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The multicopper oxidase of *Pseudomonas aeruginosa* is a ferroxidase with a central role in iron acquisition

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Summary

Recently it has been observed that multicopper oxidases are present in a number of microbial genomes, raising the question of their function in prokaryotes. Here we describe the analysis of an *mco* mutant from the opportunistic pathogen *Pseudomonas aeruginosa*. Unlike wild-type *Pseudomonas aeruginosa*, the *mco* mutant was unable to grow aerobically on minimal media with Fe(II) as sole iron source. In contrast, both the wild-type and mutant strain were able to grow either anaerobically via denitrification with Fe(II) or aerobically with Fe(III). Analysis of iron uptake showed that the *mco* mutant was impaired in Fe(II) uptake but unaffected in Fe(III) uptake. Purification and analysis of the MCO protein confirmed ferroxidase activity. Taken together, these data show that the *mco* gene encodes a multicopper oxidase that is involved in the oxidation of Fe(II) to Fe(III) subsequent to its acquisition by the cell. In view of the widespread distribution of the *mco* gene in bacteria, it is suggested that an iron acquisition mechanism involving multicopper oxidases may be an important and hitherto unrecognized feature of bacterial pathogenicity.

Introduction

With a few exceptions, iron is essential for growth in all bacteria (Ratledge and Dover, 2000). It is a component of haemoproteins, iron–sulphur proteins and a variety of other iron-containing proteins in the cell (Zaveckas *et al.*, 2000). Iron is the fourth most abundant element in the earth's crust, but this relative abundance is not matched by its availability. At neutral pH in an aerobic environment, free Fe(III) is limited to an equilibrium concentration of 10^{-17} M, much lower than the optimal concentration for growth of microbes (10^{-8} to 10^{-6} M). It is well established that free-living bacteria and some eukaryotes produce high-affinity Fe(III) chelators called siderophores that are capable of mobilizing iron for its uptake (Meyer *et al.*, 1996). Pathogenic bacteria are also confronted with an iron-restricted environment when they invade a host. Fe(III) is bound to lactoferrin and transferrin in the blood or is compartmentalized in the cell cytoplasm inside ferritin, an iron storage protein (Ratledge and Dover, 2000). Iron acquisition mechanisms associated with bacterial pathogenicity include production and uptake of siderophores (Braun and Killmann, 1999), uptake of iron from host sources (haem/haemoglobin, transferrin and lactoferrin) (Guerinot, 1994; Genco and Dixon, 2001) and direct uptake of the soluble form of iron (Fe(II)) (Braun and Killmann, 1999).

Mobilization and storage of iron in eukaryotes is a highly regulated process and involves redox reactions that interconvert the two forms of iron. Using yeast as a model eukaryotic organism, a high-affinity uptake system for iron was described that involved the initial oxidation of Fe(II) to Fe(III), followed by its transport into the cell by the iron permease Ftr1 (de Silva *et al.*, 1997; Hassett *et al.*, 1998a). These processes are catalysed by Fet3 and the iron permease Ftr1. Fet3 is a ferroxidase (de Silva *et al.*, 1995) that catalyses the reaction:

$$4Fe(II) + O_2 + 4H^+ \quad 4Fe(III) + 2H_2O$$

The Fet3 protein is a multicopper oxidase (MCO) and contains three distinct copper centres (de Silva *et al.*, 1997). Ceruloplasmin is the homologue of Fet3 that is found in the sera of all vertebrates (Sato and Gitlin, 1991) and has been assigned a number of biological functions over the last 50 years (Messerschmidt and Huber, 1990). However, it is now established that the central role for ceruloplasmin in mammals is in iron metabolism (Harris *et al.*, 1999).

In spite of the evidence that MCOs have a central role in iron metabolism in eukaryotes, the possibility that a similar system might operate in bacteria does not appear to have been considered. Indeed, the widespread presence of MCOs in prokaryotes has only recently been noted, following an analysis of bacterial genomes, in which Alexandre and Zhulin (2000) described bacterial MCOs as laccases. Laccases are a type of MCO that exhibit *p*-diphenol:O_2 oxidoreductase activity and are usu-

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ally found in plants and fungi. 'Laccase' activity is a property of almost all MCOs, and hence it is not informative in suggesting a specific physiological function. Emerging from recent studies is the indication of a link between MCOs and metal metabolism in bacteria. There is genetic and biochemical evidence that Mn(II) oxidation by manganese oxide-producing bacteria is catalysed by an MCO (Brouwers *et al*., 2000). In *Escherichia coli* it has been suggested that the MCO known as CueO (or YacK) is involved in resistance to Cu ions (Outten *et al*., 2000). The analysis of genome sequences of many pathogenic bacteria has revealed that they possess a MCO (see below), leading us to hypothesize that these MCOs might catalyse the oxidation of Fe(II) as part of an iron acquisition system. If this were the case, then they would have a function similar to the Fet3 protein of yeast. *Pseudomonas aeruginosa* was chosen to test this hypothesis. This bacterium is an important opportunistic pathogen of humans, the physiology and genetics of *P. aeruginosa* have been studied in depth and the entire sequence of the genome of *P. aeruginosa* was recently completed (*http://www.pseudomonas.com*) (Stover *et al*., 2000). In this paper we describe the phenotype of a *mco* mutant of *P. aeruginosa* and demonstrate that the MCO protein is critical for iron acquisition in this bacterium.

