Involvement of genes of the two-step protein secretion pathway in the transport of the manganese-oxidizing factor across the outer membrane of \textit{Pseudomonas putida} strain GB-1

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\textbf{Abstract}

Microorganisms can accelerate the rate of Mn$^{2+}$ oxidation by up to five orders of magnitude compared to abiotic Mn$^{2+}$ oxidation. Mn$^{2+}$ oxidation in \textit{Pseudomonas putida} strain GB-1 involves an enzyme incorporated in the outer membrane that oxidizes Mn$^{2+}$ extracellularly. This Mn$^{2+}$-oxidizing factor has to be synthesized inside the cell and transported across the outer membrane. We used a method known as transposon mutagenesis to generate two mutants that are incapable of Mn$^{2+}$ oxidation because they are unable to transport the Mn$^{2+}$-oxidizing factor across the outer membrane. However, when cells were lysed, Mn$^{2+}$ oxidation occurred, verifying that transport and not synthesis of the Mn$^{2+}$-oxidizing factor was affected. Transport of the Mn$^{2+}$-oxidizing factor was restored when normal sequences obtained from a genomic library of \textit{Pseudomonas putida} strain GB-1 were introduced into the mutant strains. By sequencing the DNA of the disrupted genes of these two mutants it was determined that the affected genes are very similar to the \textit{xpc} gene family of the related species, \textit{Pseudomonas putida} WCS358 and \textit{Pseudomonas aeruginosa}. This gene family is known to be involved in the two-step protein secretion process in Gram-negative bacteria. In one of the two mutants, the disruption occurs in the gene that encodes a subunit of a complex that spans the membrane. In the other mutant the disruption occurs in the \textit{pilD} gene, which encodes an enzyme (peptidase) that modifies the subunits that are assembled into this membrane-spanning complex. These results indicate the involvement of a two-step protein secretion pathway in the transport of the Mn$^{2+}$-oxidizing factor of \textit{Pseudomonas putida} strain GB-1.

\textbf{Introduction}

Manganese is the second most abundant transition metal in the Earth’s crust. It occurs in various minerals, including carbonates, silicates, sulfides, phosphates, and oxides. Manganese has several valence states, of which the 2+, 3+, and 4+ oxidation states prevail in nature. Whereas Mn$^{2+}$ can exist in readily soluble forms, Mn$^{3+}$ and Mn$^{4+}$ form highly insoluble oxides and oxyhydroxides. Manganese oxides are environmentally reactive as a result of their adsorptive and oxidative properties. They scavenge trace metals from the environment and affect the fate of complex organic matter like humic substances by oxidative reactions (Tebo et al. 1997; Sunda and Kiefer 1994). The cycling of manganese between soluble and insoluble forms is determined by interconversions between valence states, and thus depends on the redox conditions (Eh, pH) of the environment. In most natural aerobic environments, manganese oxidation is thermodynamically favorable (cf. Nealson et al. 1988). Without microbial interference, Mn$^{2+}$ oxidation probably involves a two-step process, in which initially formed Mn$^{3+}$ oxides or oxyhydroxides slowly disproportionate to form Mn$^{4+}$ oxides (cf. Tebo et al. 1997). In many marine and fresh waters, Mn$^{2+}$ is oxidized at much higher rates than predicted by the thermodynamic conditions of the environment (cf. Nealson et al. 1988; Tebo 1991). Analyses of the oxidation state of the oxides sedimented in such environments revealed that rapid transitions from the Mn$^{2+}$ to Mn$^{4+}$ state must have occurred, probably without the relatively stable Mn$^{3+}$ intermediates involved in abiotic Mn$^{2+}$ oxidation (Tebo et al. 1997). These phenomena can be explained by microbial mediation of the manganese oxidation- and mineral-forming processes (Tebo et al. 1997).

Microorganisms have been long known to promote the oxidation of manganese (Jackson 1901; Beijerinck 1913). They may alter the redox conditions of the (micro) environment by secretion of oxidizing or alkaline substances and thus enhance the Mn$^{2+}$ oxidation rate by indirect mechanisms. Many other evolutionary diverse and widely distributed microbial species, however, have developed extensive, apparently specific machinery to oxidize Mn$^{2+}$. One of those species is the fresh-water, Gram-negative bacterium \textit{Pseudomonas putida} strain GB-1.

