450\textsubscript{mor}) and the \textit{Fe\textsubscript{3}S\textsubscript{4}} ferredoxin (Fd\textit{mor}), encoded by \textit{morA} and \textit{morB}, respectively, have been characterized previously, whereas no evidence has hitherto been obtained for a specifically morpholine-induced reductase, which would be required to support the activity of the P450\textsubscript{mor} system. Analysis of the \textit{mor} operon has now revealed the gene \textit{morC}, encoding the ferredoxin reductase of this morpholine monooxygenase. The genes \textit{morA}, \textit{morB} and \textit{morC} were identical to the corresponding genes from \textit{Mycobacterium} sp. strain RP1. Almost identical \textit{mor} genes in \textit{Mycobacterium chlorophenolicum} PCP-1, in addition to an inducible cytochrome P450, pointing to horizontal gene transfer, were now identified. No evidence for a circular or linear plasmid was found in \textit{Mycobacterium} sp. strain HE5. Analysis of the downstream sequences of \textit{morC} revealed differences in this gene region between \textit{Mycobacterium} sp. strain HE5 and \textit{Mycobacterium} sp. strain RP1 on the one hand, and \textit{M. chlorophenolicum} on the other hand, indicating insertions or deletions after recombination. Downstream of the \textit{mor} genes, the gene \textit{orf1}, encoding a putative glutamine synthetase, was identified in all studied strains. The gene \textit{morC} of \textit{Mycobacterium} sp. strain HE5 was heterologously expressed. The purified recombinant protein FdR\textsubscript{mor} was characterized as a monomeric 44 kDa protein, being a strictly NADH-dependent, FAD-containing reductase. The \textit{Km} values of FdR\textsubscript{mor} for the substrate NADH (37 \pm 7 \pm 1 \textit{\mu}M) and the artificial electron acceptors potassium ferricyanide (14 \pm 2 \pm 1 \textit{\mu}M) and cytochrome \textit{c} (28 \pm 0 \pm 3 \pm 6 \textit{\mu}M) were measured. FdR\textsubscript{mor} was shown to interact functionally with its natural redox partner, the \textit{Fe\textsubscript{3}S\textsubscript{4}} protein Fd\textit{mor}, and with the \textit{Fe\textsubscript{2}S\textsubscript{2}} protein adrenodoxin, albeit with a much lower efficiency, but not with spinach ferredoxin. In contrast, adrenodoxin reductase, the natural redox partner of adrenodoxin, could not use Fd\textsubscript{mor} in activity assays. These results indicated that FdR\textsubscript{mor} can utilize different ferredoxins, but that Fd\textsubscript{mor} requires the specific NADH:ferredoxin oxidoreductase FdR\textsubscript{mor} from the P450\textsubscript{mor} system for efficient catalytic function.

INTRODUCTION

The degradation of the secondary cyclic amines morpholine, piperidine and pyrrolidine has been reported for different mycobacteria (Cech \textit{et al.}, 1988; Knapp & Brown, 1988; Poupin \textit{et al.}, 1998, 1999a). The detection of intermediates during morpholine degradation in \textit{Mycobacterium aurum} MO1 and in an environmental \textit{Mycobacterium} strain strongly indicates that the initial ring cleavage occurs at the C–N bond (Combourieu \textit{et al.}, 1998b, 2000; Poupin

Abbreviations: AdR, adrenodoxin reductase; Adx, adrenodoxin; CD, circular dichroism; Fd, ferredoxin; FdI, spinach ferredoxin I; FdR, ferredoxin reductase; NBT, nitro blue tetrazolium; P450, cytochrome P450 monooxygenase.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences reported in this paper from \textit{Mycobacterium} sp. strain HE5 and \textit{Mycobacterium chlorophenolicum} PCP-1 are AY816211 and AY960119, respectively.
et al., 1998). Studies have suggested that a cytochrome P450 catalyses the hydroxylation of the carbon atom of this bond (Combourieu et al., 1998a; Poupin et al., 1998, 1999b).

An environmental bacterium has been isolated in our laboratory and identified as a new Mycobacterium sp. strain HE5, which is able to utilize morpholine, piperidine and pyrrolidine as sole carbon, nitrogen, and energy source. A specifically induced expression of a cytochrome P450 is observed during the degradation of these N-heterocycles (Schräder et al., 2000), which supports the above-mentioned reports. As observed for other mycobacterial strains, no enzymic hydroxylation activity is detected in cell-free extracts of Mycobacterium sp. strain HE5. To tackle this problem, the cytochrome P450, designated P450_mor, and its proposed reduct partner, a Fe₃S₄ ferredoxin (Fd_mor), have been purified separately to homogeneity (Sielaff et al., 2001). Thus, for the first time, proteins involved in morpholine degradation could be isolated.

P450 cytochromes are able to catalyse a wide range of reactions, mainly hydroxylations (Urlacher et al., 2004). The activation of molecular oxygen species at the haem cofactor of these enzymes requires electrons, which are derived from the oxidation of NAD(P)H by an oxidoreductase. The cytochrome P450 can be reduced either directly by an FAD- and FMN-containing reductase (class II system) or indirectly by electrons transferred from an FAD-containing reductase to the cytochrome P450 via a small iron-sulphur protein (class I system). Most bacterial P450 systems belong to the latter class (Munro & Lindsay, 1996). However, a specifically induced reductase cannot be detected in cell-free extracts of Mycobacterium sp. strain HE5 (Sielaff et al., 2001) or of other mycobacterial strains (Combourieu et al., 1998a; Poupin et al., 1998; Trigui et al., 2004). The determined internal peptide of P450_mor is identical to the translated sequence of the gene pipA, encoding a P450 (CYP151) from Mycobacterium smegmatis mc²¹⁵⁵. PipA is involved in piperidine and pyrrolidine metabolism, but the pip operon lacks a reductase-encoding gene (Poupin et al., 1999b). These results have led to the assumption that the reductase is a constitutively expressed protein (Sielaff et al., 2001), as was once supposed for other P450 systems from different Actinomycetales (O’Keefe & Harder, 1991). Transcription studies have demonstrated that in Streptomyces coelicolor, three ferredoxin reductases are sufficient to support the activity of 18 P450 cytochromes (Lei et al., 2004), which is in agreement with this hypothesis.

