Identification and functional characterization of a gene for the methanol : *N,N'*-dimethyl-4-nitrosoaniline oxidoreductase from *Mycobacterium* sp. strain JC1 (DSM 3803)

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Mycobacterium sp. strain JC1 is able to grow on methanol as a sole source of carbon and energy using methanol : *N,N'*-dimethyl-4-nitrosoaniline oxidoreductase (MDO) as a key enzyme for primary methanol oxidation. Purified MDO oxidizes ethanol and formaldehyde as well as methanol. The *Mycobacterium* sp. strain JC1 gene for MDO (*mdo*) was cloned, sequenced, and determined to have an open reading frame of 1272 bp. Northern blot and promoter analysis revealed that *mdo* transcription was induced in cells grown in the presence of methanol. Northern blotting together with RT-PCR also showed that the *mdo* gene was transcribed as monocistronic mRNA. Primer extension analysis revealed that the transcriptional start site of the *mdo* gene is located 21 bp upstream of the *mdo* start codon. An *mdo*-deficient mutant of *Mycobacterium* sp. strain JC1 did not grow with methanol as a sole source of carbon and energy.

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INTRODUCTION

Alcohol dehydrogenases (ADHs) catalyse the interconversion of alcohols to aldehydes or ketones (Reid & Fewson, 1994; Rella *et al.*, 1987; von Wartburg *et al.*, 1964). Microbial ADHs are divided into three major categories: NAD- or NADP-dependent dehydrogenases, NAD(P)-independent dehydrogenases and FAD-dependent alcohol oxidases that catalyse an essentially irreversible oxidation of alcohols (Reid & Fewson, 1994). Methanol dehydrogenase (MDH) plays a key role in the oxidation of methanol in methylotrophic bacteria (Anthony, 2000). Gram-negative methylotrophic bacteria have NAD(P)-independent MDHs that contain pyrroloquinoline quinone (PQQ) as a prosthetic group (Anthony, 2000). Among Gram-positive methylotrophic bacteria, *Bacillus methanolicus* C1 uses NAD-dependent nicotinoprotein MDH (Arfman *et al.*, 1989, 1997; Hektor *et al.*, 2002), and *Amycolatopsis methanolica*, *Mycobacterium gastri* MB19, *Rhodococcus*

rhodochrous LMD 89.129 and *Rhodococcus erythropolis* DSM 1069 use *N,N'*-dimethyl-4-nitrosoaniline (DMNA)-dependent nicotinoprotein methanol:DMNA oxidoreductase (MDO) (Bystrykh *et al.*, 1993a, b; van Ophem *et al.*, 1993) for the oxidation of methanol. This MDO is similar to the NAD-dependent MDH of *B. methanolicus* C1 in quaternary structure, cofactor composition, tightly but non-covalently bound NAD(P)(H) and enzymic properties (Bystrykh *et al.*, 1993b; Hektor *et al.*, 2002; van Ophem *et al.*, 1993; Vonck *et al.*, 1991). The MDO also has enzymic properties different from those of the *B. methanolicus* C1 MDH: MDO exhibits DMNA-dependent MDH, NADH-dependent formaldehyde reductase and formaldehyde dismutase activities (Hektor *et al.*, 2000), while *B. methanolicus* C1 MDH only displays NAD-dependent MDH and NADH-dependent formaldehyde reductase activities (Arfman *et al.*, 1989). The Gram-positive bacterium *Rhodococcus* sp. NI86/21 (Nagy *et al.*, 1995) and the methanogen *Methanosarcina barkeri* (Daußmann *et al.*, 1997) also possess a DMNA-dependent nicotinoprotein alcohol:DMNA oxidoreductase, but the enzymes exhibit no activity with methanol as a substrate.

There have been extensive studies on the functions and regulation of genes involved in the oxidation of methanol in Gram-negative methylotrophic bacteria (Arps *et al.*, 1995; Harms *et al.*, 1987; Kalyuzhnaya *et al.*, 2008; Morris *et al.*, 1995; Stephens *et al.*, 1988; Toyama *et al.*, 2003; van

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Abbreviations: ADH, alcohol dehydrogenase; DMNA, *N,N'*-dimethyl-4-nitrosoaniline; MDH, methanol dehydrogenase; MDO, methanol: *N,N'*-dimethyl-4-nitrosoaniline oxidoreductase; PQQ, pyrroloquinoline quinone.