Like all Gram-negative bacteria, \textit{P. putida} GB-1 has an extended cell envelope composed of an inner and an outer membrane separated by the periplasm. In Mn$^{2+}$-con-
Gene involvement pathway

Figure 1. Approach for the identification of genes involved in Mn$^{2+}$ oxidation. Open arrow = promoter, light gray = gene A, medium gray = gene B, white = gene C, black = Tn5. Closed arrowheads indicate the location of the sequence primers. Km$^R$: kanamicin resistance. I: Mn$^{2+}$ oxidation depends on hypothetical operon containing genes A, B, and C. Expression of gene A leads to manganese oxidizing activity (Mn ox$^+$, brown colonies). II: Transposon (Tn5) insertion, selection on Km$^R$ and no manganese oxidizing activity (Mn ox$^-$, white colonies). Sequence analysis to locate site of transposon insertion using Tn5 specific primers (closed arrowheads). III: Digestion chromosomal DNA wild type (wt) and selection non-mutated sequence using mutated sequence (II) as probe. IV: complementation mutated sequence (II) by transformation of non-mutated sequence (III) to the non-manganese oxidizing mutant. Selection for manganese oxidizing activity (Mn ox$^+$, brown colonies).

In recent years, the introduction of molecular genetic methodology in geomicrobiology has significantly advanced the understanding of Mn$^{2+}$ oxidation in several bacterial species (van Waasbergen et al. 1996; Corstjens et al. 1997; Tebo et al. 1997). By interfering with the expression of genes involved in Mn$^{2+}$ oxidation, for example by introducing mutations in the genetic material, the role of the corresponding gene product in the oxidation process can be evaluated. Such an approach, summarized in Figure 1, is presented in this paper. Mutations in the bacterial chromosome can be brought about by introducing a transposon into the bacterial cells. A transposon is a mobile genetic element, which can randomly insert in the chromosome, thus interrupting its nucleotide sequence. Mutated cells that do not form brown precipitates and thus remain white in Mn$^{2+}$-containing medium will probably contain a transposon insertion in a gene directly or indirectly involved in Mn$^{2+}$ oxidation. Clearly, mutations in the gene encoding the Mn$^{2+}$-oxidizing enzyme will abolish Mn$^{2+}$ oxidation, but also mutations in genes responsible for the synthesis, possible modification, folding, or transport of the enzyme affects the oxidizing enzymatic activity. The kinetic parameters of the Mn$^{2+}$ oxidation process are typical of an enzymatic reaction and poisons that specifically affect proteins and strongly inhibit Mn$^{2+}$ oxidation (Okazaki et al. 1997). The data suggest that P. putida GB-1 produces a manganese-oxidizing enzyme, which is incorporated in the outer membrane and oxidizes Mn$^{2+}$ extracellularly. To achieve this, the bacterium has to coordinate a complex set of biochemical processes. The manganese-oxidizing factor has to be synthesized, involving the expression of genes encoding the structural protein and possible cofactors assisting in the electron transfer from Mn$^{2+}$ to oxygen. Subsequently, the enzyme has to be transported across the inner and outer membrane to reach its final destination.

One of the main pathways by which proteins are secreted from Gram-negative bacteria involves two steps (Pugsley 1993). The exoproteins are synthesized with a specific N-terminal leader sequence, the signal peptide. The signal peptide is recognized by enzymes of the Sec (secretion) machinery, which mediate the translocation of the exoprotein across the inner membrane. Upon translocation, the signal peptide is removed by a specific proteolytic enzyme, a signal peptidase. In the periplasm, a second set of transport proteins mediate translocation across the outer membrane. These macromolecules include proteins that are thought to aggregate to form port-holes in the outer envelope.

Such an extensive biochemical machinery may underlie the extracellular oxidation of Mn$^{2+}$ by P. putida GB-1, and the elucidation of the oxidizing mechanism has to take into account all steps involved. The Mn$^{2+}$-oxidizing factor has been partly purified (Okazaki et al. 1997), but it has not been chemically characterized so far. As a consequence, the evidence for the involvement of a catalytic protein in the oxidation process is supported by indirect evidence only.

