Environmental Microbiology (2007) **9**(4), 944–953 doi:10.1111/j.1462-2920.2006.01216.x

# A multicopper oxidase is essential for manganese oxidation and laccase-like activity in *Pedomicrobium* sp. ACM 3067

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## Summary

***Pedomicrobium* sp. ACM 3067 is a budding-hyphal bacterium belonging to the α-*Proteobacteria* which is able to oxidize soluble $Mn^{2+}$ to insoluble manganese oxide. A cosmid, from a whole-genome library, containing the putative genes responsible for manganese oxidation was identified and a primer-walking approach yielded 4350 bp of novel sequence. Analysis of this sequence showed the presence of a predicted three-gene operon, *mox*CBA. The *mox*A gene product showed homology to multicopper oxidases (MCOs) and contained the characteristic four copper-binding motifs (A, B, C and D) common to MCOs. An insertion mutation of *mox*A showed that this gene was essential for both manganese oxidation and laccase-like activity. The *mox*B gene product showed homology to a family of outer membrane proteins which are essential for Type I secretion in Gram-negative bacteria. *mox*BA has not been observed in other manganese-oxidizing bacteria but homologues were identified in the genomes of several bacteria including *Sinorhizobium meliloti* 1021 and *Agrobacterium tumefaciens* C58. These results suggest that *mox*BA and its homologues constitute a family of genes encoding an MCO and a predicted component of the Type I secretion system.**

## Introduction

Under aerobic conditions several species of phylogenetically distant bacteria are able to oxidize $Mn^{2+}$ to form insoluble manganese oxides. $Mn^{2+}$-oxidizing strains belonging to the genera *Bacillus*, *Cytophaga*, *Leptothrix*, *Pedomicrobium*, *Oceanospirillum* and *Pseudomonas* have been identified (Ghiorse, 1984). These bacteria can increase the rate of manganese oxidation to approximately five times that of non-biological oxidation at pH < 8.5 (Nealson *et al*., 1988; Tebo, 1991). There is also increasing evidence that manganese oxides and manganese-oxidizing and -reducing bacteria play an important role in carbon cycling in the natural environment (Nealson and Myers, 1992).

Previous research into the mechanism of bacterial manganese oxidation has concentrated on three bacterial species. These are *Pseudomonas putida* strains MnB1 and GB-1, *Leptothrix discophora* SS-1 and *Bacillus* sp. SG-1 (van Waasbergen *et al*., 1996; Corstjens *et al*., 1997; Brouwers *et al*., 1999; Francis and Tebo, 2001). In all three species manganese oxides were found deposited outside the cell and this led to the conclusion that the $Mn^{2+}$-oxidizing factor was located either extracellularly or in the outer membrane (Adams and Ghiorse, 1987; Okazaki *et al*., 1997). There is now clear experimental evidence that bacterial $Mn^{2+}$ oxidation is an enzymatic process; in all these bacterial strains an apparent Km for $Mn^{2+}$ was determined to be in the μM range and the addition of classical enzyme inhibitors such as $HgCl_2$, KCN, $NaN_3$ and *o*-phenanthroline all caused a reduction in $Mn^{2+}$-oxidizing activity. Taken together the data suggested that $Mn^{2+}$-oxidizing enzymes shared similar biochemical properties (Adams and Ghiorse, 1987; Okazaki *et al*., 1997).

The genes involved in $Mn^{2+}$ oxidation in *Ps. putida*, *L. discophora* and *Bacillus* sp. SG-1 have been identified (van Waasbergen *et al*., 1996; Corstjens *et al*., 1997; Brouwers *et al*., 1999). The genes for $Mn^{2+}$ oxidation appeared to be part of a two-gene operon, in the case of *Ps. putida,* a three-gene operon in the case of *L. discophora* and a seven-gene operon in *Bacillus* sp. SG-1. In these operons the protein encoded by *cum*A in *Ps. putida*, *mof*A in *L. discophora* and *mnx*G in *Bacillus* showed significant homology to a class of copper-binding proteins known as multicopper oxidases (MCOs).

Multicopper oxidases are a class of copper proteins that contain multiple bound copper atoms and link the four-electron reduction of molecular oxygen to the oxidation of

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a second substrate (Solomon *et al*., 1996). There are many examples of MCOs found in nature including the well-characterized human ceruloplasmin and the fungal laccases and in recent years several MCOs have also been identified in bacteria (Grass and Rensing, 2001; Huston *et al*., 2002; Lee *et al*., 2002). These MCOs have a wide-range of substrates including $Fe^{2+}$ for ceruloplasmin and Fet3p of yeast (Bonaccorsi di Patti *et al*., 2000) and phenolic compounds and manganese for the fungal laccases (Schlosser and Hofer, 2002; Nagai *et al*., 2003). It has also been demonstrated that some MCOs have the ability to oxidize more than one substrate and many can oxidize artificial substrates such as ρ-phenylenediamine (Bonaccorsi di Patti *et al*., 2000), 2,2′ azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Brown *et al*., 2002) or DMP (Sanchez-Amat *et al*., 2001).

In bacterial MCOs there are four bound copper atoms per protein molecule. Conserved histidine, cysteine and methionine residues give rise to four distinctive copper-binding motifs in the primary amino acid sequences of these proteins. It should be noted, however, that individual copper-binding motifs provide ligands to more than one of the bound copper atoms and therefore work in concert to bind the four coppers. These four motifs were named A, B, C and D in their order in the primary structure of CumA and Mof*A* (Corstjens *et al*., 1997; Brouwers *et al*., 1999).

