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cumA, a Gene Encoding a Multicopper Oxidase, Is Involved in Mn^{2+} Oxidation in *Pseudomonas putida* GB-1

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***Pseudomonas putida* GB-1-002 catalyzes the oxidation of Mn^{2+}. Nucleotide sequence analysis of the transposon insertion site of a nonoxidizing mutant revealed a gene (designated *cumA*) encoding a protein homologous to multicopper oxidases. Addition of Cu^{2+} increased the Mn^{2+}-oxidizing activity of the *P. putida* wild type by a factor of approximately 5. The growth rates of the wild type and the mutant were not affected by added Cu^{2+}. A second open reading frame (designated *cumB*) is located downstream from *cumA*. Both *cumA* and *cumB* probably are part of a single operon. The translation product of *cumB* was homologous (level of identity, 45%) to that of *orf74* of *Bradyrhizobium japonicum*. A mutation in *orf74* resulted in an extended lag phase and lower cell densities. Similar growth-related observations were made for the *cumA* mutant, suggesting that the *cumA* mutation may have a polar effect on *cumB*. This was confirmed by site-specific gene replacement in *cumB*. The *cumB* mutation did not affect the Mn^{2+}-oxidizing ability of the organism but resulted in decreased growth. In summary, our data indicate that the multicopper oxidase CumA is involved in the oxidation of Mn^{2+} and that CumB is required for optimal growth of *P. putida* GB-1-002.**

The Mn cycle in nature is strongly determined by the redox state of the metal. Generally, reduced Mn [Mn(II)] forms soluble salts, and oxidized Mn [Mn(III and IV)] precipitates as highly insoluble oxides and oxyhydroxides. Interconversions between reduced and oxidized forms are usually catalyzed by microorganisms, which strongly influence the Mn cycle (20).

Work in our laboratory has focused on the process of bacterial Mn^{2+} oxidation, which is a widespread phenomenon that occurs in many different environments and is catalyzed by a variety of microbial species. Many different oxidation mechanisms are recognized, including indirect mechanisms (which act through changes in the pH or E_h of the environment) and direct mechanisms (which involve the mediation of macromolecules, such as proteins or protein-polysaccharide complexes). The fact that macromolecules are directly involved in Mn^{2+} oxidation suggests that this process has a physiological function, but its functional significance remains unclear, even though several possibilities have been suggested. For example, Mn^{2+} oxidation may supply energy for growth (19, 28) or may be involved in scavenging of harmful oxygen species (4, 29), or accumulated Mn oxides may serve as terminal electron acceptors that support anaerobic growth (15, 16, 18). Other possible functions of Mn^{2+} oxidation include improving competition for dissolved Mn^{2+}, prolonging the viability of manganese-encrusted cells (1), and lysing of complex humic substances in order to provide small organic substrates for growth (35). A major difficulty in elucidating the mechanisms and functional significance of bacterial Mn^{2+} oxidation stems from the diversity of the species capable of Mn^{2+} oxidation. Common properties and unifying principles that underlie the oxidizing processes were not found until recently.

Recent application of molecular genetic techniques appears to have revealed a common element of the Mn^{2+}-oxidizing systems of two bacterial species. *Leptothrix discophora* SS-1, a freshwater proteobacterium, secretes an Mn^{2+}-oxidizing factor into media (2, 7). A putative *L. discophora* SS-1 operon was identified in which one of the genes, which supposedly encodes a structural component of the oxidizing factor, encodes a protein homologous to multicopper oxidases (13). *Bacillus* sp. strain SG-1, a marine gram-positive spore-forming bacterium, produces spores that are capable of oxidizing Mn^{2+} (16, 33). A sporulation-dependent operon of SG-1 was found to encode, inter alia, a protein homologous to several multicopper oxidases, and transposon mutagenesis of this operon resulted in a non-Mn-oxidizing phenotype (38).

Pseudomonas putida is a freshwater proteobacterial species, and two strains of this species (MnB1 and GB-1) have been shown to oxidize Mn^{2+} (14, 22, 31). When supplied with Mn^{2+}, the cells deposit Mn oxide outside the outer membrane in the early stationary growth phase (31). The oxidation appears to be catalyzed by an enzyme (31). Transposon mutagenesis of these two *P. putida* strains has yielded several mutants that are defective in Mn^{2+} oxidation or in secretion of the oxidizing factor(s) across the outer membrane (8, 10, 17). An analysis of a number of these mutants indicated that cytochrome *c* is involved in the oxidation of Mn^{2+} and that the specific protein secretion pathway is involved in transport of the oxidizing factor.