The GenBank/EMBL/DDBJ accession numbers for the *orf1*, *orf2*, *mdo* and *orf3* sequences of *Mycobacterium* sp. strain JC1 identified in this study are GQ161963–GQ161966.

Spanning *et al.*, 1991; Zhang & Lidstrom, 2003). On the other hand, very little is known about the physiological role and regulation of the genes for the key enzymes responsible for the oxidation of methanol in Gram-positive methylotrophic bacteria. Only the genes for NAD-dependent MDHs of *B. methanolicus* strains C1 (de Vries *et al.*, 1992) and MGA3 (Brautaset *et al.*, 2004) have been cloned and sequenced, and several genes, including the gene for MDH, involved in the methylotrophy of *B. methanolicus* MGA3 were identified to be transcriptionally upregulated in cells growing with methanol (Jakobsen *et al.*, 2006).

Mycobacterium sp. strain JC1 can grow on methanol as the sole source of carbon and energy (Cho *et al.*, 1985; Ro *et al.*, 1997b; Song *et al.*, 2002). In previous experiments (Park *et al.*, 2003; Ro *et al.*, 1997a), *Mycobacterium* sp. strain JC1 grown with methanol did not exhibit activities of the classical PQQ-containing MDH in Gram-negative bacteria, the NAD-dependent PQQ-containing MDH and cytochrome *c*-dependent MDH of *A. methanolica*, the NAD-dependent MDH of methylotrophic *Bacillus*, or the NAD-dependent glutathione-requiring MDH and methanol oxidase of methylotrophic yeasts (Anthony & Zatman, 1967; Arfman *et al.*, 1989; Duine *et al.*, 1984; Kato *et al.*, 1975; Mehta, 1975; Tani *et al.*, 1972). Instead, the bacterium exhibited MDO activity during growth on

methanol (Park *et al.*, 2003), suggesting that this bacterium may use MDO as a key enzyme for the oxidation of methanol. However, it has not been identified whether MDO is the enzyme necessary for *Mycobacterium* sp. strain JC1 to grow with methanol as sole carbon and energy sources.

In this study, we cloned and characterized the gene for MDO (*mdo*) in *Mycobacterium* sp. strain JC1 and, through complementation analysis, showed that the *mdo* gene encodes the enzyme responsible for the oxidation of methanol in this bacterium.

METHODS

Bacterial strains, plasmids, and cultivation conditions. The bacterial strains and plasmids used in this work are described in Table 1. *Mycobacterium* sp. strain JC1 DSM 3803 was grown at 37 °C in standard mineral base medium (SMB; Kim & Hegeman, 1981) supplemented with 1% (v/v) methanol (SMB-MeOH), 0.5% (w/v) methylamine (SMB-MeNH₂) or 0.2% (w/v) glucose (SMB-glucose), or in Luria-Bertani medium (LB). Cells grown with methanol were harvested in the mid-exponential growth phase, washed once with 10 mM potassium phosphate buffer (pH 7.0, standard buffer), and stored at -20 °C. For cultivation on plates, cells were grown on solid medium containing SMB supplemented with 1% (v/v) methanol, 0.5% (w/v) methylamine or 1% (w/v) glucose in the presence or