The MCOs involved in manganese oxidation, CumA, MofA and MnxG, show very little homology to each other with the exception of the four copper-binding motifs. For many MCOs the copper-binding residues are located at the N- and C-terminus. In the case of CumA and MofA, the A and B motifs are located at the N-terminus and C and D motifs at the C-terminus. However, the region separating the two pairs differs considerably in length. The MnxG of *Bacillus* SG-1 differs yet again, as the copper-binding motifs are transcribed in a different order, C and D being at the N-terminus and A and B being at the C-terminus, although these motifs are separated by a similar number of amino acids as in CumA (van Waasbergen *et al*., 1996).

*Pedomicrobium* sp. ACM 3067 is a manganese-oxidizing strain of *Pedomicrobium* which was isolated from manganese-related 'dirty water' from a water distribution system with a manganese oxide-encrusted biofilm (Sly and Arunpairojana, 1987; Sly *et al*., 1988). The genus *Pedomicrobium* comprises Gram-negative, budding-hyphal bacteria belonging to the α-*Proteobacteria* (Cox and Sly, 1997). They are found in aquatic environments and, with the exception of *Pedomicrobium ferrugineum* which oxidizes $Fe^{2+}$, all species (*Pedomicrobium americanum*, '*P. australicum*', *P. manganicum*) are able to oxidize soluble $Mn^{2+}$ to insoluble manganese oxide (Gebers, 1981; Gebers and Beese, 1988) which is accumulated on extracellular polysaccharides on the cell surface (Ghiorse and Hirsch, 1979; Sly *et al*., 1990).

Previous work on *Pedomicrobium* sp. ACM 3067 showed that manganese oxidation by this strain was an enzymatic process (Larsen *et al*., 1999). Further investigation also showed that manganese oxidation is dependent on the presence of copper ions which, when taken together with the observation that other manganese-oxidizing enzymes are homologous to MCOs, led to the conclusion that an MCO homologue may also be responsible for manganese oxidation in *Pedomicrobium* sp. ACM 3067 (Larsen *et al*., 1999).

Here we report the detection and characterization of a gene encoding an MCO homologue, *mox*A, in *Pedomicrobium* sp. ACM 3067. We also report the sequence of the proximal genes and the identification of a putative manganese oxidation (*mox*) operon. In addition, the essential role of the product of this gene in manganese oxidation is unambiguously demonstrated through the characterization of a targeted manganese oxidation-deficient mutant of *Pedomicrobium* sp. ACM 3067.

## Results

### *Detection of putative MCO gene*

Using whole cells of *Pedomicrobium* sp. ACM 3067 as template polymerase chain reaction (PCR) with the primer pair Alpha-AF/Alpha-BR2 was carried out in an attempt to amplify the A to B region of the putative MCO gene. This approach yielded an amplicon of the predicted size, approximately 150 bp. This product was cloned into the plasmid pGEM-T Easy and the insert sequenced using the universal M13F and M13R primers. Analysis of the resultant sequence showed that the region internal to the binding sites of the Alpha primers showed homology to other predicted MCO genes. This result confirmed that the amplicon obtained was most likely a portion of an MCO gene homologue. The *Pedomicrobium* sp. ACM 3067-specific sequence obtained was used as the basis for the design of a specific PCR primer, 3067-AF, which was then used in conjunction with the degenerate Alpha-CR primer in PCR. This combination yielded an approximately 400 bp amplicon which, when sequenced, showed significant homology to other MCO genes. These results confirmed that *Pedomicrobium* sp. ACM 3067 has an MCO-like gene.

### *Screening of the genomic cosmid library*

The primer combinations validated above were used to screen a whole-genome cosmid library of *Pedomicrobium* sp. ACM 3067. Several cosmid clones yielded an appropriately sized amplicon in an initial screen using the 3067-AF/3067-AR primer pair. These clones were then further screened using the 3067-AF/Alpha-CR primer pair. This approach identified a single cosmid clone within the

 