The determination of the amino acid sequences of P450_mor and Fd_mor provided the opportunity to determine the genetic basis of the P450_mor monoxygenase. Of special interest was the possibility of identifying the ferredoxin reductase of the P450_mor system. We report here the cloning of the operon encoding all structural genes of the P450_mor monoxygenase. Sequence determination of this operon region revealed a gene encoding a ferredoxin reductase, which was expressed as an enzymically active recombinant protein. This is the first report of the characterization from a P450 system of a native NADH-dependent ferredoxin reductase that is specifically required for enzymic function with the Fe₃S₄ protein Fd_mor.

### METHODS

#### Materials

All chemicals, NADH and spinach ferredoxin (FdI) were purchased from Sigma-Aldrich and Fluka. For molecular biological work, all biochemicals and enzymes other than restriction endonucleases were provided by Roche Molecular Biochemicals. Restriction endonucleases were from Fermentas or New England Biolabs, based on availability. Oligonucleotides were provided by Metabion. The Lambda ZAPII system was obtained from Stratagene. Cloning vectors were from Fermentas. Expression vectors and Ni-NTA affinity column material was from Novagen. All other column materials were obtained from Pharmacia. Purified adrenodoxin reductase (Adr) and adrenodoxin (Adx) were a kind gift from Professor Rita Bernhardt and Dr Frank Hannemann (Universität des Saarlandes).

#### Bacterial strains

Mycobacterium sp. strain HE5 (DSM 44238) was from our laboratory collection. Mycobacterium chlorophenolicum PCP-1 (DSM 43826) was kindly provided by Timo Nieminen (University of Oulu, Finland). Escherichia coli XL-1 Blue MRF² and E. coli Rosetta (DE3) were purchased from Stratagene and Novagen, respectively.

#### Preparation of whole-cell DNA

Mycobacterium sp. strain HE5 and M. chlorophenolicum PCP-1 were grown on 20 mM morpholine to OD₅₆₀ ~ 1-0 and harvested as described previously (Schuffenhauer et al., 1999). Cells (400 μg) were resuspended in 400 μl TENS buffer (50 mM Tris/HCl, pH 8-0, 100 mM EDTA, 0-3% SDS), transferred to a 2 ml tube containing 1-6 g of glass beads (0-25 μm diameter), and shaken in a bead beater (Retsch) at maximum power for 10 min. After removing the cell debris and glass beads by centrifugation at 20 000 g, the supernatant was collected and incubated with RNase A (200 μg ml⁻¹) at 37 °C for 30 min. Subsequently, proteinase K was added (200 μg ml⁻¹) and the solution incubated at 35 °C for 1 h. The following steps were standard procedures and were performed in sequence: extraction with phenol and phenol/chloroform (60:40); ethanol precipitation; and resuspension of precipitated DNA in 10 mM Tris/HCl, pH 8-0, containing 1 mM EDTA.

#### DNA techniques

Molecular procedures were either standard techniques (Sambrook et al., 1989) or those recommended by the respective manufacturers. All PCRs and sequencing reactions were performed on a Mastercycler (Eppendorf). Nucleotide sequences were determined using the dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems) and analysed using an ABI PRISM 377 DNA Sequencer. Attempts to isolate a potential circular plasmid were performed according to Larsen (2000). Plugs for usage in PFGE were prepared as described by Hughes et al. (2001). PFGE was performed on a CHEF-DR II system (Bio-Rad) at 13 °C and 170 V, and the pulse time was raised linearly over 24 h from 20 to 80 s.

#### Amplification and cloning of DNA fragments

A specific DNA fragment was amplified from whole-cell DNA using a degenerate primer (5’-CAC CAC CGS YTS CGS CGS YTS ATG AAC CC-3’) designed based on the 19 aa internal fragment of P450_mor (Sielaff et al., 2001) and a primer (5’-GGG AGT GTG TTG GTT CGG GTG TTG C-3’) derived from the C-terminal part (1154-1171) of pipA from M. smegmatis mc²¹⁵⁵ (Poupin et al., 1999b). PCR was performed according to the following standard protocol: 94 °C for 4 min; (94 °C for 15 s, 55 °C for 20 s, 72 °C for 1 min) for 10 cycles (94 °C for 15 s, 55 °C for 20 s, 72 °C for 1 min plus 5 s at each cycle) for 20 cycles. This standard protocol was used for all PCRs,
except that annealing temperatures and extension times were changed if necessary. The product P450-F1 was ligated into the pGem-T vector (Promega) and transformed into *E. coli* XL-1 Blue MRF', and for positive clones the plasmid was sequenced using M13 forward and reverse primers. From the sequence of P450-F1, two specific primers, mor6 (5'-AAA CTC ATC GGC TGC TCG CTA GC-3') and mor7 (5'-ACT CGG TGG ATG GGT CGA TG-3'), were derived and used to amplify, from whole-cell DNA, a 630 bp PCR product (annealing at 65°C) which was directly sequenced and subse- quently labelled using DIG High-Prime, yielding the probe P450m. This probe was used in Southern analysis of whole-cell DNA digests using a bank of restriction enzymes (PstI, XhoI, PvuII, SmaI and EcoRI) after separation by electrophoresis on a 0.8 % (w/v) agarose gel, transferred with a semi-dry blotter on a nylon membrane, and hybridized to the probe P450m. Chemiluminescence detection revealed a band of suitable size (4.5 kb) in the EcoRI digest. After repetition of digestion and separation, bands of appropriate size were recovered by gel extraction (Qiagen Gel Extraction Kit) and ligated into the EcoRI site of the Lambda ZAPII vector. Recombinant clones were packaged in *vitro*, and after infection of *E. coli* XL-1 Blue MRF', the resulting phage particles were screened by plaque hybridization with P450m. The phagemids of positive plaques were excised in vivo and the resulting clones were screened by colony hybridization and, as a control, by colony PCR. Because no positive clone could be detected, the recombinant clones were used as template in PCR with the primers mor6 and NRPY (modified M13 reverse primer: 5' AAT C CGC TCA GGC TCG CTA GC-3') which was directly sequenced and sub- sequently labelled using DIG High-Prime, yielding the probe P450m. The resulting clones were screened by PCR, and plasmids of positive clones were purified by using a Qiagen Plasmid Purification Kit and designated pMN21 and pMP10, respectively. These amplified DNA fragments were directly sequenced. These amplified DNA fragments were directly sequenced. 