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process. Mutant DNA can be cut with specific nucleases, and the fragment containing the transposon can be isolated and its nucleotide sequence analyzed. Comparison with protein-encoding nucleotide sequences available from worldwide databases may identify the genes of interest.

Using this approach, we demonstrated that genes involved in the protein-specific transport pathway described above are involved in the secretion of the Mn$^{2+}$-oxidizing factor of \( P. \) putida GB-1.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains and plasmids**

Bacterial strains and plasmids used in this study are listed in Table 1. \( P. \) putida GB-1 (first described in Corstens et al. 1992) was kindly provided by K.H. Nealson (Jet Propulsion Laboratory, Pasadena). This strain was first described as a \( P. \) fluorescens (Okazaki et al. 1997) but was recently identified as a \( P. \) putida (de Vrind et al. 1998). The transposon-containing plasmid pBR322::Tn5 was constructed by T. Goosen (Department of Genetics, Agricultural University, Wageningen). This construct does not replicate in \( P. \) putida GB-1.

**Media and culture conditions**

\( P. \) putida GB-1 was grown at room temperature in \( Lepthrix discophora \) (LD) medium as described previously for \( L. \) discophora SS-1 (Boogerd and de Vrind 1987). \( E. \) coli strains were cultured in LB (Miller 1972) at 37°C. Solid media were prepared by adding 18 g/L Bacto-agar (Gibco BRL) prior to autoclaving. Solid LD medium contained 100 \( \mu \)M MnCl$_2$.

\( P. \) putida GB-1 is resistant to ampicillin up to a concentration of 1 mg/mL. Inhibition is decreased by streptomycin. On streptomycin-containing media, streptomycin-resistant colonies were spontaneously generated with a frequency of about \( 5 \times 10^{-7} \). One of these colonies was subcultured (GB-1-002) and used in further experiments with streptomycin resistance as extra phenotypic marker. Antibiotics were used in the following concentrations (in micrograms per milliliter): ampicillin (Ap): 100; kanamycin (Km): 50; tetracycline (Tc): 25; and streptomycin (Sm): 100.

**Determination of Mn$^{2+}$-oxidizing activity and preparation of cell lysates**

The Mn$^{2+}$-oxidizing activity of intact cells and cell lysates was determined quantitatively with the redox dye Leucoberbellin blue (LBB) as described for \( L. \) discophora (Boogerd and de Vrind 1987). Cells in the early stationary growth phase were harvested by centrifugation. They were rinsed once with an original volume of 1 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer pH 7.5. Eventually, cells were resuspended in an original volume of 1 mM HEPES pH 7.5. Cell lysates were obtained by ultrasonication of resuspended cells for 10 min at maximum amplitude at 0°C (Vibra cell, Sonics & Materials, Inc.). Oxidation reactions were started by addition of MnCl$_2$ to a final concentration of 600 \( \mu \)M. Every 30 min samples of 100 \( \mu \)L were added to 500 \( \mu \)L of LBB. The cell material was removed by centrifugation and 200 \( \mu \)L aliquots of the supernatants were transferred to a microtitertplate. The absorbance was measured at 620 nm with a Titertek Multiskan. KMnO$_4$ was used as a standard. In the LBB assay, 240 \( \mu \)M of KMnO$_4$ is equivalent to 600 \( \mu \)M of Mn$^{2+}$ oxidized.

**Transposon mutagenesis of \( P. \) putida GB-1-002**

To generate non-oxidizing mutants, cells of early stationary phase cultures of \( P. \) putida GB-1-002 were transformed by electroporation with the plasmid pBR322::Tn5 (Bio-Rad Gene Pulser, 2.5 kV, 200 \( \Omega \), 25 \( \mu \)F, 10$^3$ cells in 100 \( \mu \)L 10 mM HEPES pH 7.5 with 1 mM MgCl$_2$). Tn5 contains a Km-resistance gene which was used for selection. The electrooporated cells were allowed to recover in 1 mL LD for 1 h, plated on Km-containing LD and grown for two days at room temperature. Km-resistant colonies
were tested for Mn$$^{2+}$$-oxidizing activity on solid LD, without Km.