Analysis of another nonoxidizing transposon mutant of strain GB-1 localized the mutation in a partially sequenced open reading frame (ORF) encoding, inter alia, two consensus Cu^{2+}-bind-

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TABLE 1. Bacterial strains and plasmids

P. putida strains GB-1	Relevant characteristics[a]	Reference
P. putida strains		
GB-1	Wild type, Ap^r Cm^r	11
GB-1-002	Spontaneous mutant of GB-1, Ap^r Cm^r Sm^r	17
GB-1-007	Non-Mn^{2+}-oxidizing Tn*5* mutant of GB-1-002, Ap^r Cm^r Sm^r Km^r	17
GB-1-010	Site-directed *cumB* gene replacement mutant of GB-1-002, Ap^r Cm^r Sm^r Km^r	This study
E. coli strains		
DH5α	Δ*lacU169* (F80*lacZM15*) *recA1*	34
GJ23	*recA* derivative of AB1157 + pGJ28	37
Plasmids		
pBR322	*ori* ColE1, Ap^r Tc^r, narrow host range	6
pBR322::Tn*5*	*ori* ColE1, Ap^r Tc^r Km^r, narrow host range	17
pUC19	*ori* ColE1, Ap^r, *lacI* 80d*lacZ*, narrow host range	40
pPLH7	11.3-kb *Eco*RI fragment (Km^r, Tn*5*) from GB-1-007 cloned in pUC19	This study
pPLH114A+B	*Eco*RI-*Bam*HI fragments from pPLH7 cloned in pUC19	This study
pPLH70	5.2-kb *Eco*RI fragment containing *cumA* and *cumB* cloned in pUC19	This study
pPLH75	5.2-kb *Eco*RI fragment from pPLH70 containing kanamycin cassette inserted in *Bsa*AI site of *cumB*, cloned in pBR322	This study

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Sm^r, streptomycin resistance; Cm^r, chloramphenicol resistance.

ing regions (17). This finding suggests that GB-1 and other Mn^{2+}-oxidizing pseudomonads also depend on a Cu^{2+}-binding protein for Mn^{2+} oxidation. In this paper we describe a detailed analysis of the mutation site in the transposon mutant and the effect of Cu^{2+} on the Mn^{2+}-oxidizing activity of *P. putida* GB-1.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *P. putida* GB-1 (which was first described by Corstjens et al. [11]) was kindly provided by K. H. Nealson (Jet Propulsion Laboratory, Pasadena, Calif.). This organism is resistant to ampicillin (17) and chloramphenicol. *P. putida* GB-1-002 is a spontaneous streptomycin-resistant (Sm^r) mutant of GB-1 (17) that was used to generate transposon mutants with Sm^r as an extra phenotypic marker. The transposon-containing plasmid pBR322::Tn*5* was constructed by T. Goosen (Department of Genetics, Wageningen Agricultural University, Wageningen, The Netherlands). This construct does not replicate in *P. putida* GB-1.

Media and culture conditions. *P. putida* GB-1 was grown at room temperature in LD (*L. discophora*) medium as described previously for *L. discophora* SS-1 (7). To measure the effects of copper, nickel, and zinc on growth and/or Mn^{2+}-oxidizing activity, $CuCl_2$, $ZnCl_2$, and $NiCl_2$ were added at concentrations ranging from 0 to 100 μM. Cell growth was monitored by determining the optical density at 600 nm. *Escherichia coli* DH5α and GJ23 were cultured in Luria-Bertani medium (27) at 37°C. Solid media contained 1.8% (wt/vol) agar (Gibco BRL).

Selection markers were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; tetracycline, 25 μg/ml; streptomycin, 100 μg/ml; and chloramphenicol, 50 μg/ml.

Determination of Mn^{2+}-oxidizing activity. The Mn^{2+}-oxidizing activity of *P. putida* GB-1-002 was determined quantitatively with the redox dye Leucoberbelin blue (LBB) as described previously for *L. discophora* SS-1 (7). LBB is oxidized by Mn with valences of +3 or higher, which results in a blue product. *P. putida* cells were harvested from a liquid culture by centrifugation. They were rinsed once with 1 volume of 10 mM HEPES buffer (pH 7.5). Eventually, the cells were resuspended in 1 volume of 10 mM HEPES (pH 7.5). Oxidation reactions were started by adding $MnCl_2$ (final concentration, 100 μM) to equal amounts of cells or lysate. At regular intervals 100-μl samples were added to 500 μl of LBB. The cell material was removed by centrifugation, and 200-μl aliquots of the supernatants were transferred to a microtiter plate. The absorbance at 620 nm was measured with a Titertek Multiskan apparatus. $KMnO_4$ was used as the standard. In the LBB assay, 240 μM $KMnO_4$ is equivalent to 600 μM MnO_2. In all cases, LBB was oxidized only after Mn^{2+} was added to the samples.

All Mn^{2+} oxidation assays were performed at room temperature.