**Cloning of morC**. For both ends of *morC*, primers were designed that contained suitable restriction sites flanked by 'spacer' nucleotides at the 5' end to facilitate efficient digestion. A PflI site was incorporated in the N-terminal primer 5'-CAGGATA CTGTA CCACC CGCGG CAGCTG-3' to allow for in-frame ligation in the *Neol* -treated vector pET28b (+) to express morC as a C-terminal His-Tag fusion protein. In the C-terminal primer 5'-CTAGA AAGCTT TGGG GAGCTG TCG-3', a HindIII site was incor- porated (restriction sites underlined). PCR was performed using whole-cell DNA as template according to the standard protocol (see above) with the annealing temperature set to 67°C. The major 1.2 kb product was cut with PflI and HindIII, extracted from the gel and ligated in the *Neol*/HindIII-digested vector pET28b (+). This plasmid was transformed in *E. coli* XL-1 Blue MRF' cells. The resulting recombinant cells were screened by PCR, and plasmids of positive clones were purified by a MonoQ column. After elution in a linear gradient of 0–1.5 M KCl in buffer B, fractions containing FdRmor were pooled and concentrated in an ultrafiltration device (Vivascience). Fractions containing FdRmor, monitored by the flavin-specific absorption at 452 nm, were pooled and concentrated in an ultracentrifuge cell (SML-Amicon) at 120 kPa, and the lysozyme was centrifuged at 18000 g for 30 min (4°C) to remove cell debris. The supernatant was loaded onto a 0.5 ml Ni-NTA His-Bind Resin flow-through column, previously equilibrated with 3 ml buffer A containing 10 mM imidazole. After washing with 5 ml buffer A containing 20 mM imidazole, FdRmor was eluted by stepwise addition of 0.25 ml buffer A containing 200 mM imidazole. Fractions containing FdRmor, monitored by the flavin-specific absorption at 452 nm, were pooled and concentrated in an ultracentrifuge cell (SML-Amicon) at 120 kPa, and the lysozyme was centrifuged at 18000 g for 30 min (4°C) to remove cell debris. The supernatant was loaded onto a 0.5 ml Ni-NTA His-Bind Resin flow-through column, previously equilibrated with 3 ml buffer A containing 10 mM imidazole. After washing with 5 ml buffer A containing 20 mM imidazole, FdRmor was eluted by stepwise addition of 0.25 ml buffer A containing 200 mM imidazole. Fractions containing FdRmor, monitored by the flavin-specific absorption at 452 nm, were pooled and concentrated in an ultracentrifuge cell (SML-Amicon) at 120 kPa, and the lysozyme was centrifuged at 18000 g for 30 min (4°C) to remove cell debris. 

**Production and purification of recombinant FdRmor**. Luria-Bertani medium (4 ml) with 30 μg kanamycin ml⁻¹ was inoculated with 5 μl of a glycerol stock of *E. coli* Rosetta (DE3) containing pMRC28 and cultured overnight at 30°C. This culture was used to inoculate four Erlenmeyer flasks (2 l), each containing 500 ml Terrific Broth with 30 μg kanamycin ml⁻¹. The flasks were incubated at 37°C until OD₆₀₀ reached 0.8 (≈5 h). The cells were then induced with 1 mM IPTG and incubated at 25°C for 18 to 20 h. Cells were harvested by centrifugation (7500 g, 20 min, 4°C) and stored at −20°C. After resuspension in 20 ml buffer A (50 mM NaH₂PO₄, pH 8–0, 300 mM NaCl, 20 %, v/v, glycerol) containing 10 mM imidazole, 0.1 mM PMSF and 5 μl Benzonase, cells were disrupted by two passages through a 20 K French pressure cell (SML-Amicon) at 120 MPa, and the lysate was centrifuged at 18000 g for 30 min (4°C) to remove cell debris. The supernatant was loaded onto a 0.5 ml Ni-NTA His-Bind Resin flow-through column, previously equilibrated with 3 ml buffer A containing 10 mM imidazole. After washing with 5 ml buffer A containing 20 mM imidazole, FdRmor was eluted by stepwise addition of 0.25 ml buffer A containing 200 mM imidazole. Fractions containing FdRmor, monitored by the flavin-specific absorption at 452 nm, were pooled and concentrated in an ultracentrifuge cell (SML-Amicon) at 120 kPa, and the lysozyme was centrifuged at 18000 g for 30 min (4°C) to remove cell debris. The supernatant was loaded onto a 0.5 ml Ni-NTA His-Bind Resin flow-through column, previously equilibrated with 3 ml buffer A containing 10 mM imidazole. After washing with 5 ml buffer A containing 20 mM imidazole, FdRmor was eluted by stepwise addition of 0.25 ml buffer A containing 200 mM imidazole. Fractions containing FdRmor, monitored by the flavin-specific absorption at 452 nm, were pooled and concentrated in an ultracentrifuge cell (SML-Amicon) at 120 kPa, and the lysozyme was centrifuged at 18000 g for 30 min (4°C) to remove cell debris. 

**Purification of FdRmor**. Culture of *Mycobacterium* sp. strain HE5 cells and preparation of crude extracts were performed as described previously (Sielaff et al., 2001). The purification protocol for FdRmor was modified. After eluting FdRmor from Q-Sepharose fast flow in a linear gradient of 0–1 M KCl in buffer B, fractions containing Fd were identified by their brownish colour and collected according to their A₂₈₀/A₂₈₂₅ value. The collected fractions were concentrated in an ultracentrifuge device and proteins were then separated on a Sephadex 75 gel filtration column using buffer B. Successively, FdRmor was applied to a MonoQ column. After elution in a linear gradient of 0–1.5 M KCl in buffer B, the protein was desalted using a PD10 column run with buffer B. FdRmor purified by this procedure was >95 % pure, as judged by SDS-PAGE analysis.