Southern analysis of non-oxidizing transposon mutants

Genomic DNA was isolated (according to Corstjens and Muyzer 1993) from two non-oxidizing mutants (GB-1-008 and GB-1-009) obtained by transposon mutagenesis. The DNA was further purified by equilibrium centrifugation in a cesium chloride-ethidium bromide gradient (Sambrook et al. 1989). EcoRI digests were electrophoresed on a 0.7% agarose gel, and they were denatured and blotted to a nylon filter (Hybond-N, Amersham) according to standard methods (Sambrook et al. 1989). Tn5-containing EcoRI fragments were hybridized with digoxigenin (DIG) labeled pBR322::Tn5. The DIG label was detected with specific antibodies and visualized autoradiographically with CSPD (Boehringer Mannheim).

Cloning and nucleotide sequence analysis of genomic Tn5-flanking regions of the transposon mutants

Complete EcoRI digests of the genomic DNA of the Tn5-carrying mutants GB-1-008 and GB-1-009 were cloned in vector pUC19 and transformed to E. coli DH5α. Because Tn5 does not have an EcoRI restriction site, recombinant clones containing the EcoRI restriction fragments in which Tn5 had integrated could be selected for on Km. This resulted in the plasmids pPLH5 and pPLH6, harboring the Tn5-containing EcoRI fragments of GB-1-008 and GB-1-009, respectively. In a subsequent step, the EcoRI fragment of pPLH6 was subcloned using the unique BamHI site of Tn5 (cf. Fig. 4). A 4.5 kilobase (kb) BamHI-EcoRI and a 13.5 kb BamHI fragment (3.1 kb Tn5 + 1.4 kb Pseudomonas DNA and 2.7 kb Tn5 + 10.8 kb Pseudomonas DNA, respectively) were cloned in vector pUC19, yielding the plasmids pPLH116A and pPLH116B, respectively. The subcloning allowed the nucleotide sequences adjacent to Tn5 to be determined with a primer (5’-CCG-TTC-AGG-ACG-CTA-CTT-GT-3’) specific for the inverted repeat present in both ends of Tn5. A similar subcloning and sequencing approach was unsuccessful with pPLH5. Restriction mapping of pPLH5 (cf. Fig. 4) showed that Tn5 was located 1.8 kb from the pUC19 multiple cloning site (MCS). Nucleotide sequencing of pPLH5 was started from the MCS, using the common pUC19 reverse primer. Subsequent sequences were determined using primer walking. With a primer (5’-ACG-CCT-GCA-GCA-GCG-GCA-CG-3’) based on the 5’ end of pilC from P. Putida WCS358 an additional sequence was obtained from the 6.1 kb Pseudomonas fragment of pPLH5. Sequence analyses were performed using automated dideoxy chain-termination technology. Resulting nucleotide sequences were further investigated using programs of the Wisconsin Genetics Computer Group (version 8.1). Obtained sequences were deposited in the genbank under nucleotide accession number AF062531 and AF062532.

Construction and screening of the genomic library of P. putida GB-1-002

Purified genomic DNA from P. putida GB-1-002 was partially digested with Sau3A. Fragments of approximately 25–30 kb were isolated using a sucrose gradient (Sambrook et al. 1989) and ligated in a BamHI-digested cosmid-vector, pLAFR3 (Staskawicz et al. 1987). The resulting constructs were packaged in vitro in phage particles using the Packagene Lambda DNA Packaging System (Promega) according to Ish-Horowicz and Burke (1981). E. coli DH5α was transfected with this mixture and colonies were selected on LB containing tetracycline and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to screen for transductants carrying cosmids with an insert. In total, 1920 cosmids (out of 7 x 10⁶) were stored as a genomic library. The total length of the inserts of the 1920 cosmids correspond to at least 10 times the size of the genome, which is estimated at 4 to 5 Mb.