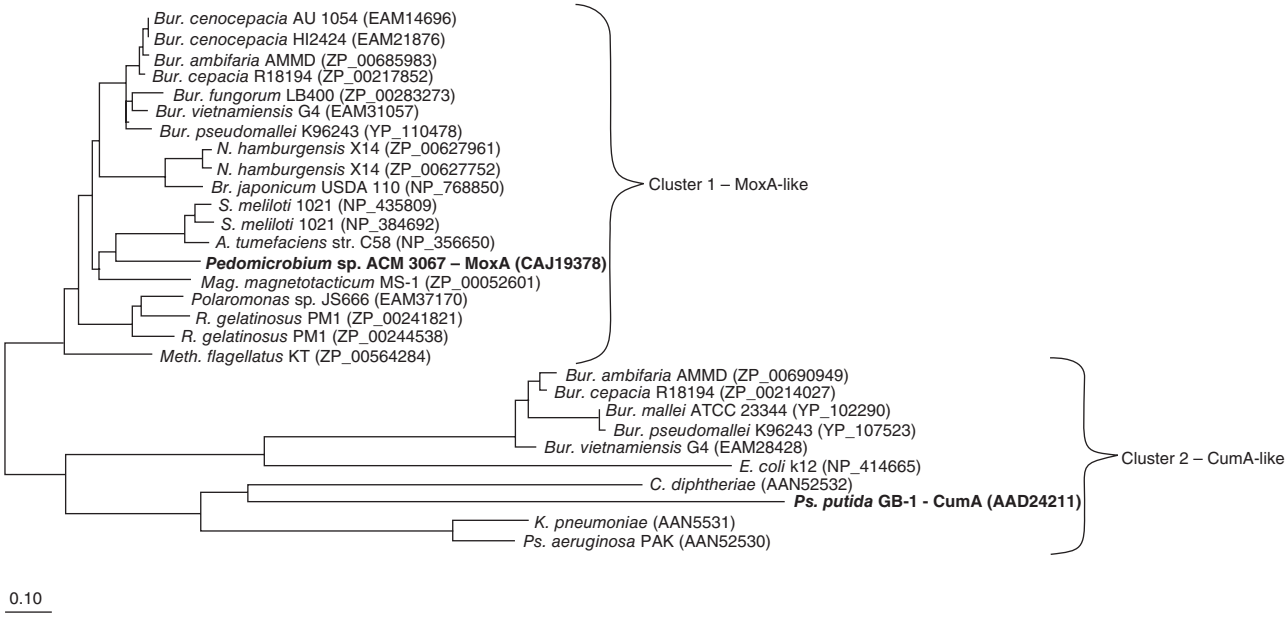


**Fig. 1.** A neighbour-joining phylogenetic tree of the aligned amino acid sequences of selected bacterial MCO homologues showing the relationship of MoxA-like (Cluster 1) and CumA-like (Cluster 2) homologues. *Bur.*, *Burkholderia*; *N.*, *Nitrobacter*; *Br.*, *Bradyrhizobium*; *S.*, *Sinorhizobium*; *A.*, *Agrobacterium*; *Mag.*, *Magnetospirillum*; *R.*, *Rubrivivax*; *Meth.*, *Methylobacillus*; *E.*, *Escherichia*; *C.*, *Corynebacterium*; *Ps.*, *Pseudomonas*; *K.*, *Klebsiella*. Accession numbers are given in brackets.

library which contained the putative MCO gene designated *mox*A.

### *Sequencing the* mox *locus*

The sequence of the 400 bp PCR amplicon was used as the basis for the design of primers to sequence the *mox*A gene and proximal genes in the cosmid clone using a primer-walking approach. Sequence data obtained were compiled into a single contiguous piece using the Staden package (Staden *et al*., 1998). Further sequencing was carried out until the entire length of 4350 bp had been sequenced for both strands with two to six times coverage. Several open reading frames (ORFs) were identified and further analysis predicted that three of these ORFs are present in a single operon, one of which was the putative MCO homologue, *mox*A.

*MoxA – a putative MCO.* An ORF (1428 bp, 476 aa) was identified in the *mox* locus which contained the MCO-like sequences obtained from the sequencing of the 150 and 400 bp amplicons. Further analysis of the predicted protein encoded by this ORF showed it to contain the four conserved copper-binding motifs indicative of an MCO and this putative gene was designated *mox*A.

Using the derived amino acid sequence of *mox*A a BLASTP search of the GenBank database identified homologous proteins. Among these were the known manganese-oxidizing proteins of *P. putida* (CumA) as well as other well-characterized MCOs such as PcoA (Lee *et al*., 2002). The manganese-oxidizing proteins of *L. discophora* SS-1 (MofA) and *Bacillus* SG-1 (MnxG) were not present in the top 500 matches. This result was not unexpected as these proteins are considerably different in length (1662 aa and 1216 aa respectively) to MoxA (477 aa). In addition to this the copper-binding motifs of MnxG are present in a different order to the other manganese-oxidizing MCOs as described above (van Waasbergen *et al*., 1996).

Overall, there was a high degree of homology seen between the copper-binding motifs of MoxA and the copper-binding motifs of CumA but the homology of the remainder of the proteins was quite poor. This is demonstrated by the large distance between MoxA and CumA in a phylogenetic analysis (Fig. 1). In MoxA the relative position of the four copper-binding motifs was quite different when compared with the arrangement seen in CumA. In CumA and indeed many other MCOs, the A, B, C and D motifs are arranged such that the A and B motifs are near the N-terminus of the protein and the C and D motifs are located at the C-terminus. In the MoxA protein the A and B motifs are located near the N-terminus as expected; however, the C and D motifs are located in a far more distal position from the C-terminus as compared with CumA (Fig. 2). It was also noted that despite CumA and MoxA differing in the relative positions of the copper-binding motifs the overall length of the protein is very similar (460 aa and 476 aa respectively).

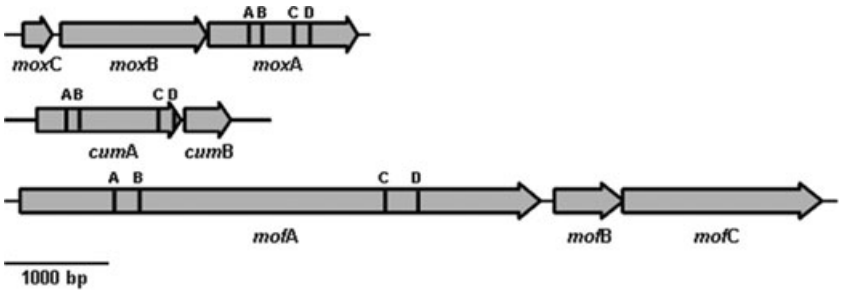


**Fig. 2.** A diagrammatic representation of the operon structure for the manganese oxidation genes of *Pedomicrobium* sp. ACM 3067 (*mox*CBA), *Pseudomonas putida* GB-1 (*cum*AB) (Brouwers *et al*., 1999) and *Leptothrix discophora* SS-1 (*mof*ABC) (Brouwers *et al*., 2000).