**Molecular characterization methods**. SDS-PAGE was carried out as described previously (Sielaff et al., 2001). Analytical gel filtration analysis was performed on a FPLC system equipped with a Superdex 75 column (Pharmacia Biotech) run with buffer B. UV/ visible spectra were recorded on an Uvikon 930 spectrophotometer (Kontron). The reduction of FdRmor with NADH was performed in a glove box (Coy) under nitrogen atmosphere at 4°C. Buffers and
solutions were made anaerobic prior to usage by several cycles of degassing and gassing with nitrogen using the sluice of the glove box. The quartz cuvette was sealed with a rubber cap. The extinction coefficient of the protein-bound flavin was determined spectrophotometrically by quantification of the FAD released from the holoprotein following SDS treatment (Aliverti et al., 1999). The identity of the enzyme-bound flavin was assessed fluorometrically. After thermal denaturation of 10 µM holoheme at 100 °C for 15 min, the released flavin was treated with 3 µM phosphodiesterase I (Aliverti et al., 1999). Emission spectra (480 nm to 600 nm) were recorded in a fluorescence cuvette of 1 cm path length on a FluoroMax2 (Jobin Yvon-Spex) at 20 °C, using an excitation wavelength of 450 nm and a slit width of 5 nm. Visible circular dichroism (CD) spectra (320 nm to 600 nm) were recorded on a JASCO J-810 spectropolarimeter with a quartz cell of 1 cm path length (scan speed 50 nm min⁻¹). Spectra were recorded five times and averaged.

Activity assays. The activity of FdRₘₗₜₜ towards the artificial electron acceptors potassium ferricyanide, cytochrome c and nitro blue tetrazolium (NBT) was determined spectrophotometrically using an Uvikon 930 spectrophotometer (Kontron). Potassium ferricyanide reduction was monitored at 420 nm (ε₄₂₀ = 1020 M⁻¹ cm⁻¹), cytochrome c reduction at 550 nm (ε₅₅₀ = 21 100 M⁻¹ cm⁻¹) and NBT reduction at 553 nm (ε₅₅₃ = 18 300 M⁻¹ cm⁻¹). Reactions were performed with 10 nM FdRₘₜₜ in 50 mM Tris/HCl, pH 8.5, at 30 °C; if not stated otherwise. For measurements of ferricyanide-reducing activities at different pH values, a buffer was used composed of 25 mM Tris and 25 mM glycine, which was adjusted to the appropriate pH with either NaOH or HCl. Activity assays of FdRₘₜₜ with Fdₘₜₜ, was performed in 50 mM glycine-buffer, pH 8.5. Adr/Adx activity was measured according to Uhlmann et al. (1994). Steady-state kinetic parameters were determined by varying the concentrations of the substrates in the standard assay. Initial velocities (v) were fitted to a hyperbolic function to obtain the kinetic parameters Kₘ and Vₘₜₜ.

RESULTS

Cloning and sequencing of the genes encoding the P450ₘₗₜₜ system

Degenerate primers were designed on the basis of an internal 19 aa fragment of P450ₘₗₜₜ and the N-terminal 30 aa fragment of Fdₘₜₜ (Sielaff et al., 2001), as it could be expected that these proteins would be encoded by adjacent genes. Primers were chosen according to codon usage in mycobacteria in order to minimize inherent degeneracy. However, no specific fragment could be amplified by PCR with genomic DNA from Mycobacterium sp. strain HE5. A different approach was eventually successful: the 19 aa P450ₘₗₜₜ fragment is identical to the corresponding translated sequence of pipA, a gene encoding a P450 that is involved in piperidine and pyrrolidine metabolism in M. smegmatis mc²₁₅₅ (Poupin et al., 1999b). The combination of a new primer designed from the 3’ end of pipA and a degenerate primer derived from P450ₘₗₜₜ produced an 895 bp fragment (P450-F1), the internal sequence of which proved to encode the P450ₘₗₜₜ fragment. Based on this sequence, internal primers were designed to produce a probe that was then used in Southern hybridization experiments with different restriction-enzyme digests. This revealed that there is only a single copy of the gene encoding P450ₘₗₜₜ in the genome of Mycobacterium sp. strain HE5. A 4·5 kb EcoRI fragment was isolated and ligated into the Lambda ZAPII vector. After in vivo excision of the phagemids of plaques giving a positive reaction in Southern hybridization experiments, no positive clone could be detected. However, use of the ligated EcoRI fragment as template in PCR with an internal primer and a vector-encoded primer yielded a specific 1373 bp fragment (P450-F2) comprising 1272 bp from the 3’ terminal portion of the cloned fragment (Fig. 1).

Based on the new sequence of P450-F2, a second probe, Fdfrm, was amplified, the sequence of which was located downstream of probable suitable restriction sites. A restriction site map of the P450ₘₗₜₜ gene region (data not shown) was obtained by Southern analysis of several DNA digests with P450m, which was located upstream of these sites, or with Fdfrm. This now allowed the cloning of specific fragments based on their location and the expected extent of new sequence information. A 2·0 kb NruI fragment was cloned, which was about 1·4 kb shorter at its 5’ terminal site than the EcoRI fragment. A positive clone was detected from this library and plasmid pM21 was isolated. Sequencing of the internal fragment revealed the upstream region of the P450ₘₗₜₜ operon. Cloning of a 2·0 kb PstI fragment led to the isolation of plasmid pMP10, which contained the downstream region of this operon. Summarizing, 4782 bp of the P450ₘₗₜₜ operon could finally be sequenced.

Analysis of the genes encoding the P450ₘₗₜₜ system

Sequencing of DNA fragments revealed a putative operon consisting of six ORFs, of which two were truncated (Fig. 1). MorA encoded a protein of 400 aa, which contained a sequence identical to that of the previously determined
internal 19 aa peptide of P450<sub>mor</sub> (Sielaff <i>et al.</i>, 2001). Thus, we concluded that <i>morA</i> encodes the cytochrome P450<sub>mor</sub>. There was a difference between the predicted molecular mass of 44 603 Da from <i>morA</i> and the molecular mass of 44 769 Da determined by mass spectrometry for P450<sub>mor</sub> (Sielaff <i>et al.</i>, 2001). P450<sub>mor</sub> proved to be N-terminally blocked in Edman degradation, suggesting an N-terminal acylation, which could account for this difference. <i>morA</i>, as well as the following genes <i>morB</i>, <i>morC</i> and <i>orf</i>′<i>1</i>, was found to be identical to corresponding genes from <i>Mycobacterium</i> sp. strain RP1 (Trigui <i>et al.</i>, 2004). This point will be dealt with later.