Results

Sequence analysis of the P. aeruginosa mco *gene*

A search for Fet3 homologues in the *P. aeruginosa* incomplete genome identified a single locus. Subsequent annotation of the complete sequence of the *P. aeruginosa* genome has identified this gene as *pcoA,* but, apart from the observation that this gene encodes an MCO-like protein, no assignment of function has been made (Stover *et al*., 2000). The *mco* gene of *P. aeruginosa* encodes a protein composed of 632 amino acids with a calculated molecular mass of 72 kDa. Figure 1 shows key segments of the deduced amino acid sequences of MCOs from *P. aeruginosa* and selected bacteria. At the N-terminus a secretory signal sequence was predicted using the Sigcleave program (Fig. 1A). The presence of a 'twin-arginine' signal sequence suggests that the MCO is secreted to the periplasm in a folded state via the Sec-independent 'twin-arginine translocation' (Tat) system (Sargent *et al*., 1998).

MCOs are characterized by three different types of Cu(II) centres (Solomon *et al*., 1996), and these have recently been observed in the crystal structure of the *E. coli* multicopper oxidase, CueO (Roberts *et al*., 2002). These redox centres enable the protein to couple four one-electron oxidation reactions to the four-electron reduction of oxygen to water. We compared the derived amino sequence of the MCO protein from *P. aeruginosa* and the CueO sequence (Fig. 1B). All of the amino acid residues that are critical for the formation of the type 1 and trinuclear copper sites in CueO are conserved in MCO protein from *P. aeruginosa*. The ligands His-101 (Cu_2), His-103 (Cu_2), His-141 (Cu_2) and His-143 (Cu_3), are conserved in both proteins. The residues His-443 (Cu_1), His-446 (Cu2), His-448 (Cu_3), His-499 (Cu_3), Cys-500 (Cu_1), His-501 (Cu_2), and Met-510 (Cu_1) found in *E. coli* are also present in the MCO of *P. aeruginosa,* although these residues can be found at amino acid residues 562, 565, 567, 610, 611, 612 and 619 respectively (Fig. 1B). The difference in the position of the two histidine/cysteine copper-binding regions (identified by residue number above) in the primary structure of *E. coli* CueO and *P.*

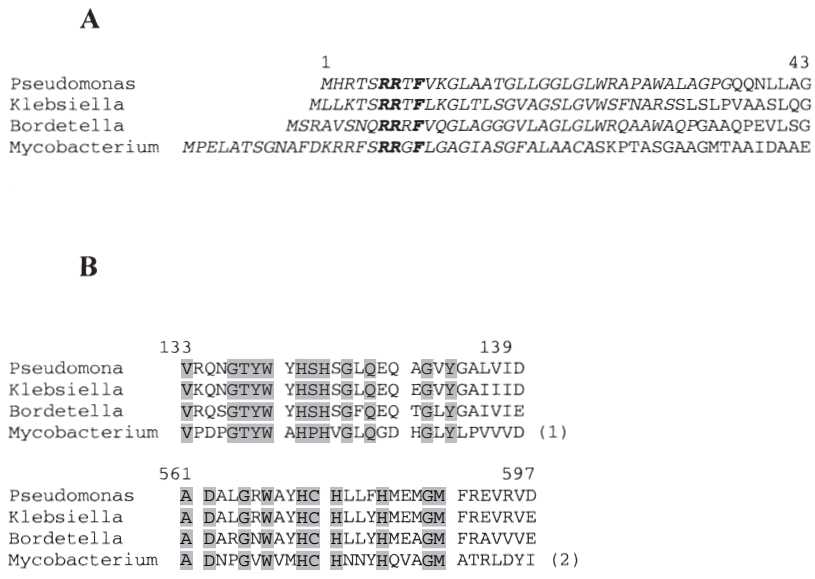

Fig. 1. Sequence analysis of multicopper oxidases from *P. aeruginosa* and selected bacteria.
A. Alignment of deduced N-terminal sequences of selected bacterial multicopper oxidases. Predicted signal sequences are shown in italics and the twin-arginine motif in bold. Sequences are numbered according to the MCO sequence (accession number AF455751).
B. The ClustlW alignment of the copper-binding segments of CueO from *E. coli*, with the homologous regions from the MCO (PcoA) protein from *P. aeruginosa.* The copper-binding ligands identified in the CueO crystal structure (PDB ID: IKV7) and the homologous residues in the MCO protein are shown in shading. Amino acid residues are numbered accordingly with CueO numbers above and MCO numbers below the alignment.

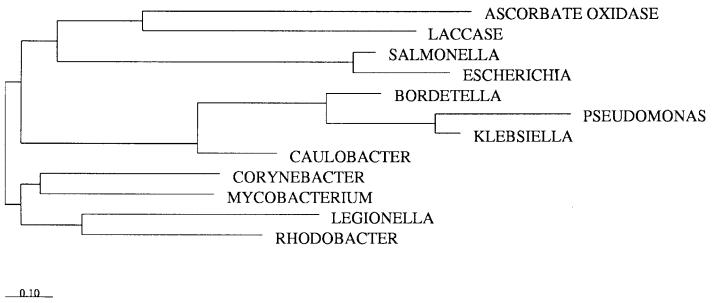

Fig. 2. Unrooted phylogenetic tree of bacterial and eukaryotic multicopper oxidases. The phylogenetic tree was constructed on the basis of amino acid level homology using the ARB program. The proteins included in the tree are: *Pseudomonas*, *P. aeruginosa* PAO1; *Mycobacterium*, *Mycobacterium tuberculosis*; *Corynebacter*, *Corynebacterium diphtheriae*; *Caulobacter*, *Caulobacter crescentus*; *Salmonella*, *Salmonella typhi*; *Escherichia*, *Escherichia coli* K-12 MG1655; *Bordetella*, *Bordetella pertussis*; *Klebsiella*, *Klebsiella pneumoniae*; *Rhodobacter*, *Rhodobacter capsulatus*; ascorbate oxidase, *Cucumis sativus* (eukaryotic: Cucumber); and laccase, *Pycnoporus cinnabarinus* (eukaryotic).