MoxA was also predicted to contain an N-terminal signal sequence which would target this protein to the periplasm/outer membrane. This signal sequence contained a motif (NRRSFMA) which showed some homology to the consensus twin-arginine translocation (TAT) motif (SRRxFLK) which would target a protein to the TAT system. A TatP analysis (http://www.cbs.dtu.dk/services/TatP-1.0/) also predicted this signal sequence to be of the TAT family.

*MoxB – a TolC-like protein.* A second ORF (1488 bp, 496 aa) was identified upstream of *mox*A and was designated *mox*B. This ORF was present in the same frame as *mox*A. Several homologues to MoxB were identified in the GenBank database although the majority of these were predicted proteins from genome-sequencing projects. It was also found that MoxB was predicted to belong to the TolC family, a family of outer membrane proteins (OMPs) which are involved in Type I secretion, and was predicted to have an N-terminal signal sequence.

*MoxC.* A small ORF (273 bp, 91 aa), *mox*C, was identified upstream of *mox*B and was predicted to be part of the operon containing *mox*BA. This ORF was found to be in an alternate frame to *mox*BA and to overlap the start of *mox*B by 4 bp. A BLASTP and BLASTN search did not identify any significant homologues to MoxC with only a poor partial alignment to uncharacterized genes of several genome-sequencing projects. Further analysis identified a potential N-terminal signal sequence but was unable to predict a potential function for MoxC.

### *Phylogenetic analysis of MoxA*

The homologues of MoxA identified by the BLASTP search and additional bacterial MCO protein sequences of similar length to MoxA were subjected to a phylogenetic analysis. The MofA and MnxG protein sequences were excluded from the analysis due to constraints on the length of the proteins included in the analysis and the rearrangement of the copper-binding motifs seen in MnxG. Inclusion of such disparate sequences would have compromised the analysis due to large, potentially ambiguous, rearrangements in the alignment of the sequences leading to artificial phylogenetic inferences. A neighbour-joining phylogenetic tree was constructed from a CLUSTALW alignment of the inferred amino acid sequences (Fig. 1). Overall, the protein sequences formed two distinct clusters. MoxA and its homologues formed a cluster (Cluster 1, Fig. 1) distinct from the CumA-like homologues (Cluster 2, Fig. 1). The homology between the amino acid sequences of Cluster 1 and those of Cluster 2 was very poor and no inference can be made with respect to the evolutionary relationship between the amino acid sequences comprising each cluster. There was a high level of homology within Cluster 1 with pairwise identity $\geq$ 58.1%. Except for the MoxA sequence from *Pedomicrobium* sp. ACM 3067, all of the sequences in Cluster 1 were from whole-genome-sequencing projects and formed several clades. MoxA formed a clade with the homologues from *Sinorhizobium meliloti* 1021, *Agrobacterium tumefaciens* C58 and *Magnetospirillum magnetotacticum* MS-1. The homologues from the *Burkholderia* spp. formed a monophyletic group. The sequences from *Nitrobacter hamburgensis* X14 grouped with the sequence from *Bradyrhizobium japonicum* USDA 110 and the sequences of *Rubrivivax gelatinosus* PM1 grouped with the sequence from *Polaromonas* sp. JS666. The sequence of the *Methylobacillus flagellatus* KT homologue was the deepest branching sequence within Cluster 1.

Two non-identical copies of the genes encoding the MoxA homologue were detected in the genome sequences of *N. hamburgensis* X14, *R. gelatinosus* PM1 and *S. meliloti* 1021. In *S. meliloti* 1021, one copy of the *mox*A-like gene occurs on the chromosome (NP_384692) and a second copy on the pSymA megaplasmid (NP_435809). In *N. hamburgensis* X14 and *S. meliloti* 1021 the two homologues group together. However, the two homologues of *R. gelatinosus* PM1 do not group together (Fig. 1). One homologue (ZP_00241821) appears to be more closely related to the MoxA homologue of *Polaromonas* sp. JS666 (82.4% identity) than to the second *R. gelatinosus* PM1 homologue (ZP_00244538, 79.7% identity).

 

The phylogenetic analysis revealed that four species of *Burkholderia* (*Bur. ambifaria* AMMD, *Bur. cepacia* R18194, *Bur. pseudomallei* K96243 and *Bur. vietnamiensis* G4) possess both MoxA-like and CumA-like homologues.

### *MoxA is required for $Mn^{2+}$ oxidation and laccase-like activity*

The function of MoxA was determined by creating a *moxA* knockout mutant. The 400 bp PCR product produced using the 3067-AF/Alpha-CR primer pair was first cloned into the vector pGEM-T Easy. An EcoRI/EcoRI fragment encompassing the *mox*A fragment was then subcloned into the vector pJP5603 and the resultant construct used to transform the conjugation competent *Escherichia coli* strain S17-1 λ*pir*. The construct was transferred into *Pedomicrobium* sp. ACM 3067 via conjugation and potential mutants were selected for on *Pedomicrobium* standard medium (PSM) Km plates.

Twelve $Km^R$ mutants were screened for manganese oxidation activity on PC plates containing $Mn^{2+}$. One mutant, ML2, which did not show the characteristic accumulation of brown precipitate, was selected for further characterization.

*Phenotypic characterization.* The ML2 mutant was characterized for its ability to oxidize $Mn^{2+}$ and the artificial laccase substrate ABTS. As can be seen in Fig. 3, ML2 was unable to form the characteristic brown precipitate in the presence of $Mn^{2+}$ and was also unable to oxidize ABTS to the characteristic dark green colour seen for the wild type. This observation confirmed the hypothesis that the *mox*A gene did indeed encode an MCO homologue and that this gene was essential for both $Mn^{2+}$ oxidation and ABTS oxidation.