The two ORFs downstream of <i>morA</i> appeared to encode the potential redox partners for a catalytically functional P450 system. <i>MorB</i> encodes the ferredoxin Fd<sub>mor</sub> (62 aa), as confirmed by comparison of the translated sequence with the N-terminal 30 aa sequence determined for the previously purified protein Fd<sub>mor</sub> from <i>Mycobacterium</i> sp. strain HE5. In addition, the predicted molecular mass of 6793 Da was in good agreement with the 6795 Da determined for Fd<sub>mor</sub> (Sielaff <i>et al.</i>, 2001). The following ORF, <i>morC</i>, encoded a 403 aa protein with a predicted molecular mass of 42 376 Da, which was, as mentioned above, identical to MorC from <i>Mycobacterium</i> sp. strain RP1, and which exhibited identities to several ferredoxin reductases, all identified from genome sequences: 39 % in different overlaps to FprC from <i>Streptomyces avermitilis</i> MA-4680 (Ikeda <i>et al.</i>, 2003) and Rx0688 from <i>Mycobacterium tuberculosis</i> H37Rv (Cole <i>et al.</i>, 1998), and 37 % to FprA from <i>S. avermitilis</i> MA-4680 (Ikeda <i>et al.</i>, 2003). FprC and FprA were identified adjacent to genes encoding Fe<sub>3</sub>S_{4} ferredoxins and the P450s CYP105Q1 and CYP147B1, respectively (Lamb <i>et al.</i>, 2003). The identification of MorC as being in fact a ferredoxin oxidoreductase was confirmed in the present study by heterologous expression of <i>morC</i> and analysis of the protein. All previously purified P450 coupled reductases have been reported to belong to the glutathione reductase family, all of which contain an FAD-binding consensus sequence (GxGxxG) in the N-terminal region (Dym & Eisenberg, 2001). In FdR<sub>mor</sub>, this motif is changed (GGSLAG), whereas a second consensus sequence (GxGxxGxExE) was found to be conserved. Sequence analysis of FprC and FprA from <i>S. avermitilis</i> revealed that they also contain the changed motif. However, despite such local differences, the overall homology of FdR<sub>mor</sub> to putidaredoxin reductase (28 % identity) from the P450<sub>cam</sub> system from <i>Pseudomonas putida</i> (Serioukova <i>et al.</i>, 2004) indicates that these proteins are nevertheless related to the glutathione reductase family.

<i>orf</i>′<i>1</i> downstream of <i>morC</i> was truncated, and the derived amino acid sequence (141 aa) was identical to the deduced 74 aa of the truncated <i>orf</i>′<i>1</i> from <i>Mycobacterium</i> sp. strain RP1, and also showed 85 % identity to the 130 aa protein encoded by the similarly truncated <i>orf</i>′<i>2</i> from <i>M. smegmatis</i> mc<sup>155</sup>. Both sequences exhibit significant identities to the N-terminal sequences of putative glutamate synthetases (Poupin <i>et al.</i>, 1999b; Trigui <i>et al.</i>, 2004).

### Identical P450 genes in different mycobacterial strains

Quite recently, the genes encoding a cytochrome P450 system involved in secondary amine utilization in <i>Mycobacterium</i> sp. strain RP1 became known (Trigui <i>et al.</i>, 2004). The analysed <i>PstI</i> fragment, exhibiting the ORFs <i>morA</i>, <i>morB</i>, <i>morC</i> and <i>orf</i>′<i>1</i>, is identical to the corresponding sequence of <i>Mycobacterium</i> sp. strain HE5. The existence of totally identical <i>mor</i> gene regions was surprising, since the homology of this gene region is higher than that of the corresponding 16S rDNA (98-0 % identity). <i>M. chlorophenolicum</i> PCP-1 is another relative of these strains, according to its 16S rDNA (98-4 % identity to <i>Mycobacterium</i> sp. strain HE5, 97-3 % to <i>Mycobacterium</i> sp. strain RP1) and is known to be capable of degrading polychlorinated phenols (Apajalathi & Salkinoja-Salonen, 1987; Häggblom <i>et al.</i>, 1994). It has now been shown in our laboratory that <i>M. chlorophenolicum</i> PCP-1 is also able to use morpholine, piperidine and pyrrolidine as sole carbon, nitrogen and energy source, and that a cytochrome P450 is induced during growth on morpholine, but not on the putative intermediate diglycolic acid (Deebb, 2003). The specific DNA fragments MC-F1, MC-F2, MC-F3 and MC-F4 (Fig. 1) could be amplified by PCR using primers derived from the <i>mor</i> operon and whole-cell DNA isolated from <i>M. chlorophenolicum</i> PCP-1 as template. Sequencing 2727 bp of these fragments revealed the nearly identical genes <i>morA</i>, <i>morB</i> and <i>morC</i> (only one nucleotide was different in <i>morB</i>) in an identical order to that of the <i>mor</i> operon from <i>Mycobacterium</i> sp. strain HE5. But a pronounced difference was detected downstream of <i>morC</i>, beginning with a changed nucleotide in the stop codon of <i>morC</i> (Fig. 2). The intergenic region between <i>morC</i> and <i>orf</i>′<i>1</i> was 66 bp longer in <i>Mycobacterium</i> sp. strain HE5. The sequence following this stretch is again almost identical to that of <i>M. chlorophenolicum</i> PCP-1, although to a lesser extent (95-5 %) than the <i>mor</i> genes. From these results, it seemed clear that these mycobacterial strains might have exchanged DNA. However, no plasmid could be detected in <i>Mycobacterium</i> sp. strain HE5, either by standard procedures for the isolation of circular plasmids or by PFGE for the detection of linear plasmids.