aeruginosa MCO arises from an additional 40 amino acid sequence found near the carboxy terminus of the *P. aeruginosa* protein sequence. This sequence is conserved in a number of the other MCO proteins that are phylogenetically closest to the MCO from *P. aeruginosa* (Fig. 2). This includes the MCOs from *Bordetella pertussis* and *Klebsiella pneumoniae*.

More distantly related bacterial MCO proteins are also identified in the unrooted evolutionary distance tree of putative bacterial MCOs (Fig. 2). This tree reveals that the MCOs from the enteric bacteria *E. coli* and *Salmonella typhi* and those from the pathogens *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae* form distinct lineages. Regardless of the phylogenetic relationships, alignment of these sequences revealed a high degree of identity in the signal sequence, indicating a common localization and also conservation of the key copper-binding ligands.

The MCO of P. aeruginosa *exhibits ferroxidase and* p*-phenylenediamine oxidase activity*

Having identified an MCO homologue in *P. aeruginosa,* we tested for activities associated with proteins of this family in cell-free extracts. No Mn(II)-oxidizing activity was detected, but Fe(II) oxidase (ferroxidase) and *p*-phenylenediamine oxidase activity was observed (data not shown). Furthermore, this ferroxidase and *p*-phenylenediamine oxidase activity could be detected in periplasmic preparations of *P. aeruginosa* PAK (data not shown), consistent with the expected localization of the MCO protein. Having established the presence of a functional MCO in *P. aeruginosa,* we purified this protein from the *P. aeruginosa mco* (pUCPSKmco) strain, as described in *Experimental procedures*. The purified MCO exhibited ferroxidase activity with a specific activity of 0.13 μmol Fe(II) oxidized mg protein^{-1} min^{-1}. Sodium fluoride (12 mM), the classical inhibitor of MCOs, caused an approximate 75% reduction in the rate of Fe(II) oxidation.

To confirm that the *mco* gene identified above encoded a protein with the observed activities and to investigate the physiological function of the MCO, a *mco* mutant was constructed in *P. aeruginosa* strain PAK. The genotype of the *mco* mutant was confirmed by polymerase chain reaction (PCR) analysis and Southern blotting. Figure 3A shows that *p*-phenylenediamine oxidase ('laccase') activity was present in the soluble extracts of wild-type cells but was absent in the *mco* mutant. In order to confirm that the loss of activity seen in the *mco* mutant strain was due to the inactivation of the *mco* gene, the mutation was complemented by introducing the *mco* gene *in trans* into the mutant strain using the construct pUCPSKmco. When this mutant was complemented, *p*-phenylenediamine oxidase activity was restored (Fig. 3A). This result confirmed that the putative *mco* gene encoded an active MCO protein with laccase activity. Figure 3B shows a Western blot of cell-free extracts of *P. aeruginosa* detected using antibody raised against recombinant *P. aeruginosa* MCO expressed in *E. coli*. The data clearly show that an immunoreactive polypeptide of apparent molecular mass 64 kDa is present in wild-type cells but is absent in the *mco* mutant. Complementation of the mutant with plasmid pUCPSKmco restored this polypeptide. These data confirm that the putative *mco* gene inactivated by mutation and complemented *in trans* encodes the MCO activity.

The mco *mutant is unable to grow aerobically on minimal media with Fe(II) as iron source*

In view of the central importance of Fet3 and its homologues in the uptake of iron in eukaryotic cells we tested whether the *P. aeruginosa* MCO also had a role in iron metabolism in this bacterium. To address this question, the growth of *P. aeruginosa* PAK*, P. aeruginosa mco* and *P. aeruginosa mco* (pUCPSKmco) was examined on RCV minimal medium with malate as carbon source. Growth experiments conducted aerobically in minimal media supplemented with Fe(II) as iron source (1.7 μM) revealed that the *mco* mutant has a dramatic phenotype; while wild-type strain PAK grew, no detectable growth of the *mco* mutant was observed (Fig. 4A). We attempted to restore aerobic growth of the *mco* mutant by supplementing the

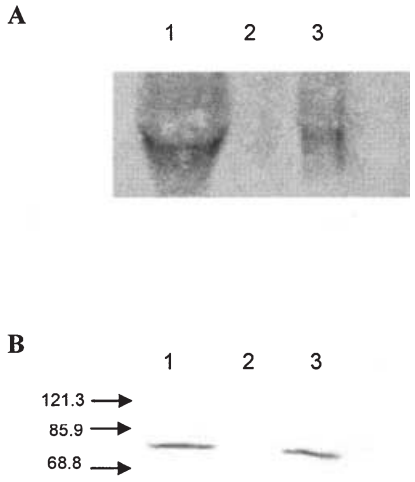

Fig. 3. Analysis of *p*-phenylenediamine oxidase activity and presence of the MCO protein in wild-type, *mco* mutant and complemented strains of *P. aeruginosa*.
A. Polyacrylamide gel of *P. aeruginosa* soluble extracts stained for *p*-phenylenediamine oxidase activity. Lane 1, *P. aeruginosa* strain PAK wild type; lane 2, *P. aeruginosa* PAK *mco*; lane 3, *P. aeruginosa* PAK *mco* (pUCPSKmco).
B. Western blot of MCO expression in *P. aeruginosa.* Lane 1, *P. aeruginosa* PAK wild type; lane 2, *P. aeruginosa* PAK *mco*; lane 3, *P. aeruginosa* PAK *mco* (pUCPSKmco). The position of molecular mass standards is indicated to the left of the figure.