*Genotypic characterization.* To confirm that the *mox*A gene was interrupted as intended a genotypic characterization of the ML2 mutant was carried out. Southern blots using either the 400 bp 3067-AF/Alpha-CR PCR product or a 1 kb PstI/PstI fragment of pJP5603 as a probe were carried out. As can be seen in Fig. 4A the *mox*A-specific probe hybridized to a single band in the *Pedomicrobium* sp. ACM 3067 wild type at approximately 1.7 kb in the SacII-digested genome and approximately 7.6 kb in the XhoI-digested genome. In the ML2 mutant the same probe bound to a band of approximately 6.3 kb in the SacII-digested genome and approximately 9.9 kb in the XhoI-digested genome. The increase in size seen for the hybridized bands was consistent with the insertion of the pJP5603-MCO plasmid (3.5 kb) into the *mox*A gene.

The insertion was further confirmed by the second Southern blot (Fig. 4B) where the pJP5603-specific probe did not hybridize to the wild-type genome and hybridized to bands in the ML2 genome which were of the same size as those seen in the *mox*A-specific blot. These observations confirmed that the *mox*A gene had indeed been interrupted by the insertion of the pJP5603-MCO plasmid.

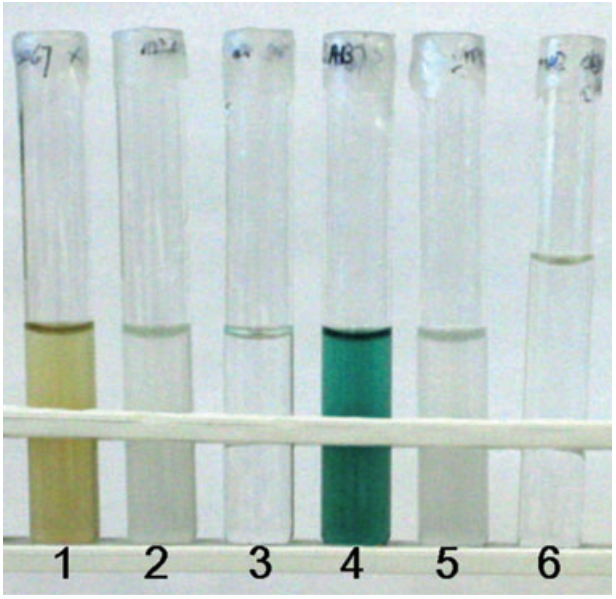


**Fig. 3.** Phenotypic observation of Mn oxidation and laccase-like activity of wild-type *Pedomicrobium* sp. ACM 3067, knockout mutant *Pedomicrobium* sp. ML2 and control. Tube 1, ACM 3067 plus $Mn^{2+}$ tube 2, ML2 plus $Mn^{2+}$ tube 3, 10 mM Hepes plus $Mn^{2+}$ tube 4, ACM 3067 plus ABTS; tube 5, ML2 plus ABTS; tube 6, 10 mM Hepes plus ABTS.

## Discussion

The results above represent the first identification and characterization of a metabolic gene in the budding-hyphal bacterium *Pedomicrobium*. We have shown that this gene has homology to MCOs and is essential for both manganese oxidation and laccase-like activity in *Pedomicrobium* sp. ACM 3067. We have demonstrated that this gene, *mox*A, and its homologues have a distinct form and are present in a gene arrangement not observed for other manganese-oxidizing MCOs. We have also shown that the *mox*BA gene pair has uncharacterized homologues in several bacteria, none of which have been reported to oxidize manganese. We therefore suggest that *mox*BA homologues constitute an emerging family of genes.

### *Cellular location of MoxA and MoxB*

It has been shown in the other bacterial manganese oxidation systems that the factors responsible for manganese oxidation are found either in the outer membrane or extracellularly (Adams and Ghiorse, 1987; Brouwers

 

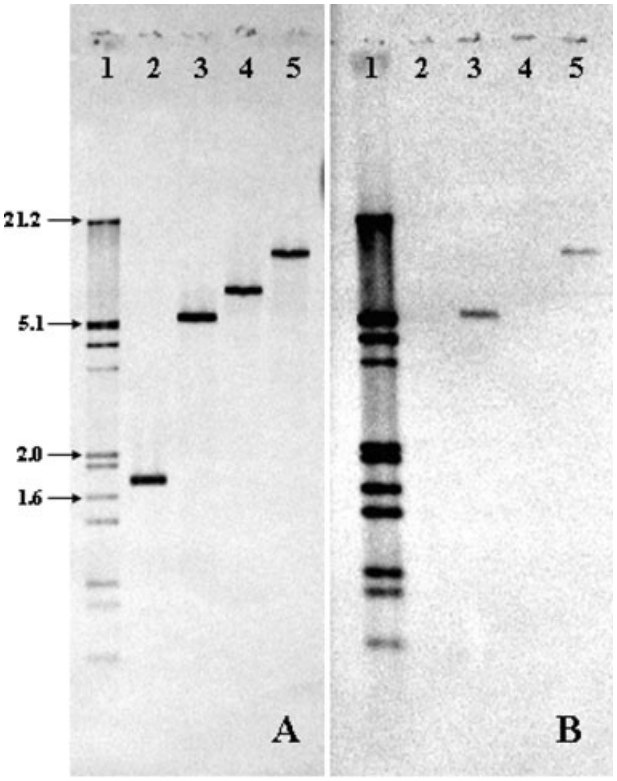


**Fig. 4.** A Southern blot hybridization of SacII- or XhoI-digested wild-type *Pedomicrobium* sp. ACM 3067 and knockout mutant *Pedomicrobium* sp. ML2 genomic DNA probed with DIG-labelled MCO fragment (A) and PstI fragment of pJP5603 (B). Lane 1, DIG-labelled molecular weight marker III (Roche Applied Science); lane 2, ACM 3067 digested with SacII; lane 3, ML2 digested with SacII; lane 4, ACM 3067 digested with XhoI; lane 5, ML2 digested with XhoI. Molecular weights of representative bands of the marker are given in kb.