### Analysis of the upstream region of <i>morA</i>

The 3-9 kb <i>PstI</i> fragment from <i>Mycobacterium</i> sp. strain RP1 (see restriction sites in Fig. 1) contained a 914 bp sequence upstream of <i>morA</i> that was previously identified as non-coding (Trigui <i>et al.</i>, 2004). In this work, a larger extent of this region was sequenced. This enabled the identification of a stop codon upstream of <i>morA</i> at position 560 of the sequenced <i>mor</i> operon (Fig. 1), which terminated a truncated ORF, designated ′<i>morR</i>. The translated 186 aa showed identities of 80 % to a putative regulatory protein encoded by the gene pipR from <i>M. smegmatis</i> mc<sup>155</sup> (Poupin <i>et al.</i>, 1999b), and of 39 % (in a 177 bp overlap) to SAV1742, a putative GntR-family regulator from <i>S. avermitilis</i> MA-4680 (Ikeda <i>et al.</i>, 2003). PipR has been shown to be involved in
the regulation of piperidine and pyrrolidine metabolism, which involves the cytochrome P450 CYP151 encoded by the gene pipA (87% identity to morA) found downstream of pipR. Between pipR and pipA, an insertion element (IS1096) has been identified (Poupin et al., 1999b), which was not present between morR and morA in Mycobacterium sp. strain HE5. Instead, an ORF was identified in which the start codon overlapped with the stop codon of morR. This ORF, designated orfX, encoded a polypeptide of 260 aa, which showed identities of 31% (in a 158 aa overlap) to the hypothetical proteins SAV1740 and SAV1124 from S. avermitilis MA-4680 (Omura et al., 2001) and to a low extent to chemotactic transducers from different bacteria. No function can be assigned to the hypothetical protein (260 aa) encoded by orfX, or to SAV1740 (265 aa) and SAV1124 (278 aa), as they lack, for example, the C-terminal portion of chemotactic transducers, which are composed in general of 600 to 700 aa. Interestingly, SAV1740 is found 39 bp downstream of SAV1742, which shows significant identities to the polypeptide encoded by morR. A possible ORF homologous to orfX could also be identified at the same position in the sequence from M. smegmatis mc²155, but two additional nucleotides are present in the latter at position 1111 (position 273 of orfX) that were not detected in the corresponding sequences of Mycobacterium sp. strain HE5 and Mycobacterium sp. strain RP1. If these two nucleotides were deleted in M. smegmatis mc²155, the predicted polypeptide (125 aa) would exhibit an identity of 65% to that of orfX. However, in M. smegmatis mc²155, this possible ORF was disrupted after 375 bp by the IS element.

**Production and purification of morC**

MorA and morB have now been shown to encode the previously isolated proteins P450mor and Fdmor, thus establishing them as part of the morpholine-hydroxylating P450 system. A specific, morpholine-induced reductase could not be detected in Mycobacterium sp. strain HE5 by the methods employed (Sielaff et al., 2001), but we have now identified morC as encoding the ferredoxin reductase FdRmor of the P450mor system. Direct proof of this has been provided in a separate publication by the reconstitution of all three isolated proteins in an enzymically active morpholine monooxygenase (Sielaff & Andreesen, 2005). MorC was expressed as a C-terminal His-Tag fusion protein to study its characteristics as a ferredoxin reductase and to enable a comparison to previously purified ferredoxin reductases from other bacterial P450 systems.

An additional protein band was clearly visible in SDS-PAGE after growth at 37°C of E. coli Rosetta (DE3) harbouring pMRC28 and induction with 1 mM IPTG. But nearly all of the protein was found to form inclusion bodies. Lowering the growth temperature after induction significantly increased the amount of soluble protein, which was found to be highest when cells were grown at 25°C for 18 to 20 h. This protein, which from now on is called FdRmor, was isolated by chromatography on a Ni²⁺ affinity column and subsequent gel filtration on Sephadex G75. The purified protein was judged to be about 90% homogeneous in SDS-PAGE (Fig. 3). Attempts to further purify FdRmor by anion-exchange chromatography on MonoQ resulted in the loss of the flavin cofactor and therefore of its activity. The cofactor could not be restored, either by the addition of
FAD or of FMN. The amount of purified FdRmor was calculated to be about 30 nmol [~1·2 mg (1 culture)⁻¹], using the estimated extinction coefficient for FdRmor (see below).

Molecular properties of FdRmor

FdRmor showed an $M_r$ of about 50 000 in denaturing PAGE (Fig. 2), which appeared to be in the same range as the calculated mass of 43 523 Da (42 376 calculated from the sequence of morC and 1147 from the linker sequence). The $M_r$ of native FdRmor was determined by gel filtration to be 50 000, indicating that the protein was a monomer under these conditions. The pure FdRmor enzyme exhibited in its oxidized state spectral features typical of flavin-containing enzymes, with spectral maxima at 273, 378 and 452 nm. Shoulders were observed at 422 and 473 nm (data not shown). A value of 10·0 was calculated for the ratio of protein to flavin-specific absorption ($A_{273}/A_{452}$). Addition of excess sodium dithionite or NADH under anaerobic conditions led to full reduction of the flavin. No spectral signals attributable to flavin semiquinone species could be detected (data not shown). The non-covalently bound flavin in FdRmor was identified as FAD. The fluorescence of the released flavin increased about tenfold after addition of phosphodiesterase, as expected for the conversion from FAD or of FMN. The amount of purified FdRmor was calculated to be about 30 nmol [~1·2 mg (1 culture)⁻¹], using the estimated extinction coefficient for FdRmor (see below).

Catalytic properties of FdRmor

FdRmor was capable of oxidizing NADH and reducing the electron acceptors potassium ferricyanide and cytochrome $c$. The reduction of these acceptors by FdRmor was strictly dependent on NADH, and no activity was obtained using NADPH as substrate. The addition of FAD to the assay had no enhancing effect on the activity of FdRmor. The pH optimum for the NADH-dependent reduction of potassium ferricyanide by FdRmor was found to be 9·4. At pH 7·5, the activity of the enzyme declined to about 50 %. The optimal temperature for this reaction was found to be around 30 °C (data not shown). No NADH oxidase activity of FdRmor could be observed under these conditions. The steady-state kinetic parameters of FdRmor (Table 1) for the substrate NADH were determined with saturating concentrations of excess sodium dithionite or NADH under anaerobic conditions led to full reduction of the flavin. No spectral signals attributable to flavin semiquinone species could be detected (data not shown). The non-covalently bound flavin in FdRmor was identified as FAD. The fluorescence of the released flavin increased about tenfold after addition of phosphodiesterase, as expected for the conversion from FAD or of FMN. The amount of purified FdRmor was calculated to be about 30 nmol [~1·2 mg (1 culture)⁻¹], using the estimated extinction coefficient for FdRmor (see below).