Table 1. Bacterial strains, phages and plasmids used in this study

Strain, phage or plasmid	Genotype or description	Reference or source
<i>Mycobacterium</i> sp.		
JC1	Wild-type (DSM 3803), Hyg ^s	Song <i>et al.</i> (2002)
MT3m	<i>mdo</i> -deficient mutant of JC1, Hyg ^r	This study
MT3c	MT3m carrying plasmid pHP7	This study
<i>E. coli</i>		
EPI100-T1 ^R	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>dlacZ</i> ΔM15 Δ <i>lacX74 recA1 endA1 araD139</i> Δ(<i>ara, leu</i>)7697 <i>galU galK λ⁻ rpsL nupG tonA</i>	Epicentre
DH5α	<i>supE44 lac169(φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Gibco BRL
Phages		
Lambda clone 1	Lambda clone containing <i>mdo</i> gene of <i>Mycobacterium</i> sp. strain JC1	This study
Plasmids		
pWEB	8179 bp cosmid vector, Amp ^r Neo ^r	Epicentre
pBluescriptII KS+	2961 bp plasmid derived from pUC19, Amp ^r	Stratagene
pGEM-T easy	3015 bp linear plasmid for direct subcloning of PCR product, Amp ^r	Promega
pCR2.1	3929 bp plasmid for TA cloning of PCR product, Amp ^r Kan ^r	Invitrogen
pDAS1	8621 bp reporter vector containing a promoterless <i>lacZ</i> gene, Hyg ^r	Seo <i>et al.</i> (2007)
pGOAL19	P _{Ag85} - <i>lacZ</i> P _{hsp60} - <i>sacB</i> <i>Pacl</i> cassette vector, Amp ^r Hyg ^r	Parish & Stoker (2000)
pNBV1	5.8 kb high-copy-number plasmid with pAL5000 replicon derived from p16R1, Hyg ^r	Howard <i>et al.</i> (1995)
pHP1	pCR2.1 harbouring 352 bp PCR product used as a template to synthesize random-primed <i>mdo</i> gene probes	This study
pHP2	pBluescript II KS+ containing a 4.4 kb <i>Bam</i> HI fragment from lambda clone 1	This study
pHP3	pBluescript II KS+ containing a 6.8 kb <i>Xho</i> I fragment from lambda clone 1	This study
pHP4	pDAS1 containing the putative promoter region of <i>mdo</i>	This study
pHP5	pBluescript II KS+ containing 1.0 kb <i>Sac</i> II fragment from <i>mdo</i> gene	This study
pHP6	pHP5 containing 1.5 kb <i>Kpn</i> I fragment from pGOAL19	This study
pHP7	pNBV1 containing 1352 bp PCR product for complementation of MT3m	This study

absence of 100 µg hygromycin ml⁻¹. *Escherichia coli* strains were grown at 37 °C in LB. *E. coli* DH5α was used as host for all plasmid constructions. For phage infection, cultures were grown in LB medium containing 0.2 % (w/v) maltose and 10 mM MgSO₄.

Purification of MDO. MDO was purified at 4 °C from 50 g of thawed cells, employing sonic treatment, ultracentrifugation, dialysis after ammonium sulfate fractionation (30–60 % saturation with respect to ammonium sulfate), concentration (Amicon Ultra-4) after gel filtration (Sephacryl S-300), DEAE-Sepharose column chromatography, and hydroxyapatite column chromatography. The final preparation contained 2.3 mg purified MDO. Protein concentrations were determined using the Bradford assay (Bradford, 1976) with BSA as a standard.

DNA manipulation. Chromosomal DNA was isolated from *Mycobacterium* sp. strain JC1 by the method of Goldberg & Ohman (1984). Plasmid DNAs were isolated from *E. coli* by the alkaline lysis method (Sambrook & Russell, 2001). Electrotransformation of *Mycobacterium* sp. strain JC1 was carried out with a Gene Pulser apparatus (Bio-Rad) at 2.5 kV, 800 Ω and 25 µF. Competent cells were prepared by the method of Dower *et al.* (1988). Phage lysate and DNA were prepared according to the manufacturer's instructions (Epicentre).

Random-primed probe synthesis. Two synthetic primers, MDO-5 and MDO-3, were synthesized to amplify the region covering the *mdo* gene of *Mycobacterium* sp. strain JC1, based on the amino acid sequences NQIWDF and LGNALY that are present near the N-terminus and in an internal region, respectively, of the purified MDO. Primers MDO-5 and MDO-3 were 2- and 32-fold degenerate 20-mer (5'-AACCAAATCTGGGAYTTCCC-3') and 18-mer (5'-GTASAGSGCGTTNCCSAG-3'), respectively. Peptides for internal sequence analysis were prepared by trypsin digestion of the purified MDO, followed by HPLC on a NovaPak HR C18 column (3.9 mm × 300 mm; Waters). Sequences of the native and peptide fragments were analysed as described by Edman & Begg (1967) using the Procise 491 sequencing system (Applied Biosystems). The PCR mixture contained 100 ng genomic DNA and 50 pmol of each primer, and 1.5 U Han-Taq polymerase (Genenmed) in a final volume of 50 µl. Amplification was carried out as follows: after primary denaturation for 3 min at 95 °C, 30 cycles each of denaturation for 40 s at 95 °C, annealing for 40 s at 52 °C and elongation for 40 s at 72 °C were performed, and then post-elongation was carried out for 10 min at 72 °C. The 0.7 kb PCR products digested with *Eco*RI were eluted from agarose gel after electrophoresis, purified with the QIAquick gel extraction kit (Qiagen), and cloned into pCR2.1 for sequencing. The production of random-primed probes of less than 0.4 kb was based on the sequence of the 0.7 kb cloned fragment using two nested primers, MDO-R5 (5'-CAAGGAGTTCCATCCGTTCC-3') and MDO-R3 (5'-GAACTCGTTGATGTTGCGGC-3'), with no degeneracy. Amplification was carried out using the same mixture and conditions as described above. The PCR products digested with *Eco*RI were purified from agarose gel and cloned into pCR2.1 for sequencing or used as templates to synthesize random-primed probes labelled with a digoxigenin (DIG)-high prime DNA labelling kit (Roche) for plaque hybridization, Southern blotting and Northern blot analysis.