Molecular genetic techniques. Transposon mutagenesis of *P. putida* GB-1-002 was performed as described previously (17). One of the nonoxidizing mutants obtained was designated GB-1-007. An 11.0-kb Tn*5*-containing *Eco*RI fragment of the genomic DNA of mutant GB-1-007 was cloned in pUC19, resulting in plasmid pPLH7 (Fig. 1). A 5.9-kb *Bam*HI-*Eco*RI fragment and a 5.1-kb *Bam*HI fragment (3.1 kb of Tn*5* plus 2.8 kb of *Pseudomonas* DNA and 2.7 kb of Tn*5* plus 2.4 kb of *Pseudomonas* DNA, respectively) were cloned in vector pUC19, yielding plasmids pPLH114A and pPLH114B, respectively. The subcloning procedure allowed the nucleotide sequences adjacent to Tn*5* to be determined with a primer (5′-CCG-TTC-AGG-ACG-CTA-CTT-GT-3′) specific for the inverted repeats of Tn*5*. Subsequently, sequences were determined by using M13/pUC19 forward and reverse primers and primer walking. A sequence analysis was performed by using automated dideoxy chain termination technology. The resulting nucleotide sequences were analyzed further with programs of the Wisconsin Genetics Computer Group (version 8.1) and the Pseudomonas Genome Project.

Construction, screening of the genomic library of *P. putida* GB-1-002 to select constructs that hybridized with digoxigenin-labeled pPLH7, and complementation were performed as described previously (8, 17). To obtain a *P. putida* GB-1 *cumB* mutant (Fig. 1), the 5.2-kb hybridizing *Eco*RI fragment from one of the constructs selected was cloned in pUC19, resulting in pPLH70 (which was identical to pPLH7 lacking Tn*5* [Fig. 1]). A unique *Bsa*AI site in *cumB* was used to insert a 1.2-kb kanamycin cassette (Pharmacia Biotech) after the 3′ overhang was removed by using T4 DNA polymerase. The *Eco*RI fragment containing the kanamycin cassette was subcloned in pBR322, resulting in pPLH75. This construct was mobilized to *P. putida* GB-1-002 by using *E. coli* GJ23 as described by Van Haute et al. (37). The *P. putida* GB-1-002 *cumB* mutants were selected on the basis of kanamycin resistance (recombination) and chloramphenicol resistance (loss of *E. coli* GJ23). Tetracycline sensitivity was used to select for double recombination events. *cumB* gene replacement was confirmed by PCR by using *cumB*-specific primers.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the GenBank database under accession no. AF086638.

RESULTS

Analysis of the Tn*5* insertion site in the nonoxidizing mutant GB-1-007. Transposon mutagenesis of *P. putida* GB-1-002 resulted in several classes of nonoxidizing phenotypes, some of which were defective in secretion of the Mn^{2+}-oxidizing factor and some of which were completely devoid of Mn^{2+}-oxidizing activity (17). One of the latter mutants was designated GB-1-

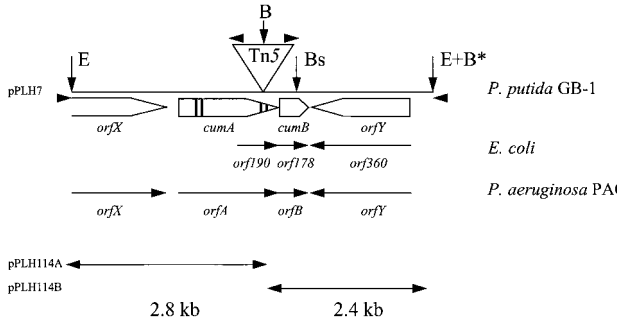
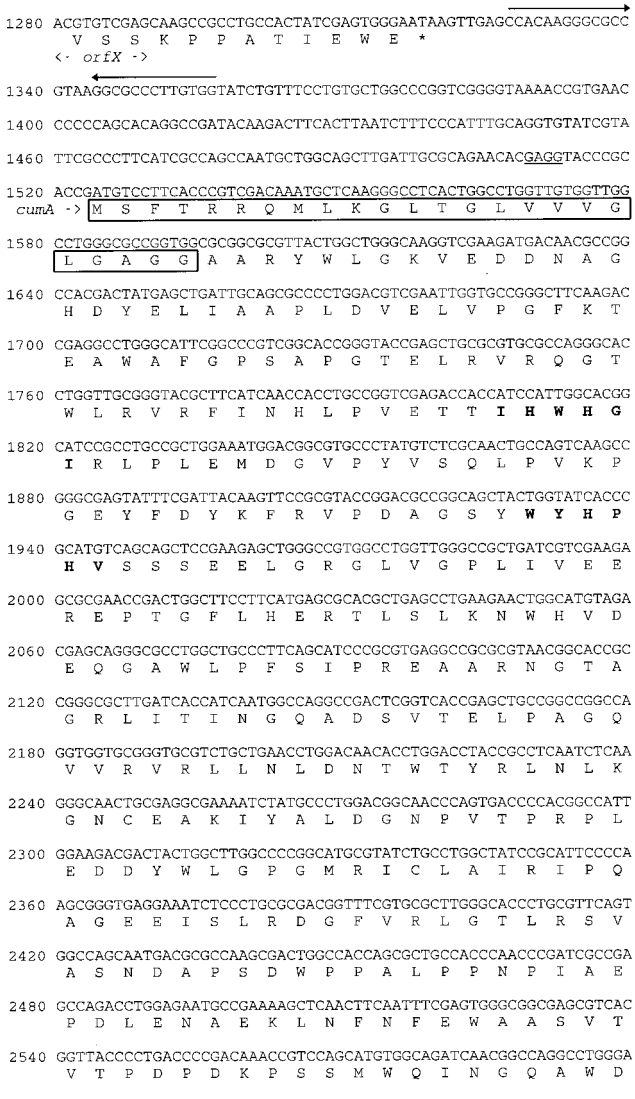