media with high concentrations of Fe(II). As observed, there was no apparent growth of the mutant with 1.7 μM Fe(II) but slow growth, compared with wild-type cells, was observed with the addition of 170 μM Fe(II) (Fig. 4B). The complemented strain showed similar growth to that of the wild type (Fig. 4B) at all concentrations of Fe(II) tested.

The mco *mutation is conditional and can be suppressed by Fe(III) under aerobic conditions and by anaerobic conditions*

The above data were consistent with a central role for the MCO in Fe(II) acquisition under aerobic conditions in *P. aeruginosa*. As it is established that *P. aeruginosa* has an uptake system for Fe(III), we tested growth of wild-type cells and the *mco* mutant on minimal media under aerobic conditions with Fe(III) as sole iron source. In contrast to the observed differences in growth on Fe(II), the growth rate of wild-type and the *mco* mutant under aerobic conditions with Fe(III) did not greatly differ (Fig. 5A), although the rate of growth of the *mco* mutant was slightly lower, probably as a consequence of the presence of tetracycline in the medium**.** *P. aeruginosa* is a facultative aerobe, having the capability to grow anaerobically via denitrification (Philippot *et al.*, 2001). In *Helicobacter pylori* it has been demonstrated that the transporter FeoB is required for Fe(II) uptake under microaerophilic conditions (Velayudhan *et al.*, 2000). *P. aeruginosa* has a FeoB homologue, annotated in the genome as *feoB* (Stover *et al.*, 2000). The wild-type and *mco* mutant strains of *P. aeruginosa* both grew anaerobically on minimal media supplemented with 1.7 μM Fe(II) as an iron source in the presence of 15 mM KNO_3, although again the doubling time of the *mco* mutant was slightly higher (Fig. 5B). This demonstrated that the phenotype of the *mco* mutant was apparent only under aerobic conditions, consistent with the fact that the MCO would only function in iron acquisition under aerobic conditions as it requires molecular oxygen as a substrate. The above results are consistent with a central role for the MCO in the acquisition of Fe(II) for cell growth under aerobic conditions, analogous to the Fet3 ferroxidase of yeast and ceruloplasmin in mammalian systems (Hassett *et al.*, 1998b).

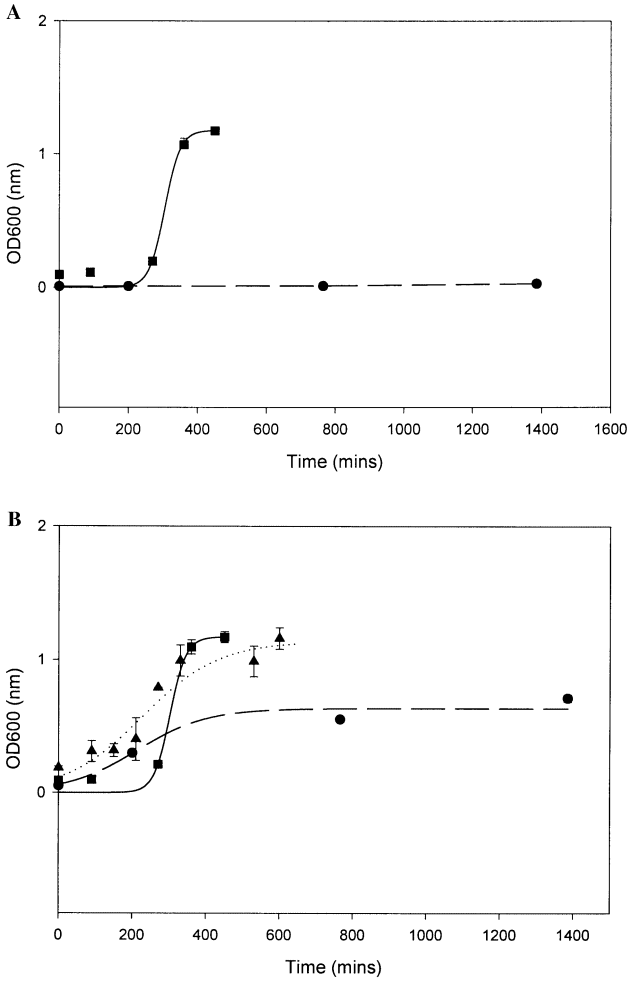

Fig. 4. Growth of *P. aeruginosa* strains at various Fe(II) concentrations in minimal media.
A. Fe(II) concentration: 1.7 μM; *Ps. aeruginosa* PAK wild-type: ■ (solid line); ● *P. aeruginosa mco*: (dashed line).
B. Fe(II) concentration: 170 μM; *Ps. aeruginosa* PAK wild-type: ■ (solid line); ● *P. aeruginosa mco*: (dashed line); *P. aeruginosa* PAK *mco* (pUCPSKmco) ▲ (dotted line).