Table 1. Steady-state kinetic parameters for NADH-dependent ferricyanide- and cytochrome c-reducing activities of FdRmor

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}/K_m$ (s⁻¹ μM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferricyanide</td>
<td>60·2 ± 0·9</td>
<td>14·2 ± 1·1</td>
<td>4·24</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>4·5 ± 0·2</td>
<td>28·0 ± 3·6</td>
<td>0·16</td>
</tr>
<tr>
<td>NADH</td>
<td>−</td>
<td>37·7 ± 4·1</td>
<td>−</td>
</tr>
</tbody>
</table>

Measurements were performed in triplicate in 50 mM Tris/HCl, pH 8·5, with 10 nM FdRmor. Kinetic parameters were obtained by varying substrate concentrations in the standard assay. Standard errors for the obtained parameters are included.

Analysis of the mor operon in this study revealed that the P450mor monooxygenase is a class I system, composed of three components: the NADH-oxidizing ferredoxin reductase FdRmor, the ferredoxin Fdmor as electron-transfer protein and the cytochrome P450mor, which acts as monooxygenase. Thus, FdRmor should be able to interact catalytically with its proposed natural redox partner Fdmor. The reduction of Fdmor by FdRmor was directly monitored by the decrease of the ferredoxin peak at 412 nm in the spectrum of FdRmor after the addition of NADH and a catalytic amount of FdRmor (Fig. 5). Subsequently, the ability of FdRmor to mediate the FdRmor-catalysed reduction of different electron acceptors was studied. The addition of FdRmor had no effect on the FdRmor-dependent reduction of ferricyanide, whereas FdRmor enhanced the reaction of...
Fd\textsubscript{mor} towards cytochrome c up to fivefold. In addition, it has been reported previously that the presence of recombinant Fd\textsubscript{mor} enables the reduction of NBT by FdR\textsubscript{mor}, which cannot be catalysed by FdR\textsubscript{mor} on its own (Sielaff & Andreesen, 2005). Altogether, these results proved that FdR\textsubscript{mor} reduces Fd\textsubscript{mor} which acts as an electron shuttle to different artificial electron acceptors. Thus, the systematic name for FdR\textsubscript{mor} should be NADH:ferredoxin oxidoreductase.

The natural redox partners of previously characterized ferredoxin reductases have always been Fe\textsubscript{3}S\textsubscript{2} ferredoxins. In contrast, it has been clearly demonstrated that Fd\textsubscript{mor} contains a Fe\textsubscript{3}S\textsubscript{4} cluster (Sielaff & Andreesen, 2005). To elucidate whether or not FdR\textsubscript{mor} and Fd\textsubscript{mor} specifically require ferredoxin reductases have always been Fe\textsubscript{3}S\textsubscript{2} ferredoxins. In contrast, it has been clearly demonstrated that FdR\textsubscript{mor} reduces Fd\textsubscript{mor} which acts as an electron shuttle to different artificial electron acceptors. Thus, the systematic name for FdR\textsubscript{mor} should be NADH:ferredoxin oxidoreductase.

DISCUSSION

Cloning and sequencing of the mor operon from Mycobacterium\textit{sp.} strain HE5 revealed six ORFs, of which three were found to encode the components of the P450\textsubscript{mor} system: mor\textsubscript{A}, encoding the cytochrome P450\textsubscript{mor}; mor\textsubscript{B}, encoding the Fe\textsubscript{3}S\textsubscript{4} ferredoxin Fd\textsubscript{mor}; and mor\textsubscript{C}, encoding the NADH:ferredoxin reductase FdR\textsubscript{mor}. These genes were found to be identical to the corresponding genes from Mycobacterium\textit{sp.} strain RP1. Only the gene mor\textsubscript{A} from Mycobacterium\textit{sp.} strain RP1 has recently been expressed, and the protein can convert the heterocycles piperidine, pyrrolidine and morpholine in a heterologous system with the alternative ferredoxin NADP\textsuperscript{+} oxidoreductase and ferredoxin from spinach (Trigui \textit{et al.}, 2004). MorA exhibits high identities to PipA, a cytochrome P450 which has been shown to be involved in piperidine and pyrrolidine metabolism of \textit{M. smegmatis} mc\textsuperscript{155} (Poupin \textit{et al.}, 1999b).

In this study, almost identical mor\textsubscript{A}, mor\textsubscript{B} and mor\textsubscript{C} genes were also identified in \textit{M. chlorophenolicum} PCP-1. Differences were detected downstream of these genes, where a shorter intergenic region is present in \textit{M. chlorophenolicum} PCP-1. The following gene, orf\textsubscript{9}, is also highly conserved, but the lower identity compared to the mor genes correlates much better to the lower identity of the 16S rDNA. This indicates that only the mor genes have integrated recently into this gene region. In contrast, no differences between Mycobacterium\textit{sp.} strain HE5 and Mycobacterium\textit{sp.} strain RP1 were observed downstream of mor\textsubscript{C}, suggesting that the mor genes have been exchanged together with downstream sequences including orf\textsubscript{1}'. Subsequently, this gene region might have undergone deletions or insertions, thus indicating a lesser importance for the putative glutamine synthetase encoded by orf\textsubscript{1}' for the degradation of morpholine. Transcription studies should indicate if orf\textsubscript{1}' is functionally related to the mor operon, for example, by scavenging the nitrogen. The identification of identical mor genes in different mycobacterial strains suggests that this P450 system is more widely distributed within this genus. In fact, a number of different mycobacteria, all able to degrade morpholine, piperidine and pyrrolidine, have been shown to specifically express a cytochrome P450 during growth on these heterocycles (Poupin \textit{et al.}, 1999a). It seems very likely that these enzyme systems are also identical to the P450\textsubscript{mor} system, or at least that they exhibit high identities to it. Interestingly, the distribution of this P450 system does not seem to follow the degree of relationship between mycobacterial strains. Mycobacterium gilvum, which has been identified as the closest relative of Mycobacterium\textit{sp.} strain HE5, is not able to grow on any of the heterocycles metabolized by the P450\textsubscript{mor} system (Schräder \textit{et al.}, 2000). Similar results have been obtained for five distinct haloalkane-utilizing \textit{Rhodococcus} strains, which all share the completely conserved gene dha\textsubscript{A} encoding a haloalkane dehalogenase. The
highly conserved gene region is detected on the chromosome as well as on plasmids in all these strains (Poelarends et al., 2000). It has been suggested that an ancestral plasmid was transferred and subsequently integrated into the chromosome. A plasmid could also meet the requirements for horizontal gene transfer in morpholine-degrading mycobacterial strains, but no evidence for any sort of plasmid was found in *Mycobacterium* sp. strain HE5.