Southern hybridization. DNA digested with restriction enzymes was subjected to electrophoresis and then transferred to Hybond-N⁺ membranes (Amersham) by capillary blotting. Hybridization with DIG-labelled probe and washing steps were carried out at 68 °C, according to the manufacturer's recommendations (Roche). Detection of positive bands or plaques was performed as described by Sambrook & Russell (2001).

Cloning and DNA sequencing. A genomic DNA library of *Mycobacterium* sp. strain JC1 was constructed in *E. coli* EPI100-T1^R using a pWEB cosmid cloning kit (Epicentre), following the manufacturer's instructions. Plaque replicas were screened with the labelled random-primed probes. DNA fragments were sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977). DNA and protein sequence analysis and comparisons were carried out using the ExPASy proteomics server (<http://ca.expasy.org/>), and the National Center for Biotechnology Information (NCBI) program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

RNA isolation. Total RNA was isolated from cells harvested at the mid-exponential growth phase by using TRIzol reagent (Invitrogen) as previously described (Seo *et al.*, 2007).

Northern blot analysis and primer extension. Northern blotting (Sambrook & Russell, 2001) was performed using the random-primed probes and total RNA. Primer extension (Sambrook & Russell, 2001) was performed with the avian myoblastosis virus reverse transcriptase primer extension system (Promega) and a 31-mer primer (5'-AGGGTGCCTTGAAACCCAGGTTCTTGGCCT-3') complementary to nucleotide positions 104–134 bp downstream of the *mdo* start codon.

RT-PCR. Three synthetic primer sets, ORF2-F and MDO-R1, MDO-F1 and MDO-R2, and MDO-F2 and ORF3-R, were synthesized to elucidate the transcription unit of the *mdo* gene by RT-PCR. ORF2-F (5'-AATCGAGCAGTCGGCGAACAG-3'), MDO-F1 (5'-ACCCC-AAGGACTACAACGTCA-3') and MDO-F2 (5'-TGACTCGTA-TGACAAGAACCG-3') were 21-mers corresponding to nucleotide positions 765–785 bp downstream of the *mdo* translation start site and 239–259 bp and 1077–1097 bp downstream of the *mdo* translation start site, respectively. MDO-R1 (5'-GAACGGATGGAACCTTGAT-3'), MDO-R2 (5'-GGCTGTGCGAAGAAGGCACTGA-3') and ORF3-R (5'-CGACAGGAACCTCTCGGTGTA-3') were 21-mers complementary to nucleotides 37–57 bp and 848–868 bp downstream of the *mdo* translation start site and 122–142 bp downstream of the *orf3* translation start site, respectively. The reaction mixture (13 µl) for RT-PCR contained 1 µg total RNA, 120 ng primer, 1 µl each of 10 mM dNTPs, 1 µl 0.1 M DTT and 200 U SuperScript II reverse transcriptase (RT; Invitrogen) in First-Strand buffer (Invitrogen). Reverse transcription was carried out following the manufacturer's instructions (Invitrogen). No-RT control reactions were set up with all ingredients except the RT. Chromosomal DNA was used as a positive control for the PCR. The resulting cDNA covering a part of the *mdo* gene only, the cDNAs that possibly contained parts of *orf2* and *mdo*, and the cDNAs of *mdo* and *orf3* were directly used for PCR. The PCR mixture contained 20 pmol of each primer, 100 ng cDNA template and 0.5 U Ex Taq polymerase (Takara) in a final volume of 20 µl. Amplification was carried out using the conditions described above for the random-primed probe synthesis.