FIG. 1. Map of the Tn*5*-containing *Eco*RI fragment from the *P. putida* mutant GB-1-007. The triangle indicates the site of the Tn*5* insertion (length of Tn*5*, 5.8 kb). The open arrows indicate predicted gene locations and orientations. The vertical bars in *cumA* indicate consensus Cu^{2+}-binding regions. The solid arrowheads indicate the locations of the forward and reverse M15/pUC19 primers and the Tn*5* sequence primer. The small vertical arrows indicate restriction enzyme sites used in constructing pPLH7 and pPLH114A+B. Abbreviations for restriction enzymes: E, *Eco*RI; B, *Bam*HI; Bs, *Bsa*AI (site of kanamycin cassette insertion). The *Bam*HI restriction site in the MCS of pUC19 is indicated by B*. The GenBank accession number of the *E. coli* sequence is X61396.



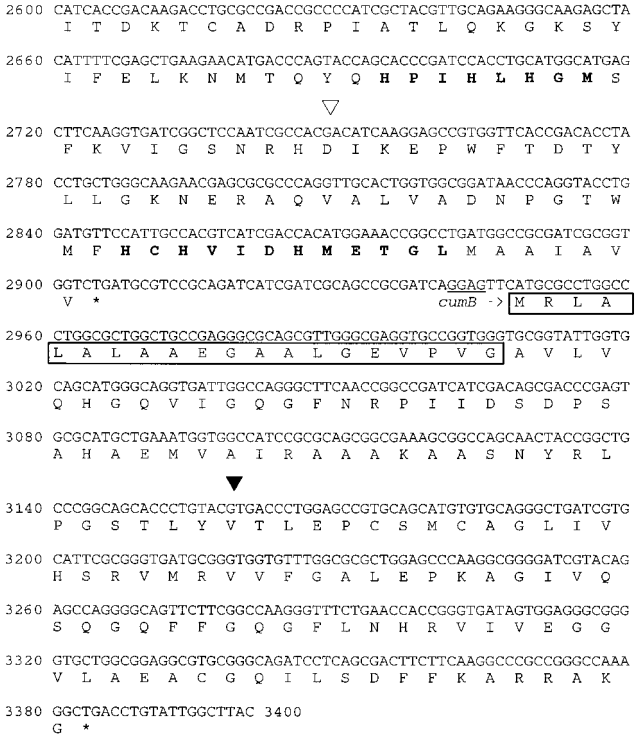


FIG. 2. Nucleotide sequence of part of the 11.0-kb pPLH7 *Eco*RI fragment. The amino acid sequences of the potential gene products are also shown. Possible Shine-Dalgarno sequences are underlined. Inverted repeats which might represent transcription terminators are indicated by arrows. The predicted CumA and CumB signal peptides are enclosed in boxes, and the predicted copper-binding regions are indicated by boldface type. The site of transposon insertion is indicated by an open arrowhead, and the kanamycin cassette insertion site in *cumB* is indicated by a solid arrowhead.