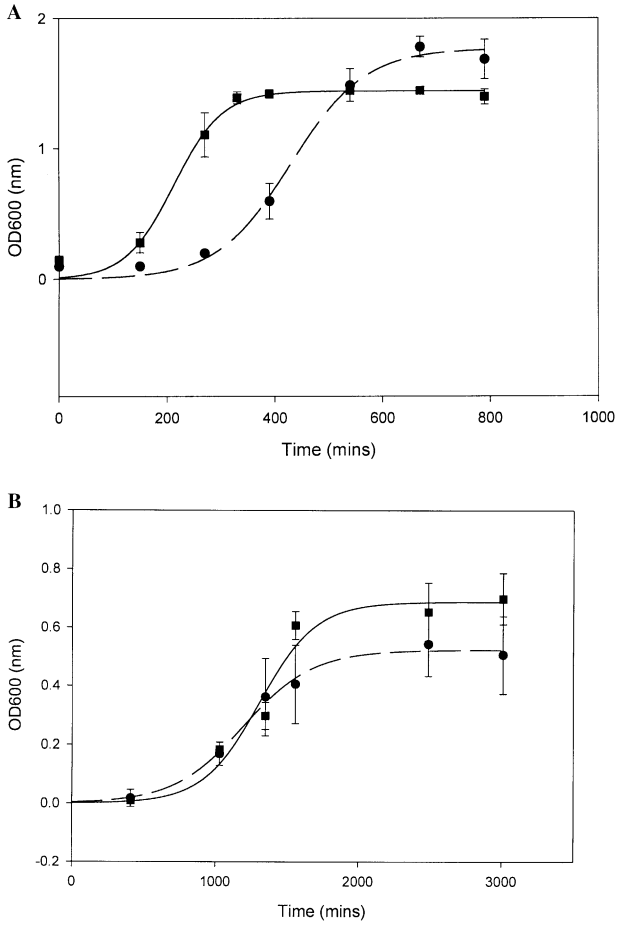

Fig. 5. Growth of *P. aeruginosa* strains under aerobic conditions with Fe(III) and anaerobic conditions with Fe(II) in minimal medium.
A. Aerobic growth in minimal medium. Fe(III) concentration: 1.7 μM; *P. aeruginosa* PAK wild-type: ■ (solid line); *P. aeruginosa mco*: ● (dashed line).
B. Anaerobic growth in minimal medium. Fe(II) concentration: 1.7 μM; *P. aeruginosa* PAK wild-type: ■ (solid line); *P. aeruginosa mco*: ● (dashed line).

The mco *mutant is defective in Fe(II) acquisition under aerobic conditions*

If the MCO is essential for Fe(II) acquisition under aerobic conditions, then this leads to the prediction that the rate of Fe(II) acquisition would be low in the *mco* mutant compared with wild-type *P. aeruginosa*. To test this, we grew wild-type and *mco* mutant strains and ran a series of assays to measure iron uptake using the ferrozine assay to monitor depletion of Fe(II) or Fe(III) from the medium. As described in *Experimental procedures*, cells of the two strains were first grown in Luria–Bertani (LB) medium supplemented with Fe(III) to attain a high cell density. Cells were then harvested by centrifugation and resuspended in the minimal casamino acid (CAA) medium. Following addition of Fe(II), its removal from CAA medium by wild-type *P. aeruginosa* over a 60 min period is shown in Table 1. In contrast, over the same time period the removal of Fe(II) by the *mco* mutant under identical conditions was fourfold lower. Fe(III) acquisition was similar in wild-type cells and the *mco* mutant, indicating that this iron uptake pathway was not affected by the *mco* mutation.

The mco *gene in* P. aeruginosa *does not appear to have a central role in copper tolerance*

It has been suggested that in *E. coli* the multicopper oxidase, CueO, is a component of a system involved in tolerance to low levels of copper ions (Outten *et al.*, 2001). This conclusion was based on the measurement of the final optical density reached by the aerobic cultures of *E. coli* wild-type and *cueO* mutant. It was observed that there was a slightly lower amount of biomass formed by the *cueO* mutant when the concentration of Cu(II) in the medium reached about 1 mM. To test whether the MCO of *P. aeruginosa* also had a role in resistance to copper ions, we carried out growth experiments at the critical concentration (1 mM Cu) at which the difference was previously reported (Outten *et al.*, 2001), measuring final OD. At this concentration no difference was observed between the wild-type and *mco* mutant. This leads us to conclude that there is no obvious role for the MCO protein in copper tolerance in *P. aeruginosa*.

Discussion

In this study we sought to test the hypothesis that the MCO of *Ps. aeruginosa* is a ferroxidase involved in iron acquisition. The loss of MCO activity in the *mco* mutant and reappearance of the activity in the complemented strain confirm that the MCO activity identified is encoded by the *mco* gene. Purification and characterization of the enzyme further confirmed the MCO as a ferroxidase; more detailed characterization of the enzyme will be presented in a future publication. The *P. aeruginosa* MCO contains all of the amino acids required to form the type 1 and trinuclear copper sites as found in the crystal structure of CueO, strongly suggesting that the active site in

Table 1. comparison of iron uptake in *P. aeruginosa* PKA and *mco* mutant.