*et al*., 1998; Francis *et al*., 2002; de Vrind *et al*., 2003). It has also been previously shown in *Pedomicrobium* sp. ACM 3067 that manganese oxidation activity is associated with the membrane fraction (Larsen *et al*., 1999). The presence of a putative N-terminal signal sequence in both MoxA and MoxB is therefore not unexpected. It is interesting to note that the MoxA signal sequence shows high homology to the TAT family of signal sequences. This family differs from the sec-dependent pathway in that proteins targeted to the TAT pathway are exported to the periplasm in a folded state (Palmer *et al*., 2005). The presence of a predicted TAT pathway signal is a common feature of many other MCOs.

### *Conservation of the* mox*BA pairing*

During the course of analysis of the *mox*A and *mox*B gene products it was noted that many of the high-ranking homologues identified for each of the genes were from the same genome-sequencing projects. Further investigation showed that four recently completed genomes, *A. tumefaciens* C58 (Wood *et al*., 2001), *Br. japonicum* USDA 110 (Kaneko *et al*., 2002), *Bur. pseudomallei* K96243 (Holden *et al*., 2004) and *S. meliloti* 1021 (Galibert *et al*., 2001), and 10 unfinished genomes in GenBank, *Burkholderia cenocepacia* AU 1054, *Bur. cenocepacia* HI2424, *Bur. cepacia* R18194, *Bur. fungorum* LB400, *Bur. vietnamiensis* G4, *Mag. magnetotacticum* MS-1, *Meth. flagellatus* KT, *N. hamburgensis* X14, *Polaromonas* sp. JS666 and *R. gelatinosus* PM1, contained homologues to both MoxA and MoxB and that the relative arrangement of these was identical, in that the MoxB homologue was present immediately upstream of the MoxA homologue.

In many cases further analysis identified a small gene upstream of the MoxB homologues which, similar to MoxC in *Pedomicrobium* sp. ACM 3067, overlapped the start of the MoxB homologues. However, there appeared to be very little homology between any of these small genes at the amino acid level.

To date none of these species has been reported to oxidize manganese although, to the best of our knowledge, there are no reports in which manganese-oxidizing ability has been tested. In our hands *S. meliloti* 1021 is unable to oxidize $Mn^{2+}$ when used to inoculate media containing $MnCl_2$ (J.P. Ridge, unpubl. obs.). This leads to the question as to what role these genes play in these bacteria.

### *A new form of MCO?*

The high level of homology seen between the MoxA homologues and the lower homology seen between these proteins and other known bacterial MCOs (Fig. 1) leads us to suggest that this form of MCO, where the A/B and C/D motifs are located close together on the primary sequence and are followed by a relatively long C-terminal region, may constitute a distinct family of MCOs. This hypothesis is given some weight by the apparently common arrangement of the *mox*BA genes in a variety of bacteria. This arrangement differs markedly from the other known manganese oxidation operons where the MCO is transcribed first followed by one or two secondary genes. The differences are further highlighted by the fact that *mox*B in *Pedomicrobium* shows no significant homology to *cum*B of *Ps. putida* GB-1, or *mof*B and *mof*C of *L. discophora* SS-1. Although there appears to be little homology among them, *mox*C and its equivalent genes appear to be a feature of some of these gene clusters.

It must also be noted that in the genome sequences of *N. hamburgensis* X14, *R. gelatinosus* PM1 and *S. meliloti* 1021 the *mox*BA-like genes occur twice and that the two copies are not completely identical. Further, in *S. meliloti*

 

**Table 1.** Strains and plasmids used.

| Strain | Characteristics | Source/reference |
|---|---|---|
| *Escherichia coli* | | |
| DH5α | General cloning strain | Invitrogen |
| XL1-Blue MR | Cloning strain for genome cosmid library | Stratagene |
| JM109 λ*pir* | JM109 with λ*pir* on chromosome | Penfold and Pemberton (1992) |
| S17-1 λ*pir* | Conjugative strain λ*pir* and mobilizing factors on chromosome | Penfold and Pemberton (1992) |
| *Pedomicrobium* sp. | | |
| ACM 3067 | Wild-type isolate | Sly and colleagues (1988) |
| ML2 | Knockout mutant of *Pedomicrobium* sp. ACM 3067, $Km^r$, $MnOx^-$ | This study |
| Plasmid and vectors | | |
| SuperCos1 Cosmid vector | Cosmid library vector, $Ap^r$ | Stratagene |
| pGEM-T-Easy | General cloning vector, $Ap^r$ | Promega |
| pJP5603 | Mobilizable suicide vector, $Km^r$ | Penfold and Pemberton (1992) |
| pGEM-T-Easy-MCO | pGEM-T-Easy vector containing 400 bp putative MCO fragment from *Pedomicrobium* sp. ACM 3067, $Ap^r$ | This study |
| pJP5603-MCO | pJP5603 suicide vector containing 420 bp EcoRI fragment from pGEM-T-Easy-MCO, $Km^r$ | This study |

1021, one copy of the *mox*BA-like genes occurs in the chromosome and a second closely related copy occurs on the pSymA megaplasmid.