007. The location of Tn*5* in GB-1-007 and the nucleotide sequences of the adjacent regions were determined (Fig. 1). The translation products of some of the ORFs detected are shown in Fig. 2. The transposon was inserted in an ORF identified as a gene encoding a protein homologous to multicopper oxidases based on the presence of predicted Cu^{2+}-binding regions (Fig. 2 and 3). This gene was designated *cumA* (Cu protein involved in manganese oxidation). Multicopper oxidases are found in a wide variety of organisms and are characterized by their conserved Cu^{2+}-binding sites (Fig. 3). The predicted *cumA* translation product is a 459-amino-acid protein that has a molecular weight of approximately 50,500 and contains an N-terminal signal peptide (Fig. 2). The overall level of identity with an ORF of *Pseudomonas aeruginosa* (referred to below as *orfA*), as determined on the basis of the amino acid sequence, was 67%. *cumA* is preceded by a possible Shine-Dalgarno sequence (Fig. 2). Upstream of *cumA* an ORF designated *orfX* was detected. *orfX* exhibited homology to *guaA*, which codes for GMP synthase (level of identity with 400 amino acids encoded by the 3′ end of the gene encoding the *Bacillus subtilis* GMP synthase [GenBank accession no. P29727] [25], 55%). Immediately downstream from *orfX* strong secondary structures indicate that a transcription termination site is present (Fig. 2).

Downstream from *cumA*, two other ORFs, designated *cumB* and *orfY*, were identified (Fig. 1 and 2). *cumB*, which is preceded by a possible Shine-Dalgarno sequence, is located next to *cumA*. Since *cumB* and *orfY* have the same orientation and

	2 3	3 3	1 2 3	313 1 1
CumA	95 IHWHGI	136 WYHPHV	391 HPIHLHGM	442 HCHVIDHMETGL
Pcum	93 IHWHGI	134 WYHPHL	390 HPIHLHGM	440 HCHVIDHMETGL
MofA	304 IHLHGG	384 WYHDHT	1174 HPVHFHLL	1279 HCHILGHEENDF
MnxG	527 MHIHFV	572 FFHDHL	281 HVFHYHVH	334 HCHLYPHFGIGM
Fet3	8 MHFMGL	52 WYHSHT	341 HPFHLHGH	411 HCHIEWHLLQGL
CopA	99 IHWHGL	140 WYHSHS	542 HPIHLHGM	590 HCHLLYHMEMGM
PcoA	99 IHWHGI	140 WYHSHS	537 HPIHLHGM	586 HCHLLYHMEMGM
Lacc	78 VHWHGL	121 WYHSHY	508 HPIHKHGN	585 HCHIASHQMGGM
Hcp	119 FHSHGI	178 IYHSHI	994 HTVHFHGH	1039 HCHVTDHIHAGM

FIG. 3. Alignment of the amino acid sequences of the copper-binding sites in CumA, the *P. aeruginosa* PAO1 homologue, and several other multicopper oxidases. The copper-binding residues are designated 1, 2, and 3 on the basis of the types of copper which they potentially bind. Abbreviations: CumA, *P. putida* GB-1 CumA (this study); Pcum, *P. aeruginosa* PAO1 CumA homologue (*Pseudomonas* Genome Project); MofA, *L. discophora* SS-1 MofA (GenBank accession no. Z25774) (13); MnxG, marine *Bacillus* sp. strain SG-1 MnxG (GenBank accession no. U31081) (38); Fet3, *S. cerevisiae* ferroxidase (GenBank accession no. P38993) (5); CopA, *P. syringae* copper resistance protein (GenBank accession no. M19930) (26); PcoA, *E. coli* plasmid pRJ1004 copper resistance protein (GenBank accession no. X83541) (9); Lacc, *Neurospora crassa* laccase (GenBank accession no. P10574) (21); Hcp, human ceruloplasmin (GenBank accession no. M13699) (23).

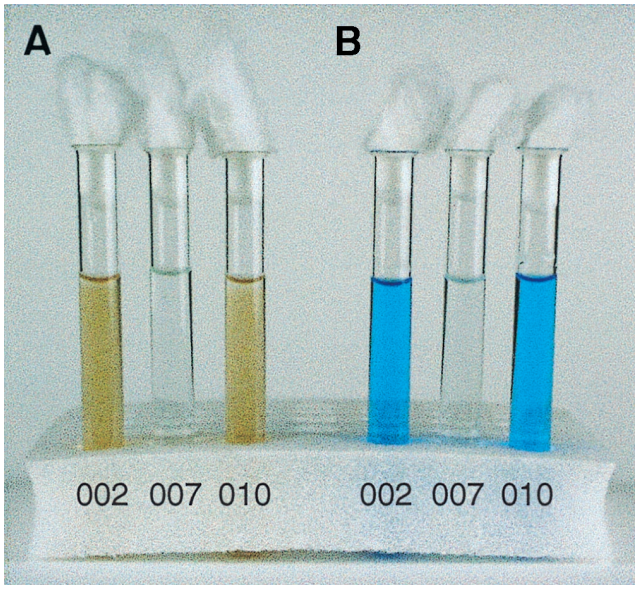


FIG. 4. (A) Manganese-oxidizing activities of *P. putida* GB-1-002 and GB-1-007 and *cumB* mutant GB-1-010 in LD medium containing 100 μM $MnCl_2$. (B) Same three samples after the redox dye LBB was added.