The main discrepancy between the P450 systems in *Mycobacterium* sp. strain HE5, *Mycobacterium* sp. strain RP1, *M. chlorophenolicum* PCP-1 and *M. smegmatis* mc²155 on the genomic level is the lack of a ferredoxin reductase-encoding gene in the last strain. In the *pip* operon of *M. smegmatis* mc²155, the gene *orf1*, encoding the ferredoxin, is immediately followed by the gene *orf2′*, encoding a putative glutamine synthetase (Poupin et al., 1999b). Sequencing the genome of *M. tuberculosis* has revealed 22 genes encoding P450 cytochromes (Cole et al., 1998), and the genome of *M. smegmatis* mc²155 has been shown to exhibit 40 P450 genes (Jackson et al., 2003). This is the highest number found in a bacterium so far, but large sets of CYP genes have also been identified in the genomes of other actinobacteria. Many of these CYP genes have an isolated position in the genome, while a lower number are close to genes encoding ferredoxins. Only a few CYP genes are organized in operons that include genes encoding both reductase and ferredoxin. For instance, in the genome of *S. avermitilis*, 33 CYP genes have been identified, but only two are linked to ferredoxin and ferredoxin reductase genes (Lamb et al., 2003). Interestingly, both these reductases show significant homologies to FdR<sub>mor</sub>. Gene expression studies with *S. coelicolor* reveal that only three reductases and six ferredoxins seem to be sufficient to support the activity of the 18 P450 cytochromes of this organism, which are all expressed during the life cycle (Lei et al., 2004). Thus, it seems likely that in *M. smegmatis* mc²155, the missing reductase is functionally replaced by another ferredoxin reductase. The organization of the P450<sub>mor</sub> system, recruiting a specific ferredoxin reductase, is consistent with that of the classical bacterial P450<sub>cam</sub> system (Koga et al., 1985). Other biodegradative P450s, such as P450<sub>terp</sub> (Peterson et al., 1992), P450<sub>cin</sub> (Hawkes et al., 2002) and P450<sub>BRI</sub> (Nagy et al., 1995), are also organized into operons with their electron transfer proteins. This might imply that xenobiotic-metabolizing P450s generally utilize specific reduct partners to ensure efficient functionality.

The amino acid sequence of FdR<sub>mor</sub> showed identities to different ferredoxin reductases from different *Streptomyces* and *Mycobacterium* strains. So far, all of these have only been derived from nucleotide sequences and have not been characterized at the protein level. *Mor* has now been expressed as a C-terminal His-Tag fusion protein and the recombinant enzyme FdR<sub>mor</sub> characterized as an NADH-dependent, FAD-containing ferredoxin reductase and shown to interact functionally with the Fe<sub>2</sub>S<sub>2</sub> ferredoxin Fd<sub>mor</sub>. FdR<sub>mor</sub> shows some instability, which might explain the fact that no specifically induced ferredoxin reductase could previously be identified in crude extracts of *Mycobacterium* sp. strain HE5, which led us to propose that the reductase was constitutively formed (Sielaff et al., 2001). Instability, as well as a low level of expression, might be the reason for the paucity of reports on purified reductases of bacterial P450 systems. One of the best-known examples is the putidaredoxin reductase from *Pseudomonas putida*, which uses putidaredoxin as electron transfer protein to reduce P450<sub>cam</sub> (Koga et al., 1985). This reduct system is similar to the mammalian one, in which AdR and Adx reduce mitochondrial P450 cytochromes (Schiffler & Bernhardt, 2003). FprA from *M. tuberculosis* was identified as an AdR homologue, and the heterologously expressed flavoprotein was able to reduce Adx, the Fe<sub>2</sub>S<sub>2</sub> protein FdI from spinach and a 7Fe ferredoxin from *M. smegmatis* (Fischer et al., 2002). While AdR is clearly an NADPH-dependent ferredoxin reductase, FprA also oxidizes NADH, although with a lower efficiency compared to NADPH. This distinguishes these proteins from FdR<sub>mor</sub>, which is strictly NADH-dependent and cannot use NADPH as reductant. A soybean flour-induced NADH-dependent ferredoxin reductase has been purified from *Streptomyces griseus* and shown to couple electron transfer to cytochrome P450<sub>smeg</sub> in the presence of a 7Fe ferredoxin from *S. griseus* (Ramachandra et al., 1991). This 7Fe ferredoxin and those used in the studies of FprA are not the natural redox partners of these reductases. So far, only the specific reduct partners of AdR and putidaredoxin reductase have been purified and characterized. Both proteins, Adx and putidaredoxin, contain an Fe<sub>2</sub>S<sub>2</sub> cluster, distinguishing them from FdR<sub>mor</sub>, which has been clearly identified as a Fe<sub>2</sub>S<sub>4</sub> ferredoxin (Sielaff & Andreesen, 2005). In contrast to FprA, FdR<sub>mor</sub> is not able to interact functionally with Fdl. FdR<sub>mor</sub> is able to utilize Adx in the NBT reduction, but the low catalytic efficiency of this reaction indicates a high specificity of FdR<sub>mor</sub> for its natural reduct partner, Fd<sub>mor</sub>. This is supported by the measured low *Kₐ* value (5−6 nM) of FdR<sub>mor</sub> for Fd<sub>mor</sub> in the reduction of NBT (Sielaff & Andreesen, 2005). Interestingly, the reduction of cytochrome *c* by FdR<sub>mor</sub> is not enhanced by the addition of Adx, although this electron acceptor is widely used to investigate AdR−Adx interactions (Grinberg et al., 2000). Furthermore, Fd<sub>mor</sub> cannot replace Adx enzymically in AdR−Adx activity assays with cytochrome *c*. Similarly, putidaredoxin and Adx cannot substitute each other in activity assays of their respective reductases, although these ferredoxins share 37% homology in their sequences (Geren et al., 1986). In summary, we conclude from these data that for higher enzymic efficiency, Fd<sub>mor</sub> requires the specific NADH:ferredoxin reductase FdR<sub>mor</sub>, thus reflecting the genomic organization of this P450 system, in which all genes are found adjacent in the same operon.

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