Construction of reporter plasmid and transformation. Amplification of the putative promoter region of *mdo* that included an inverted repeat (GCAGCGTGCTGC) present 39–50 bp upstream of the *mdo* transcription start site and the putative –35 and –10 regions of the gene was carried out using two synthetic primers, each with a *Xba*I site (underlined lower-case type), designated LZ-F (5'-tctagaATCTACGAACCGTTCCACTTCGGCT-3') and LZ-R (5'-tctagaTCGTCATCAGCAGGGTGCCTTGAA-3'). These primers corresponded to nucleotide positions 209–233 bp upstream (LZ-F) and were complementary to nucleotides 121–145 bp downstream of the *mdo* translation start site (LZ-R). The PCR mixture and amplification conditions were the same as those used for the RT-PCR, except the mixture contained 100 ng genomic DNA template. The 377 bp PCR products were eluted from agarose gel after

electrophoresis, purified with the QIAquick gel extraction kit and cloned into the *Xba*I site of the promoterless pDAS1 vector (Seo *et al.*, 2007) to create a translational fusion to the *lacZ* gene resulting in reporter plasmid pHP4. Vectors were introduced into *Mycobacterium* sp. strain JC1 by electroporation.

Construction and complementation of an *mdo*-deficient mutant. A 1.0 kb *Sac*II fragment in pHP2 containing a partial *mdo* gene was cloned into pBluescript II KS+ (Stratagene) digested with *Sac*II, resulting in subclone pHP5. A 1.3 kb *Kpn*I fragment in pGOAL19 (Parish & Stoker, 2000), which contains a *hyg*^r gene, was then inserted into pHP5 digested with *Kpn*I to yield the suicide vector pHP6. pHP6 was introduced into *Mycobacterium* sp. strain JC1 by electroporation. An *mdo*-deficient mutant resistant to hygromycin and unable to grow with methanol was selected and designated MT3m. The fidelity of the marker insertion event in MT3m was confirmed by PCR using primers Mid-fw (5'-ATCAAGGAGT-TCCATCCGTT-3'), which corresponded to nucleotides 37–56 bp downstream of the *mdo* translation start site, and Mid-rv (5'-TCCTGCACGACTTCGAGGTGT-3'), which is complementary to nucleotides 83–103 bp downstream of the translational start codon of the *hyg* gene in pGOAL19.

The entire length of *mdo* was amplified using two synthetic primers that contained a *Pst*I site (underlined lower-case type), namely CM-F (5'-ctgcagAGTGGCTCGGTTTCATGGAC-3') and CM-R (5'-ctgcagGTAGCATTGCAGCCGGATCA-3'), which corresponded to nucleotide positions 89–108 bp upstream and to the complement of nucleotides 192–211 bp downstream of the *mdo* translational start and stop sites, respectively. The PCR products were cloned into pNBV1 digested with *Pst*I, resulting in plasmid pHP7. Plasmid pHP7 was then introduced into MT3m by electroporation and the complemented mutant, MT3c, was selected by the ability to grow with methanol in the presence of hygromycin.

Enzyme activity assay. MDO activity was assayed by measuring the methanol-dependent reduction of DMNA ($\epsilon_{440}=35\,400\text{ M}^{-1}\text{ cm}^{-1}$) as described by van Ophem *et al.* (1993). Methanol or other substrates were added to the reaction mixture to a final concentration of 50 mM. One unit of enzyme activity was defined as the amount of protein required to reduce 1 μmol DMNA min^{-1} . β -Galactosidase assays were performed as described previously (Seo *et al.*, 2007). Activity was expressed as Miller units per microgram of total cell protein. Cell-free extracts were prepared by the method of Seo *et al.* (2007).

RESULTS AND DISCUSSION

Purification of catalytically active MDO

The MDO in *Mycobacterium* sp. strain JC1 was purified 48-fold, with a yield of 6.4 %. The estimated molecular mass of the purified MDO protein was approximately 46 kDa. The purified MDO exhibited a specific activity of 0.48 μmol DMNA reduced $(\text{mg protein})^{-1}\text{ min}^{-1}$; it had comparable activity with ethanol (0.37 U mg^{-1}) and formaldehyde (0.35 U mg^{-1}), and weak activity with methylamine (0.04 U mg^{-1}) as substrates, but did not oxidize 1-butanol, propanol, acetaldehyde or glucose. In contrast, the MDO proteins from *A. methanolica* and *M. gastri* effectively oxidize butanol as well as propanol (Bystrykh *et al.*, 1993a).