because the short intergenic region does not contain a transcription termination site, these genes are assumed to be part of an operon (designated Cum). The predicted *cumB* translation product is a 145-amino-acid protein that has an estimated molecular weight of 16,000 and contains a potential signal peptide (Fig. 2). CumB exhibited homology (level of identity, 67%) with the protein encoded by the ORF (*orfB*) downstream from *orfA* in *P. aeruginosa* (Fig. 1). It also exhibited homology with Orf178 of *E. coli* (level of identity, 52% [32]) (Fig. 1) and Orf74 of *Bradyrhizobium japonicum* (level of identity, 45%; GenBank accession no. L34743 [39]). The orientation of *orfY* is opposite that of *cumA* and *cumB*, and its predicted translation product (length, 485 amino acids; estimated molecular weight, 53,500) exhibited homology (level of identity, 65%) with the product of the *P. aeruginosa* ORF (*orfY*) downstream from *orfB* (Fig. 1). This protein also exhibited homology with Orf360 of *E. coli* (level of identity, 38% [32]), but in contrast to both *P. putida* and *P. aeruginosa*, *E. coli* does not contain a *cumA* homologue preceding *orf178*.

Screening of the *P. putida* genomic library with digoxigenin-labeled pPLH7 resulted in isolation of 12 positive clones. The DNA inserts of nine of these clones contained a 5.2-kb *Eco*RI fragment that hybridized with pPLH7. Mobilization of these nine constructs to mutant GB-1-007 did not result in restoration of Mn^{2+}-oxidizing activity.

As complementation did not succeed, we could not eliminate the possibility that inhibition of Mn^{2+} oxidation in the mutant GB-1-007 resulted from a polar effect of the Tn*5* insertion in *cumA* on *cumB*. A gene replacement study was performed to eliminate the possibility that *cumB* is involved in the oxidation of Mn^{2+}. The mutant obtained (designated GB-1-010) was tested for Mn^{2+}-oxidizing activity, and the growth rate was compared to the growth rates of the wild type and mutant GB-1-007. GB-1-010 retained the ability to oxidize Mn^{2+} (Fig. 4), but growth defects similar to those of mutant GB-1-007 were observed (data not shown).

Effect of Cu^{2+} on the Mn^{2+}-oxidizing activity of *P. putida* GB-1-002 and on the growth rates of *P. putida* GB-1-002 and GB-1-007. The effect of a mutation in a gene (*cumA*) encoding a multicopper oxidase on Mn^{2+} oxidation in *P. putida* may indicate that Mn^{2+} oxidation is Cu^{2+} dependent. Therefore, we cultured cells with different concentrations of exogenously added Cu^{2+} and determined the Mn^{2+}-oxidizing activities of the cultures in the early stationary growth phase. We observed that Cu^{2+} had a clear stimulating effect on the oxidation of Mn^{2+} (Fig. 5). A maximum Mn^{2+} oxidation rate of 0.52 nmol/ml · min was observed in the presence of 40 μM Cu^{2+}, which was approximately fivefold greater than the rate observed in medium without extra Cu^{2+}. At Cu^{2+} concentrations greater than 40 μM the stimulating effect decreased.

To determine whether stimulation of the oxidizing activity was specific for Cu^{2+} ions and not for addition of divalent cations in general, the effects of Zn^{2+} and Ni^{2+} were studied (Fig. 5). Stimulation of the oxidation of Mn^{2+} was not observed. In contrast, at Zn^{2+} concentrations greater than 20 μM a decrease in oxidation was observed. The effect of Ni^{2+} was even more pronounced. A decrease in Mn^{2+} oxidation was observed at an Ni^{2+} concentration of 10 μM. These results indicate that Cu^{2+} is specifically involved in oxidation of Mn^{2+} in *P. putida*.

Because the Mn^{2+}-oxidizing activity of *P. putida* is growth phase dependent (31), the effects of Cu^{2+} on the growth rates of both the *P. putida* wild type and the mutant were determined (Fig. 6). No significant differences in the growth rate of the wild type were detected with Cu^{2+} concentrations up to 100 μM. Cells entered the logarithmic growth phase at approximately the same time after inoculation and reached the stationary growth phase simultaneously. Similar results were obtained with the mutant. However, compared to the wild type, the mutant had a longer lag phase and reached a lower maximum cell density (independent of the Cu^{2+} added). Addition of Zn^{2+} or Ni^{2+} at concentrations up to 100 μM did not have any effect on the growth rate of either the wild type or the mutant (data not shown).