Strain	Fe(II) activity	Fe(III) activity
P. aeruginosa PAK	1.21 (± 0.23)	0.61 (± 0.12)
P. aeruginosa mco	0.35 (± 0.14)	0.62 (± 0.21)

Iron uptake from CAA medium is shown in nmol min^{-1} mg^{-1} protein. Standard deviation is given in parentheses. A paired *t*-test confirmed the difference between the FE(II) uptake of *P. aeruginosa* PAK and *P. aeruginosa* (*P*-value of 0.0065).

the MCO protein is similar. The lack of aerobic growth with Fe(II) as an iron source in the *mco* mutant could not be completely reversed by the addition of very high concentrations of Fe(II) to the minimal media. This observation is consistent with the very low Fe(II) uptake rate observed in the *mco* mutant. These results establish that the MCO has a central role in the acquisition of Fe(II) during aerobic growth of this bacterium. We propose that under aerobic conditions Fe(II) entering the periplasm is oxidized by the MCO to Fe(III). Fe(II) is freely soluble and could reach the periplasm via porins in the outer membrane.

Pseudomonads are well known for their production of siderophores to chelate and transport extracellular Fe(III) into the cell (Venturi *et al*., 1995). It appears that this Fe(III) acquisition pathway operates independently of the MCO as the *mco* mutant was able to grow aerobically when Fe(III) was provided as an iron source. Siderophores appear to have a role in the virulence of *P. aeruginosa* (Venturi *et al*., 1995). However, a recent clinical study of *P. aeruginosa* isolated many strains from cystic fibrosis patients that lacked these siderophores (de Vos *et al*., 2001). This study raised the question of how iron might be acquired in these virulent isolates in the absence of siderophores. The MCO-mediated iron acquisition system identified here has the potential to explain the ability of siderophore-deficient *P. aeruginosa* isolates to acquire iron, although there may also be a role for a FeoB-linked Fe(II) acquisition system under anaerobic/microaerophilic conditions. Although most iron available to free-living and host-adapted *P. aeruginosa* is in the form of Fe(III), we note that redox mediator pyocyanin has been observed to mobilize iron from transferrin by reduction of sequestered Fe(III) to Fe(II). Indeed, Cox (1986a) raised the possibility that 'pyocyanin was participating in iron-solubilizing reactions and -releasing reactions and the Fe(II) product is somehow accumulated by the bacteria'. We propose that the MCO fulfils the missing link in this process by catalysing conversion of Fe(II) to Fe(III) in the periplasm. Hence, the MCO of *P. aeruginosa* may function as a component of a critically important iron uptake system that operates independently of siderophore-mediated iron uptake in *P. aeruginosa*. The MCO-dependent iron acquisition requires an Fe(III) permease. We found no evidence for the presence of a homologue of the yeast Ftr1 Fe(III) permease in the *P. aeruginosa* genome. However, an operon encoding a potential ABC cassette permease related to the Fe(III) transporters HitABC from *Haemophilus influenzae* and SfuABC from *Serratia marcescens* (Angerer *et al*., 1990; Adhikari *et al*., 1995) was identified (*Pseudomonas* genome: gene PA4687-PA4688) (W. M. Huston, M. P. Jennings and A. G. McEwan, unpublished observations).

The results reported herein lead us to propose that, in addition to its essential role in aerobic growth of free-living *P. aeruginosa* with Fe(II) as iron source, the MCO may also be a key component of the iron acquisition system during colonization of an animal host. This bacterium commonly causes infections in environments where oxygen would be available, for example burned skin or in the lungs. Colonization of the lungs of cystic fibrosis patients by *P. aeruginosa* and the related bacterium *P. cepacia* is a particular problem (Tablan *et al*., 1987). Under these conditions the MCO could be involved in iron acquisition from Fe(II) that is released as a consequence of inflammation-induced tissue damage.

Our phylogenetic analysis, as well as recent surveys done by other groups (Alexandre and Zhulin, 2000), has confirmed that MCOs are widely distributed in bacteria. Some of these proteins clearly have a role in Mn(II) oxidation (Larsen *et al*., 1999; Francis and Tebo, 2001). However, we note that the genes homologous to MCO in several of the pathogenic bacteria examined are highly conserved and possess many of the key features of the *P. aeruginosa* enzyme. All have twin-arginine signal sequences, suggesting a periplasmic localization, and contain Cu-binding domains typical of MCOs. It seems likely that many of these MCOs also have ferroxidase activity and may participate in a hitherto unrecognized iron acquisition mechanism. Indeed, it is already established that purified CueO from *E. coli* has a substantial ferroxidase activity that is of the same order of magnitude as its phenoloxidase activity (Kim *et al*., 2001). The evidence that CueO from *E. coli* is involved in copper tolerance is not convincing; the *cueO* mutant is only marginally different from wild-type cells in its copper tolerance profile (Outten *et al*., 2001). Our finding that there is no link between the presence of an MCO and growth at high Cu concentrations under aerobic condi-tions in *P. aeruginosa* reinforces this view. Although we cannot rule out a role for the MCO in oxidation of Cu(I) in the periplasm, we suggest that in fact the connection between the MCO and the regulators of copper homeostasis arises primarily from the requirement for Cu at the active site in the MCO. In *E. coli, cueO* expression is activated by copper and is dependent upon the trans-criptional activator CueR (Outten *et al*., 2000, 2001). CueR also activates expression of the copper efflux system encoded by *copA* (Stoyanov *et al*., 2001). The co-regulation of *cueO* and *copA* at low concentrations of copper may ensure that the CueO protein is present to bind copper that is expelled by the CopA system. Although this may impart slightly enhanced resistance to copper, which is gratuitous, our results for *P. aeruginosa* suggest that the primary role of CueO in *E. coli* and homologous multicopper oxidases in many other bacteria may also be Fe(II) uptake.