Cloning and genetic characterization of the *mdo* gene

Using primers MDO-5 and MDO-3, which were synthesized based on the amino acid sequences of the N-terminal region and an internal region of purified MDO, a 0.7 kb DNA fragment of *Mycobacterium* sp. JC1 was amplified, cloned, and sequenced. A high degree of homology was found to parts of the 5'-region of the *mdo* gene from *A. methanolica* (data not shown). A 0.35 kb DNA fragment was then amplified to generate templates for random-primed probes using the two nested primers, MDO-R5 and MDO-R3, and the 0.7 kb DNA fragment as a template. Two positive clones were obtained by plaque hybridization of the lambda library with the random-primed probes. Southern blot hybridization of the insert DNA prepared from the lambda clones with the random-primed probes revealed that the 4.4 kb *Bam*HI and 6.8 kb *Xho*I fragments in lambda clone 1, which overlap by 1.7 kb, hybridized with the probes. The 9 kb region of the insert DNA containing the 4.4 kb *Bam*HI and 6.8 kb *Xho*I fragments was then subjected to restriction mapping (Fig. 1). Based on the restriction map, subclones pHP2 and pHP3 were constructed in pBluescript II KS+ for sequence analysis

Fig. 1. Genetic map of 9 kb region of insert DNA in lambda clone 1 containing the *mdo* gene of *Mycobacterium* sp. strain JC1. *Bam*HI (B), *Eco*RV (E), *Pst*I (P), *Sac*I (S), *Sal*I (A), *Sma*I (M) and *Xho*I (X) were used for restriction mapping. The probe binding site, a 6.8 kb *Xho*I fragment in pHP3 and a 4.4 kb *Bam*HI fragment in pHP2 are indicated below the map. The ORF for *mdo* is indicated with a filled arrow, and other ORFs found upstream and downstream of *mdo* are indicated with open arrows.

using the 4.4 kb *Bam*HI and 6.8 kb *Xho*I fragments, respectively.

Sequencing of the *Bam*HI and *Xho*I fragments revealed that the sequence of 2145 bp at the 3'-end of the *Xho*I fragment, which contained a putative *mdo* gene, was identical to that at the 5'-end of the *Bam*HI fragment, indicating that the 3'- and 5'-end regions of the *Xho*I and *Bam*HI fragments, respectively, are overlapping and contiguous (Fig. 1). The putative *mdo* gene consisted of 1272 bp and encoded a protein with a calculated molecular mass of 46 223 Da and a pI of 5.43. Parts of the amino acid sequence deduced from the nucleotide sequence of the ORF were identical to the N-terminal amino acid sequences of internal peptide fragments of the purified MDO protein (data not shown). The calculated molecular mass of the protein encoded by the cloned gene and the estimated molecular mass of a subunit of the purified enzyme indicate that the cloned gene was in fact the structural gene for MDO. The deduced amino acid sequence of MDO in *Mycobacterium* sp. strain JC1 was highly homologous (92.7 %) to that of *R. erythropolis* NI86/21 ThcE (Nagy *et al.*, 1995; GenBank accession no. AAB80771), for which no methanol-dependent activity was detected. However, the *Mycobacterium* sp. strain JC1 MDO sequence showed lower identity (67.0 %) to that of the MDO of *A. methanolica* (van Ophem *et al.*, 1993; AAF21473). The discrepancy between the level of identity and substrate specificity in these three enzymes suggests that the amino acid sequences in regions other than the active centre have changed more rapidly than those in the active centre during evolution of the DMNA-dependent ADHs.