DISCUSSION

Previous studies have indicated that Mn^{2+} oxidation in *P. putida* GB-1 is catalyzed by an outer membrane enzyme or, more likely, an enzyme complex (8, 17, 31). Electrophoretic analyses of cell extracts (12, 31) revealed the presence of Mn^{2+}-oxidizing factors with molecular weights ranging from 250,000 to 130,000, which were assumed to represent the oxi-

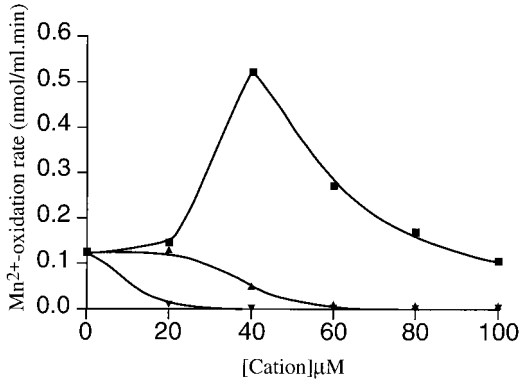


FIG. 5. Effects of different Cu^{2+} (■), Zn^{2+} (▲), and Ni^{2+} (▼) concentrations on the Mn^{2+} oxidation rate of *P. putida* GB-1, as determined by the LBB assay. For experimental details see the text.

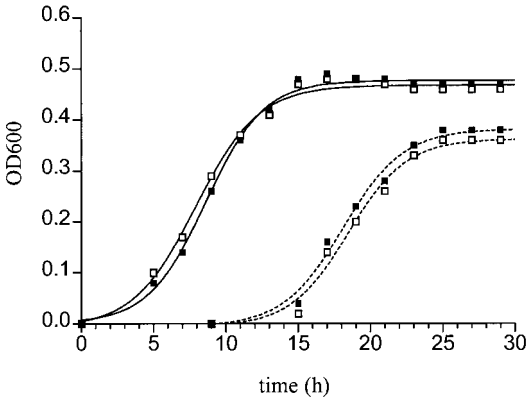


FIG. 6. Effects of no Cu^{2+} (■) and 100 μM Cu^{2+} (□) on the growth rates of *P. putida* GB-1-002 (——) and GB-1-007 (– – –). For experimental details see the text. OD600, optical density at 600 nm.

dizing complex or parts of the oxidizing complex. We propose that an important constituent of the oxidizing complex is a Cu-dependent oxidase, the product of *cumA*. The genomic organization of the regions adjacent to *cumA* indicates that this gene constitutes an operon with the downstream ORF *cumB*. *cumA* is preceded by inverted repeat sequences that are able to form a stem-loop structure, the transcription termination site of the preceding gene, *orfX*, which is a homologue of the GMP synthase gene. We propose that the DNA region between *orfX* and *cumA* contains the promoter sequence. As the short intergenic region between *cumA* and *cumB* does not contain transcription termination sequences, these genes are probably transcribed from the same promoter. *cumB* is followed by an ORF (*orfY*) that clearly belongs to another operon in view of its opposite orientation. Transposon insertion in *cumA* abolished the Mn^{2+}-oxidizing activity of the organism, whereas mutation of *cumB* had no such effect, clearly indicating that *cumA* is involved in Mn^{2+} oxidation. The gene product CumA contains a signal sequence, in accordance with the outer membrane location of the oxidizing factor. We found that growth of cells on media with exogenously added Cu^{2+} stimulated the Mn^{2+}-oxidizing activity compared to the activity of cells grown with no Cu^{2+} addition, whereas neither Zn^{2+} nor Ni^{2+} enhanced the activity. The suggestion that a Cu^{2+}-dependent oxidase is involved in Mn^{2+} oxidation in *P. putida* is supported by evidence that the multicopper oxidases MofA and MnxG are involved in Mn^{2+} oxidation in two other oxidizing organisms, *L. discophora* SS-1 and *Bacillus* sp. strain SG-1, respectively (13, 38). In the latter organism the oxidizing activity could also be stimulated by adding Cu^{2+} (38). In *L. discophora* SS-1, the effect of Cu^{2+} on Mn^{2+} oxidation has not been studied yet.

We demonstrated that the opportunistic pathogen *P. aeruginosa* PAO1 contains an ORF (*orfA*) that is very similar to *cumA* of *P. putida* GB-1. Preliminary experiments in our laboratory showed that in principle, logarithmic liquid cultures of *P. aeruginosa* are able to oxidize Mn^{2+} (data not shown), although it was difficult to reproducibly demonstrate this activity. In spite of the uncertainty, it is tempting to correlate this oxidizing activity with the presence of the *cumA* homologue *orfA*.