Cells from the library were transferred to a nylon membrane (Hybond N, Amersham) on solid LB with Tc and grown overnight at 37°C. The resulting colonies were lysed, and the DNA was denatured and bound to the filter according to standard methods (Sambrook et al. 1989). Filters were hybridized with DIG-labeled probes of pPLH5 and pPLH6. The DIG-labeled probes were detected with specific antibodies and visualized colorimetrically with 4-Nitrobluetetrazoliumchloride and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim). Colonies containing hybridizing cosmids were selected for complementation experiments.

Complementation of non-oxidizing transposon mutants

The non-oxidizing mutants GB-1-008 and GB-1-009 were transformed with the cosmids selected from the library by triparental mating. To this aim, mixtures of 100 µL were prepared containing 10⁸ cells each of the donor, the acceptor and the helper strain. The latter strain (E. coli HB101) contained plasmid pRK2013 (Figurski and Helinski 1979). The mixtures were transferred to solid LB and allowed to grow overnight at 28°C. The cells were then suspended in LD medium, plated on LD containing Tc, Sm, Km, and Ap and incubated overnight at room temperature. Resistant colonies were transferred to LD plates without antibiotics to screen for Mn$$^{2+}$$-oxidizing activity.

RESULTS

Transposon mutagenesis of P. putida GB-1-002

Transposon mutagenesis yielded 1000–2000 Km-resistant colonies per microgram pBR322::Tn5. Since pBR322 cannot replicate in P. putida, Km-resistant colonies could only result from insertion of the transposon in the bacterial genome. The antibiotics Km, Sm, and Tc inhibit Mn$$^{2+}$$ oxidation. To screen for non-oxidizing phenotypes, the Km-resistant transformants had to be transferred to media without antibiotics. Colonies that remained white on MnCl₂-containing media (Fig. 2) were subcultured.
two of them, Mn$^{2+}$-oxidizing activity was recovered after cell disruption. They were called GB-1-008 and GB-1-009. Their cell lysates oxidized Mn$^{2+}$ at similar rates as intact cells of GB-1-002 (see below). Apparently these two mutants were affected in the transport of the Mn$^{2+}$-oxidizing factor.

**Southern analysis and complementation of non-oxidizing transposon mutants GB-1-008 and GB-1-009**

Southern blots of complete EcoRI digests of genomic DNA of GB-1-008 and GB-1-009 were hybridized with DIG-labeled pBR322::Tn5. The size of the Tn5-containing fragment of mutant GB-1-008 was approximately 14.0 kb (Fig. 3). In GB-1-009 two hybridizing fragments of approximately 9.0 and 18.0 kb were detected. Because Tn5 does not contain an EcoRI restriction site these two signals apparently resulted from a double transposon insertion.

The 14.0 kb GB-1-008 and the 18.0 kb GB-1-009 fragment could be cloned in the vector pUC19 and were called pPLH5 and pPLH6, respectively. The cosmid library was screened with DIG-labeled pPLH5 and pPLH6. Four positive clones were detected with probe pPLH5. GB-1-008 was transformed with the corresponding cosmid harboring the Tn5 insertion. Ten positive clones were detected with probe pPLH6. The Tn5 insertions were mapped and characterized. The results suggested that Tn5 was inserted into the chromosome of GB-1-008 and GB-1-009 in two different sites. The restriction analyses suggested that Tn5 was inserted into the chromosome of GB-1-008 and GB-1-009 in two different sites. The restriction analyses suggested that Tn5 was inserted into the chromosome of GB-1-008 and GB-1-009 in two different sites.

The Mn$^{2+}$-oxidation assays had to be performed on media without antibiotics. Consequently, the non-oxidizing phenotypes obtained in the complementation experiments might result from loss of complementing cosmids during growth on these media. However, all cells were able to grow when transferred to Tc-containing media.