The *mdo* gene of *Mycobacterium* sp. strain JC1 starts and stops with ATG and TGA, respectively, and the G+C ratio of the cloned *mdo* was 64.3 mol%. Codon usage analysis showed a strong codon bias for Gs and Cs in the third position. The amino acid sequence deduced from the nucleotide sequence of the strain JC1 *mdo* gene was 67.6 % identical to that of the *A. methanolica* *mdo* gene (GenBank accession no. AAF21473). A potential Shine–Dalgarno sequence, AGAAGG, was found 9 bp upstream of the *mdo* translation initiation codon. An inverted repeated sequence (underlined), GCAGCGTGCTGC, was found 71–60 bp upstream of the *mdo* translational start site. Further analysis of the sequence also identified two complete upstream ORFs, 678 bp (*orf1*) and 1392 bp (*orf2*), and a complete downstream ORF (969 bp, *orf3*) located adjacent to the *mdo* ORF (Fig. 1). *orf1* is transcribed divergently from *mdo*, *orf2* and *orf3*, starts with the GTG located 1494 bp upstream of the *mdo* start codon, and codes for a protein of 225 amino acids with 40 % identity to the amino acid sequence of the TetR family transcriptional regulator of *Kribbella flavida* (GenBank accession no. EEJ20442). *orf2* and *orf3* encode proteins of 463 and 322 amino acids with 33 and 78 % identities to the amino acid sequences of the flavin-containing amine oxidase of *Rhodococcus opacus* (GenBank accession no. BAH48700) and the MoxR-like

ATPase of *Mycobacterium smegmatis* (GenBank accession no. ABK74204), respectively. *orf2* starts with the ATG located 1429 bp upstream of the *mdo* start codon, and *orf3* overlaps 23 bp with *mdo* to start with the ATG located 20 bp upstream of the *mdo* stop codon.

The *mdo* gene is transcribed into a monocistronic mRNA

Northern blot analysis using a 0.35 kb *Eco*RI insert in pHP1 labelled with DIG to probe *mdo* transcript in total RNA prepared from glucose- and methanol-grown cells revealed a band of less than 1.4 kb, which is close to the size of the *mdo* gene (1272 bp). This suggests that the *mdo* gene of *Mycobacterium* sp. strain JC1 is transcribed as a monocistronic message (Fig. 2a). RT-PCR using primers MDO-F1 and MDO-R2 (Fig. 3a) for PCR with total RNA as template produced a 0.6 kb product (Fig. 3b, lane 3) that is almost identical in size to the PCR product (629 bp) estimated from the nucleotide sequence of the *mdo* gene. However, no PCR products were generated using the primer sets ORF2-F and MDO-R1 or MDO-F2 and ORF3-R (Fig. 3a) with total RNA as the template (Fig. 3b, lanes 2 and 4, respectively). These results further support the conclusion from the Northern blot data that the *mdo* gene is transcribed independently of *orf2* and *orf3* located upstream and downstream of the gene, respectively (Fig. 2a).

Fig. 2. Inducible transcription of *mdo* in cells grown in the presence of methanol. (a) Northern hybridization with random-primed probes synthesized by using the 0.35 kb *Eco*RI fragment in pHP1 as templates and total RNA (20 μ g each) prepared from cells grown on glucose (GL) or methanol (ME). (b) β -Galactosidase assays of *mdo* promoter-*lacZ* fusions in *Mycobacterium* sp. strain JC1. β -Galactosidase activity was measured with cells harbouring pDAS1 (black bars) and pHP4 (grey bars), which contain a promoterless *lacZ* gene and a putative *mdo* promoter-*lacZ* fusion fragment, respectively, after growth on glucose (GL) or methanol (ME). Units of β -galactosidase activity are nmol ONPG hydrolysed min⁻¹ (mg protein)⁻¹.

Fig. 3. RT-PCR of *mdo* and other ORFs in *Mycobacterium* sp. strain JC1. (a) Positions of primers used for RT-PCR to identify the transcriptional unit of *mdo*. (b) Production of DNA fragments after RT-PCR with appropriate primers. RT-PCR was carried out using total RNA from cells grown with methanol, with primers ORF2-F and MDO-R1 (lane 2), MDO-F1 and MDO-R2 (lane 3), and MDO-F2 and ORF3-R (lane 4) to detect the *orf2*-*mdo*, *mdo* and *mdo*-*orf3* transcripts, respectively. RT-PCR using primers MDO-F1 and MDO-R2 in the absence of reverse transcriptase (lane 1) was used as a negative control. The extra bands at the bottom of lanes 1–4 are the primers.

Primer extension analysis identified the transcriptional start site of *Mycobacterium* sp. strain JC1 *mdo* as the nucleotide A located 21 bp upstream of the *mdo* start codon (Fig. 4). Investigation of the upstream region of the transcriptional start site revealed that sequences similar to those of the group A promoter in mycobacteria were present in the –10 (TTCACG) and –35 (TGAAGT) regions of the *mdo* gene (Gomez & Smith, 2000). An inverted repeat sequence (underlined), GCAGCGTGCTGC, which may act as a binding site for a transcriptional regulator, was also identified 39–50 bp upstream of the transcriptional start site (Fig. 4). A putative transcriptional termination sequence was not found in the analysis of the 200 bp region downstream of the *mdo* stop codon.