Bioinformatic and phylogenetic analyses (Fig. 1) also revealed that several bacteria including *Bur. ambifaria* AMMD, *Bur. cepacia* R18194, *Bur. pseudomallei* K96243 and *Bur. vietnamiensis* G4 possess MCO-like homologues of both the MoxA and CumA forms.

Considered together, these results suggest that the contribution of MCOs to bacterial metabolism is more complex than previously appreciated and that more knowledge of the function and regulation of these different enzymes is required.

### *A putative transport role for MoxB?*

It is interesting to hypothesize that MoxB may play a role in protein secretion in *Pedomicrobium* sp. ACM 3067. MoxB shows significant homology to a family of proteins which are involved in the secretion of target proteins from the cytoplasm bypassing the periplasmic space. The archetypal protein in this family is the well-studied TolC protein of *E. coli* (Koronakis *et al*., 1997). TolC is an OMP which exists as a trimer and interacts with a cytoplasmic membrane bound complex consisting of ATP-binding cassette (ABC) proteins and membrane fusion proteins (MFP). This OMP:ABC:MFP complex allows the secretion of substrate proteins bypassing the periplasmic space (Delepelaire and Wandersman, 2001).

### *Conclusion*

In conclusion, we have proven that the *mox*A gene is essential for both manganese oxidation and laccase-like activity in *Pedomicrobium* sp. ACM 3067. We have shown that MoxA differs markedly from the previously characterized bacterial MCOs. We have also shown that *mox*A and *mox*B have closely related genes in the genome sequences of other bacteria and that these are also present in the same *mox*BA arrangement seen in *Pedomicrobium* sp. ACM 3067. We propose that the MCO and predicted OMP encoded by *mox*A and *mox*B, respectively, and the homologues seen in other bacteria, may belong to an emerging family of MCO genes in which the secretion of the MCO may involve a Type I secretion system. Further research will be required to confirm this hypothesis. The findings highlight that while the ability to oxidize manganese is a common feature of putative MCOs in several different species of bacteria the genes and proteins involved appear to be quite different.

## Experimental procedures

### *Microorganisms and culture conditions*

Bacteria, plasmids and vectors used in this study are shown in Table 1. *Pedomicrobium* sp. ACM 3067 was grown shaking at 28°C in a modified PSM containing 10 mM sodium acetate, 0.5 g of yeast extract (Difco Laboratories) and 10 ml of vitamin supplement per litre (Gebers, 1981) or in PC medium containing 0.02 g of $MnSO_4 \cdot 4H_2O$ and 0.05 g of yeast extract per litre (Tyler and Marshall, 1967). *Escherichia coli* strains were grown at 37°C in Luria–Bertani (LB) medium. When required, agar was added to 1.5% (w/v) prior to autoclaving. Antibiotics were added when required at the following concentrations: ampicillin, 100 μg $ml^{-1}$; kanamycin, 50 μg $ml^{-1}$.

### *DNA purification*

For genomic DNA preparations 3-day-old *Pedomicrobium* cells grown in PSM broth were recovered by centrifugation at 2000 *g*, and DNA was extracted using the Promega Wizard Genomic DNA Purification Kit. Cosmids were typically purified from an overnight 500 ml culture of *E. coli* using a Qiagen Plasmid Midi Kit following the manufacturer's instruction. Plasmid DNA was purified using a Qiagen Plasmid Mini Kit following the manufacturer's instructions.

 

**Table 2.** Primers used in this study.

| Primer | Sequence 5′–3′ | Target | Reference |
|---|---|---|---|
| Alpha-AF[a] | WSIWBIRTICAYTGGCAYGG | α-*Proteobacteria* MCO Copper-binding motif A | This study |
| Alpha-BR2[b] | GGRTGRTACMWRWAISWICC | α-*Proteobacteria* MCO Copper-binding motif B | This study |
| Alpha-CR | CCRTGIAIRTGIAWIGGRTG | α-*Proteobacteria* MCO Copper-binding motif C | This study |
| 3067-AF | GGGCAGACTCTGCCCTGCGG | *Pedomicrobium* MCO Copper-binding motif A | This study |
| 3067-BR | CACCATGGTCTTGCCGGGC | *Pedomicrobium* MCO Copper-binding motif B | This study |
| M13F | GTAAAACGACGGCCAG | Sequencing primer | Promega |
| M13R | CAGGAAACAGCTATGAC | Sequencing primer | Promega |

**a.** F represents forward primer.
**b.** R represents reverse primer.

### *Design of PCR primers and phylogenetic analysis*

Based on amino acid sequence of the *Ps. putida* GB-1 MCO, CumA, a BLASTP search (http://www.ncbi.nlm.nih.gov/blast/) was performed in the GenBank sequence database to identify copper-binding motifs of MCOs in the *Proteobacteria*. The amino acid sequences of the four conserved copper-binding motifs designated A, B, C and D of the MCOs were aligned according to their predicted protein sequences using the ARB software (Ludwig *et al*., 2004). The degenerate primers Alpha-AF, Alpha-BR2 and Alpha-CR were designed to target the conserved copper-binding motifs A, B and C, respectively, of MCOs of the α-*Proteobacteria* (Table 2). Following sequencing of the 150 bp PCR product generated from *Pedomicrobium* sp. ACM 3067, genomic DNA using the degenerate Alpha-AF/Alpha-BR2 primer pair, the primers 3067-AF and 3067-BR were designed to specifically amplify an 80 bp region of the MCO homologue.

For phylogenetic analysis, deduced amino acid sequences of the relevant proteins were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/). Sequences were aligned using CLUSTALW and manually checked for validity. The ARB software (Ludwig *et al*., 2004) was used to generate a neighbour-joining tree based on the CLUSTALW alignment.