**Mn$^{2+}$-oxidizing activity of GB-1-002 and non-oxidizing mutants**

The Mn$^{2+}$-oxidizing activities of cells and lysates of the (complemented) mutants GB-1-008 and GB-1-009 were compared with the activity of GB-1-002. Cells of GB-1-002 oxidized all available Mn$^{2+}$ within 4.5 h at an initial rate of 2.4 nmol/mL min (Fig. 4A, closed squares). Intact cells of GB-1-009 did not oxidize Mn$^{2+}$ at all (Fig. 4A, diamonds), but lysed cells oxidized at a similar rate as GB-1-002 cells (Fig. 4A, open squares). Similar results were obtained with cells and lysates of GB-1-008 (not shown). In complemented cells of GB-1-002 and GB-1-009 the Mn$^{2+}$-oxidizing activity of intact cells was restored to 1.9 nmol/mL min, which approximated the level of GB-1-002 cells (Fig. 4B).

**Mapping and sequence analysis of the insertion site of Tn5 in the non-oxidizing mutants**

Plasmids pPLH5 and pPLH6 were analyzed to map and characterize the locations of Tn5 in mutants GB-1-008 and GB-1-009.
**Figure 4.** Mn^{2+}-oxidizing activity in the early stationary growth phase. At time point zero 600 μM MnCl₂ was added. For further experimental details see experimental procedures. (A) cells of *P. putida* GB-1-002 (closed squares), cells of mutant GB-1-009 (diamonds), cell lysate of mutant GB-1-009 (open squares). (B) cells of complemented mutant GB-1-008 (filled circles) and mutant GB-1-009 (open circles).

**Figure 5.** Map of the Tn₅ containing EcoRI fragment from *P. putida* mutant GB-1-008 (A) and GB-1-009 (B). The triangle indicates the site of transposon insertion. Open arrows indicate predicted gene location and orientation. Closed arrows show the genes of the pil operon of *P. putida* WCS358. Closed arrowheads indicate the location of the reverse pUC19(A) and the Tn₅ (B) sequence primer. Small vertical arrows indicate restriction enzyme sites. Abbreviations restriction enzymes; E, EcoRI; S, SalI; H, HindIII; Sm, SmaI; K, KpnI; B, BamHI. The BamHI restriction site in the MCS of pUC19 is indicated by B*.

and GB-1-009, respectively. Restriction analysis of the EcoRI insert of pPLH5 showed that Tn₅ was flanked by fragments of 6.1 and 1.8 kb (Fig. 5A). The entire 1.8 kb fragment was sequenced, starting from the multiple cloning site of the vector. One partial and two complete open reading frames (which are long DNA sequences uninterrupted by a stop codon that encode part or all of a protein; ORF) were identified. Tn₅ had inserted into an ORF that was preliminary identified as pilD, also designated xcpA, based on strong homology to the *P. putida* WCS358 pilD/xcpA gene encoding the enzyme prepilin peptidase (de Groot et al. 1994). The computer program GAP revealed 82% identity on amino acid (aa) level in 152 aa residues of the C-terminus.

The two other ORF downstream of the putative pilD/xcpA gene were oriented in the same direction (Fig. 5A). The first ORF was homologous to orfX of *P. putida* WCS358 (de Groot et al. 1994) and to orfX of *Neisseria*
gonorrhoeae (Freitag et al. 1995). The potential translation product of the P. putida GB-1-002 orfX gene is composed of 207 aa with a molecular weight of 22 800. It showed high similarity to the orfX translation product of P. putida WCS358 (92% identity, Fig. 6) and N. gonorrhoeae (42%). A striking feature of OrfX is the presence of a predicted nucleotide binding site or Walker box A (Walker et al. 1982) close to the N-terminus (Fig. 6). The second ORF was homologous to orfY of N. gonorrhoeae (Freitag et al. 1995). The putative translation product of orfY was a 7.3 kD polypeptide of 66 aa with 47% identity to the potential N. gonorrhoeae OrfY protein. In P. putida WCS358, orfY has not yet been identified.