Transcription of *mdo* is upregulated in cells growing on methanol versus cells growing on glucose

The expression pattern of the gene for MDH in Gram-negative facultative methylotrophic bacteria is known to be variable. MDH from *Paracoccus denitrificans* is undetectable in cells grown with multi-carbon compounds, but expressed in cells grown under limited carbon conditions

Fig. 4. Analysis of the *mdo* promoter region. (a) Primer extension assay. The transcriptional site of the *mdo* gene was identified by primer extension analysis using a 32 P-labelled 31-mer oligonucleotide primer complementary to nucleotide positions 104–123 bp downstream of the *mdo* start codon. Extension products were analysed (lane PE) in parallel with the sequencing ladder (lanes G, A, T, and C) primed with the same primer. The asterisk indicates the transcription start site. (b) Promoter region of *mdo*. The inverted repeat is represented in bold with symbols (> or <). The transcriptional start site of *mdo*, as determined by primer extension, is shown in bold with an arrow. The putative Shine–Dalgarno sequence is indicated in bold, and the start codon of *mdo* is shown in bold italic.

(de Vries *et al.*, 1988). On the other hand, the MDH in *Methylobacterium* strains was expressed in cells grown with multi-carbon substrates and was present at higher levels in cells grown with methanol (Lidstrom & Stirling, 1990; Xu *et al.*, 1993). The expression of the MDH gene in the Gram-positive bacterium *B. methanolicus* MGA3 was also upregulated in cells growing with methanol (Jakobsen *et al.*, 2006). Northern blot analysis revealed constitutive expression of the *mdo* transcript in *Mycobacterium* sp. strain JC1 grown with glucose or methanol (Fig. 2a). The higher intensity of the band in RNA prepared from methanol-grown cells indicates that the *mdo* gene is inducible in cells growing with methanol, similar to the genes for MDHs in *Methylobacterium* strains and *B. methanolicus* MGA3. The β -galactosidase assays with *Mycobacterium* sp. strain JC1 harbouring a reporter plasmid containing a translational fusion of the *lacZ* gene to the 377 bp putative *mdo* promoter region indicated that the activity in cells grown on methanol was 98-fold higher than that of the cells grown on glucose (Fig. 2b), which further confirms that the *mdo* gene of *Mycobacterium* sp. strain JC1 is constitutively expressed yet significantly induced in the presence of methanol.

Fig. 5. Schematic diagram showing homologous recombination of the *mdo* gene in *Mycobacterium* sp. strain JC1. The mutant candidates capable of growing in the presence of hygromycin were further examined by cultivation in SMB-MeOH and confirmed by PCR using primers Mid-fw and Mid-rv (1.3 kb product is arrowed).

An *mdo*-deficient mutant cannot grow on methanol as sole source of carbon and energy and is complemented by introducing an intact *mdo* gene

An *mdo*-deficient strain of *Mycobacterium* sp. strain JC1, strain MT3m, was generated by homologous recombination of the *mdo* gene with plasmid pHP6 containing *hyg^r* and a partial sequence of *mdo* (Fig. 5). The MT3m strain, which contained the *mdo* gene interrupted by pHP6 DNA, grew on SMB-glucose, but not on SMB-MeOH, in the presence of hygromycin. This indicated that MDO was responsible for the oxidation of methanol in *Mycobacterium* sp. strain JC1. Complementation of strain

MT3m with plasmid pHP7, containing an intact *mdo* gene, restored the ability to grow with methanol as the sole carbon and energy source (Fig. 6), further supporting the conclusion that MDO is the key enzyme for the oxidation of methanol in *Mycobacterium* sp. strain JC1. The complementation tests also identified that there was no polar effect in the mutant strain being caused by the mutation in the *mdo* gene, since an active *mdo* gene alone was sufficient to allow MT3m to grow with methanol.

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Fig. 6. Growth of *Mycobacterium* sp. strain JC1 in the presence of methanol. Wild-type (●), *mdo*-deficient mutant MT3m (▼) and mutant MT3m complemented with an intact *mdo* gene (MT3c, ○) were cultivated aerobically at 37 °C in SMB-MeOH.

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