Experimental procedures

Bacterial strains and growth conditions

Strains used were: *E. coli* JM109 (Yanish-Perron *et al*., 1985), *E. coli* S17-1 λpir (Penfold and Pemberton, 1992) and *P. aeruginosa* PAK (Australian Culture Collection). All strains were routinely cultured on Luria–Bertani (LB) medium at 37°C. Antibiotics were used as follows: ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; tetracycline, 25 μg ml^{-1} for *E. coli* and carbenicillin, 300 μg ml^{-1}; tetracycline, 250 μg ml^{-1} for *P. aeruginosa*. *P. aeruginosa* strains were grown on modified RCV minimal medium (Weaver *et al*., 1975) at 37°C for all experiments involving physiological analysis and protein purification. This medium was prepared as described in the literature, except that $FeSO_4$ was omitted. Iron assays revealed that the concentration in 'iron-free' RCV medium was less than 1 μM. RCV medium, where specified, was also supplemented with filter-sterilized $FeSO_4{\cdot}7H_2O$ or $FeCl_3$. Aerobic growth of *P. aeruginosa* strains was conducted at 37°C in 250 ml conical flasks with shaking at 190 rpm. Anaerobic growth of *P. aeruginosa* using RCV medium supplemented with 15 mM KNO_3 was conducted with standing cultures in completely filled 30 ml McCartney bottles. Copper tolerance experiments were conducted in RCV medium containing 5 μM Fe(III) aerobically; appropriate concentrations of copper were supplemented to the media in the form of cupric sulphate. The *P. aeruginosa mco* mutant, complemented with the *mco* gene was grown in the presence of 0.5 mM isopropyl-β-D-thiogalactoside (IPTG).

Genetic techniques

An *mco* mutation was created in *P. aeruginosa* PAK by single crossover insertional mutagenesis. To achieve this, a 380-nucleotide internal region of the *mco* gene (annotated in the *P. aeruginosa* PAO1 genome sequencing project as PA2065 or *pco*A) (Stover *et al*., 2000) was generated by PCR amplification, using the primers Gene1F1 5′-ggtgaacctcagcggctcg-3′ and Gene1R1 5′-gcggcttctcgtccgacc-3′ and cloned into the suicide plasmid pJP5608 (Penfold and Pemberton, 1992) to create plasmid pWHmco. The mutant was generated by conjugation of *P. aeruginosa* with *E. coli* S17-1 λpir (pWHmco) as described by Saunders *et al*. (1984) with selection for colonies resistant to tetracycline at 250 μg ml^{-1}. The genotype of the *P. aeruginosa mco* mutant was confirmed by Southern blot and PCR analysis. The *mco* mutant was complemented by transformation with the plasmid pUCPSK-mco using the method of Saunders *et al*. (1984). This plasmid was generated by PCR amplification of the entire *mco* gene, including its putative promoter region, using primers PseudoF1 5-cgggatccgtccatgccattgtcctgcgc-3′ and PseudoR1 5′-gcgaattcgttcgtcaaaggcctcgccgc-3′ followed by directional cloning into pUCPSK, an IPTG-inducible vector (Watson *et al*., 1996), using the restriction enzymes *Bam*HI and *Eco*RI *(*underlined).

In order to purify the MCO for antibody production, the *mco* gene was cloned into plasmid expression vector pPROEX-hta (Gibco BRL). The *mco* gene was amplified using the primers gene1tagf 5′-gtggatccgctcggcggactgggtctctgg-3′ and gene1tag 5′-ggactagttcatgcgttgtctccttcgtctaccc-3′ and cloned into the vector using the restriction enzymes *Bam*HI and *Spe*I *(*underlined) to create plasmid pPROEXmco. The *mco* gene was sequenced from this construct using the ABI Big Dye Terminator System.

Cell fractionation and enzyme activity measurements

Cell pellets were obtained from cultures by centrifugation at 8000 ***g*** for 15 min and washed twice in 50 mM Tris pH 8.0 prior to resuspension in the same buffer. Total soluble cell-free extracts were prepared from cell suspensions by breakage in a French press (18 000 psi). Periplasmic and cytoplasmic fractions were prepared essentially as described by Hanlon *et al*. (1996) except that the lysozyme concentration used was 10 μg ml^{-1}. Membranes were removed from total soluble, periplasmic and cytoplasmic fractions by ultracentrifugation at 105 600 ***g*** for 90 min

Cell fractions from whole-cell lysates or periplasmic fractions were assayed for laccase activity via an in-gel assay. Following separation of samples using non-denaturing polyacrylamide gel electrophoresis (PAGE), the gel was incubated in 50 mM sodium acetate pH 5.7 for 30 min followed by 10 mM *p*-phenylenediamine, 50 mM sodium acetate pH 5.7 until activity was visible (Sato and Gitlin, 1991). The oxidation of Fe(II) to Fe(III) by the purified MCO was measured spectrophotometrically by monitoring the production of Fe(III) at 315 nm in a Hitachi U-3000 spectrophotometer. The assays were conducted using 5 mM ferrous ammonium sulphate as the substrate in 100 mM sodium acetate buffer (pH 5) at 30°C (Hassett *et al*., 1998b). Inhibition of ferroxidase activity was achieved by addition of NaF (Curzon, 1960).