### *Construction of* Pedomicrobium *sp. ACM 3067 whole-genome cosmid library*

The SuperCos 1 kit (Stratagene) was used to construct a whole-genome cosmid library according to the manufacturer's instructions. In brief, *Pedomicrobium* sp. ACM 3067 genomic DNA was extracted from a 3-day-old culture and partially digested with Sau3A (NEB). The digested fragments (size range of 30–42 kb) were ligated into the SuperCos1 BamHI cloning site. SuperCos1 Cosmid vector was digested with BamHI then phosphatase treated (Calf Intestinal Phosphatase, Stratagene) to minimize concatenation of vector during ligation to insert DNA. The ligation mixture was packaged using the Gigapack III XL packaging extract (Stratagene) and transfected into *E. coli* XL1 Blue MR cells grown at 37°C with shaking for 4.5 h. A library consisting of 800 clones was obtained and predicted to have full coverage of the *Pedomicrobium* genome.

### *Detection of* Pedomicrobium *sp. ACM 3067 putative MCO by PCR*

The primer pairs Alpha-AF/Alpha-BR2 and Alpha-AF/Alpha-CR (Table 2) were used to amplify fragments of the putative *Pedomicrobium* sp. ACM 3067 MCO. The PCR reactions were carried out in a total volume of 25 μl containing: 1× PCR buffer (Biotech International), 3 mM $MgCl_2$, 300 pmole of each primer, 0.3 mM dNTPs and 50 ng of *Pedomicrobium* sp. ACM 3067 genomic DNA as template. The reaction was heated to 95°C in a PTC-100 Programmable Thermal Controller (MJ Research) for 5 min, temperature held at 72°C, and 1 U of *Taq* DNA polymerase (Biotech International) was added to each tube. The PCR cycling conditions were: 35 cycles of 1 min at 95°C, 1 min at 48°C, 2 min 40 s at 72°C and a final elongation at 72°C for 10 min.

### *Cloning and sequencing*

Polymerase chain reaction products were cloned into the pGEM-T Easy Vector (Promega) according to the manufacturer's instructions and the resultant constructs sequenced. The purified PCR products or plasmid constructs were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Kit (Applied Biosystems) with the appropriate primer (Table 2). The sequences were determined on an Applied Biosystems Model 377 automatic DNA sequencer at the Australian Genome Research Facility (AGRF), The University of Queensland, Brisbane, Australia. To sequence the entire *mox* locus a primer-walking approach was used. Additional sequencing primers were designed as required using the novel sequence obtained as template. The sequence data obtained from this approach were compiled using the Staden package (Staden *et al*., 1998). The DNA sequence data generated in this study have been deposited in the EMBL Nucleotide Sequence Database under the Accession No. AM049177.

Gene and operon predictions were made using the FGENESB (http://www.softberry.com/) and WEBGENEMARK (http://opal.biology.gatech.edu/GeneMark/genemark24.cgi) programs. Homologous sequences were identified using the BLAST search at NCBI (http://www.ncbi.nlm.nih.gov/blast/).

 

Protein functional analyses were performed using the INTERPROSCAN program (http://www.ebi.ac.uk/InterProScan/).

### *Generation of MCO knockout mutant*

A 400 bp fragment of the putative MCO gene from *Pedomicrobium* sp. ACM 3067 was generated by PCR using the primers 3067-AF and Alpha-CR and cloned into the pGEM-T Easy Vector to form pGEM-T Easy-MCO. An EcoRI restriction enzyme cut fragment of this construct encompassing the MCO fragment was then cloned into phosphatase-treated pJP5603 (Penfold and Pemberton, 1992). This modified vector was called pJP5603-MCO and used to transform *E. coli* JM109 λ*pir*. pJP5603-MCO was extracted from *E. coli* JM109 λ*pir* then used to transform *E. coli* S17-1 λ*pir*. Finally this plasmid was introduced into *Pedomicrobium* sp. ACM 3067 by homologous recombination. Transconjugants were selected on PSM agar containing kanamycin.

### *Southern blotting*

Genomic DNA isolated from wild-type *Pedomicrobium* sp. ACM 3067 and knockout mutant *Pedomicrobium* sp. ML2 were each digested separately with the restriction enzymes SacII and XhoI and the fragments separated by electrophoresis on a 0.7% TAE agarose gel. The DNA was transferred from the gel to positively charged nylon membrane (Roche) using a vacuum blotter (Pharmacia LKB VacuGene XL). DNA was fixed to the membrane by exposure to UV light for 5 min. The membrane was then hybridized with the appropriate probe, labelled with DIG (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions, at 60°C overnight. Following hybridization and stringency washing, DIG-labelled probe hybridized to the immobilized DNA was detected using anti-DIG-alkaline phosphatase conjugate with colorimetric (NBT-BCIP) detection.

### *Phenotypic testing for Mn oxidation activity and laccase activity*

Three-day-old *Pedomicrobium* cells grown in 100 ml of PSM broth were recovered by centrifugation at 2000 *g*. The cells were resuspended in 10 mM Hepes buffer (pH 7) to an $OD_{540}$ of 0.5 and dispensed into glass test tubes. For manganese oxidation activity, $MnSO_4$ was added to give a final concentration of 10 p.p.m. Mn. Manganese oxidation was monitored by the formation of brown/black manganese oxide and confirmed by a positive blue reaction with leucoberbelin blue reagent (Krumbein and Altmann, 1973). For laccase-like activity ABTS was added to a concentration of 1 mM. ABTS oxidation was monitored by the formation of a dark green colour (Francis and Tebo, 2001).

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