The data obtained in this study strongly support the notion that involvement of multicopper oxidases in Mn^{2+} oxidation is common in Mn^{2+}-oxidizing bacteria (36). However, several questions remain to be answered. The first question is related to the fact that complementation of the mutant has not been successful so far. In a previous study (17) we found a single transposon insertion in the mutant GB-1-007, which showed that the lack of complementation cannot be due to other possible insertions. The transposon insertion is located near the 3′ end of *cumA* between two copper-binding regions. It is possible that this location of the transposon still allows production of large amounts of almost complete CumA which may compete with CumA expressed from the complementing fragment (for instance, in the formation of the oxidizing complex). Because the essential fourth copper-binding region is missing in mutated CumA, a nonfunctional Mn^{2+}-oxidizing complex should be formed. We will use site-specific gene replacement in *cumA* to resolve this question.

The site-directed gene replacement in *cumB* eliminated the possibility that *cumB* is involved in Mn^{2+} oxidation and confirmed that the decreased growth rate of the mutant GB-1-007 was the result of a polar effect of the transposon on *cumB* transcription, which supported the suggestion that *cumA* and *cumB* are cotranscribed from the same promoter. The involvement of *cumB* in growth is consistent with the observation that a *cumB* homologue in *B. japonicum*, *orf74*, is required for optimal free-living growth (39). Like the mutant GB-1-007 and the *cumB* mutant, mutants in which *orf74* was disrupted had a longer lag phase and reached a lower cell density than the wild type. In *E. coli*, another *cumB* homologue (*orf178*) seems to be involved in the cell-killing function of members of the *gef* gene family in a manner that so far is not known (32). *P. putida* GB-1 is sensitive to the *gef* gene family (32), which may be the result of the product of *cumB*. This gene has not been found in *P. putida* GB-1 previously.

A second question to be resolved concerns the stimulating effect of Cu^{2+} on Mn^{2+} oxidation in *P. putida*. We found that the presence of low amounts of Cu^{2+} in the culture medium specifically enhanced the oxidizing activity of the cells. However, it is not clear yet whether the stimulating effect should be ascribed to Cu^{2+}-enhanced transcription of the oxidizing factor (presumed to be encoded by *cumA*), to production of a more active factor as a result of optimal Cu^{2+} incorporation, or to a combination of these effects. Why stimulation of Mn^{2+} oxidation decreased at Cu^{2+} concentrations higher than the optimum concentration (40 μM) also must be explained. It is possible that at supraoptimal concentrations, Cu^{2+} ions also occupy Mn^{2+}-binding sites. Competition for Mn^{2+}-binding sites and/or Cu^{2+}-binding sites may explain the inhibiting effects of Zn^{2+} and Ni^{2+} on Mn^{2+} oxidation. Studies of the effect of Cu^{2+} on the expression of *cumA* and the effects of specific Cu^{2+}-chelating agents on Mn^{2+} oxidation followed by reconstitution experiments should provide more insight into these questions.

Finally, it is possible to speculate about the physiological functions of multicopper oxidases in Mn^{2+}-oxidizing bacteria. Multicopper oxidases occur in a wide variety of organisms and can have different cellular functions. The multicopper oxidase family includes the CopA proteins of *Pseudomonas syringae* (26) and *Xanthomonas campestris* (24) and the PcoA protein of the *E. coli* plasmid pRJ1004 (9), all of which are involved in Cu^{2+} resistance. The *P. putida* GB-1 CumA protein probably is not involved in Cu^{2+} resistance, as mutation of the corresponding gene did not result in Cu^{2+}-sensitive growth of mutant cells. Other members of the multicopper oxidase family are the *Saccharomyces cerevisiae* Fet3 protein (5) and the human ceruloplasmin (23), both of which act as ferroxidases involved in high-affinity iron uptake. White rot fungi produce the multicopper oxidase laccase, which produces strongly oxidizing Mn(III) chelates that are used in the oxida-

tion of lignin compounds (3). Production of strong oxidizing agents that release nutrients from resistant organic compounds may be an important function of Mn^{2+}-oxidizing multicopper oxidases in nutrient-poor environments (35). However, metabolically inert structures, like the Mn^{2+}-oxidizing spores of *Bacillus* sp. strain SG-1, do not obviously benefit from such a process. It is possible that the multicopper oxidases of Mn^{2+}-oxidizing bacteria have primary cellular functions other than Mn^{2+} oxidation. These oxidases may have the ability to oxidize Mn^{2+}, which allows the bacteria to use the products depending on the organism and circumstances and thus to gain a selective advantage. Such an advantage may be exploited at the cellular level (by generation of nutrients, production of alternative electron acceptors, etc. [see above]), but an advantage at the ecological level may also be envisaged. In some environments, oxidation and reduction of Mn^{2+} (and Fe^{2+}) are coupled to oxidation and reduction of carbon, which permits efficient cycling of nutrients and reduction equivalents in stable ecosystems (30). Mn^{2+}-oxidizing bacteria play an important role in such ecosystems by guaranteeing the supply of an electron sink to the reducing zones. This may contribute to the widespread occurrence of Mn^{2+}-oxidizing bacterial species in nature.

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