Expression of the P. aeruginosa *MCO in* E. coli *and generation of antibodies*

Ps. aeruginosa MCO was expressed in *E. coli* JM109 (pPROEXmco) as a histidine-tagged protein according to the manufacturer's instructions. The histidine-tagged MCO (ht-MCO) protein was purified from inclusion bodies as previously described (Genco and Dixon, 2001) followed by nickel-affinity chromatography under denaturing conditions as described by the manufacturer (Qiagen). The purified protein was dialysed against 50 mM Tris-HCl (pH 8.0) prior to immunization. Lop rabbits were immunized with 200 μg of pure protein in adjuvant (MPL + TDM + CWS) (Sigma) and boosted until a high-titre response was achieved. The MCO protein was analysed by Western blot using rabbit polyclonal sera. Soluble extracts were run on a 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel and transferred to nitrocellulose membrane using semidry transfer (Hoeffer semidry transfer cell) according to the manufacturer's instructions. Polyclonal sera was used at a dilution of 1:4000 and secondary antibody at 1:10 000 (goat anti-rabbit IgG–alkaline phosphate conjugate, Sigma). Activity of the alkaline phosphatase (AP)-conjugated secondary antibody was detected by incubation with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate with a development time of 20 min at room temperature.

Purification of the MCO from P. aeruginosa *PAK*

The *P. aeruginosa mco* mutant complemented with plasmid pUCPSKmco was used for purification of the MCO. Periplasmic extracts from 3 l of cells were prepared, as described above, and the MCO was precipitated in a 40–60% ammonium sulphate fraction. The precipitate was dialysed in 50 mM Tris pH 8.0 at 4°C. The dialysed sample was fractionated by gel filtration (Pharmacia HiLoad, 16/60 Superdex 200). Fractions containing the MCO were identified by ferroxidase activity and Western blotting. The fractions containing MCO were further purified using anion exchange chromatography (Poros HQ, Boehringer Mannheim, Germany). The MCO eluted from the column at approximately 800 mM NaCl. The protein sample was then dialysed in 50 mM Tris pH 8.0.

Iron uptake analysis

The uptake of iron by *P. aeruginosa* strains was examined in CAA media (Cox, 1986). Cultures grown on LB media were harvested at late exponential phase of growth and washed three times in CAA media prior to resuspension in the media to a high cell density and storage on ice. For iron uptake assays all strains were resuspended in CAA medium to the same density (OD_{600} = 0.8). The iron uptake assay was conducted in 4 ml of resuspended bacteria. The cell resuspension was incubated at 37°C, with shaking at 190 rpm, and the assay was started by addition of ferrous sulphate or ferric chloride. Samples were taken from the assay at timed intervals to determine the amount of iron remaining in the supernatant. The supernatant was prepared from the samples collected by removal of the cells by centrifugation for 4 min at 13 000 *g* in the microfuge. The cell-free supernatant was then assayed for iron concentration using the ferrozine assay, as previously described (Stookey, 1970). Standard curves were conducted using ferrous sulphate or ferric chloride prepared in CAA media. Media-only control experiments showed that the levels of Fe(II) and Fe(III) remained constant in CAA media for the duration of the experiment.

MCO sequence alignment and phylogenetic analysis

Sequences of the putative bacterial MCOs described in this paper were identified in public databases (NCBI) following a tBLASTx search (Altschul *et al*., 1997) using the *Saccharomyces cerevisiae* Fet3 gene sequence (GenBank accession number 6323703) (Hassett *et al*., 1998b). Sequences used in the tree and alignments were also obtained as described above from NCBI as well as from the appropriate databases for completed genome sequences using the following gene names and GenBank accession numbers: *P. aeruginosa* PAO1:*pcoA (*15597261) (Stover *et al*., 2000), *Mycobacterium tuberculosis* H37Rv (15607986) (Cole *et al*., 1998), *Yersinia pestis* (16123558), *Caulobacter crescentus* (16125216), *Salmonella typhi* LT2 (16418670) and *Escherichia coli* YacK: (2506227). Representative eukaryotic multicopper oxidase sequences were also obtained from NCBI and are included in the phylogenetic tree. These include an ascorbate oxidase from *Cucumis sativus* (cucumber) and laccase from *Pycnoporus cinnabarinus* (yeast). A number of additional sequences used during the investigation are from contigs in early release data which remain to be annotated. These have been submitted to GenBank under the following accession numbers. *Bordetella pertussis* (AF455754) sequence data were produced by the *Bordetella pertussis* Sequencing Group at the Sanger Centre. Preliminary sequence data were obtained from The Institute for Genomic Research for the potential multicopper oxidase of *Corynebacterium diphtheriae* (AF455753) and from the Genome Sequencing Centre at Washington University for the potential multicopper oxidase of *Klebsiella pneumonia* (AF455752). The sequence of the *mco* from *P. aeruginosa* strain PAK is deposited under accession number AF455751 (this study). ClustlW alignment was used to compare amino acid level homology, enabling identification of key residues in the protein sequences, including the proposed signal sequence and the comparison of CueO and MCO. The phylogenetic tree was constructed as a neighbour joining tree, using ARB sequence editor (*http://www.mikro.biologie.tu-muenchen.de/*) on the basis of amino acid similarity. The tree was generated on the basis of a ClustlW alignment of the protein sequences.

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