The similarity of the C-terminus of the putative PilD/XcpA of P. putida GB-1-002 to other PilD/XcpA proteins and the genomic organization downstream the corresponding partial gene indicated that the identified ORF belong to the pil operon of P. putida GB-1-002. To confirm the identification of the Tn5-inserted gene as pilD/xcpA, we analyzed part of the 6.1 kb pPLH5 fragment flanking the other side of the transposon (Fig. 5A). Assuming that the pil operon of P. putida GB-1-002 would closely resemble that of P. putida WCS358, we designed a primer based on the 5’ end of the pilC gene of the latter organism (de Groot et al. 1994). Using this primer we obtained a preliminary sequence of 0.7 kb of the 6.1 kb pPLH5 fragment (Fig. 5A). Two partial ORF in opposite directions were found. Their translation products showed strong similarity to the N-terminal parts of PilA and PilC of P. putida WCS358, respectively (cf. Fig. 7). The orientations of pilA and pilC relative to pilD/xcpA, and the length of the intergenic region between the two genes was similar in both organisms. These data emphasize the identification of PilD/XcpA in P. putida GB-1-002.

The location of Tn5 in plasmid pPLH6 was analyzed by sequencing the subclones pPLH116A and 116B (Fig. 5B). The DNA regions adjacent to the transposon were analyzed using the primer specific for the Tn5 inverted repeats. The ORF thus detected (Fig. 7A) was identified as xcpT, based on homology with the P. aeruginosa (Bally et al. 1992) and P. putida WCS358 (de Groot et al. 1996) xcpT genes. The xcpT gene encodes a pseudopilin, so called because of the strong similarity of its N-terminus with that of prepilin, the product of the pilA gene (Hobbs and Mattick 1993). The putative P. putida GB-1-002 xcpT gene encodes a 15.7 kD protein of 143 aa, with 46% identity to XcpT of P. aeruginosa (Fig. 7A). Like other pseudopilins, the P. putida GB-1-002 XcpT is characterized by its N-terminal signal peptide, showing sequence homology to that of the prepilins (Fig. 7B). It also includes the conserved phenylalanine and glutamate residues at positions 1 and 5 of the mature protein, and the N-terminal region of the mature protein consists mainly of conserved hydrophobic residues (Fig. 7B). A proline residue characteristic of XcpT homologues (Kagami et al. 1998) was present at position 81 (Fig. 7A). These results indicate that mutant GB-1-009 contained a transposon insertion in a homologue of xcpT, a gene shown to be involved in protein secretion across the outer membrane of Gram-negative bacteria (Hobbs and Mattick 1993).

Discussion

Pseudomonas putida strain GB-1 produces a Mn\(^{2+}\)-oxidizing factor that is supposed to be located in the outer membrane and oxidizes Mn\(^{2+}\) extracellularly (Okazaki et
Transposon mutagenesis yielded, inter alia, two independent non-oxidizing mutants (GB-1-008 and GB-1-009) in which the Mn$^{2+}$-oxidizing activity could be recovered by cell disruption. As the mutant lysates oxidized Mn$^{2+}$ at similar rates as the intact wild-type cells, it was hypothesized that the transport of the oxidizing factor to the outer membrane was affected. This was sustained by sequence analysis of the loci in which the transposon had inserted. In strain GB-1-008 the insertion was localized in a homologue of the pilD (also called xcpA) gene of P. aeruginosa (Nunn et al. 1990) and P. putida WCS358 (de Groot et al. 1994). A gene homologous to xcpT of P. aeruginosa (Bally et al. 1992) and P. putida WCS358 (de Groot et al. 1996) was mutated in strain GB-1-009.

The xcp gene products belong to a large family of homologous proteins, generally called secretins, which are widely distributed among Gram-negative bacteria (Hobbs and Mattick 1993; Genin and Boucher, 1994). They are involved in the type II pathway (or the main terminal branch of the general secretory pathway, MTB-GSP) for protein secretion. In P. aeruginosa, the products of at least 12 genes, xcpP-Z, and pilD/xcpA constitute the MTB-GSP machinery. The role of the Xcp proteins, with the exception of PilD/XcpA, has not been clarified in detail. XcpQ, the only outer membrane protein, was shown to form oligomeric rings with a cavity permitting large proteins to pass (Bitter et al. 1998). It was suggested that these cavities might be gated by plug-like structures collectively formed by the XcpT, U, V, and W proteins (Bitter et al. 1998).

XcpT-W and possibly XcpX (Bleves et al. 1998) are so-called pseudopilins. Their N-termini show strong sequence similarity with PilA, the structural subunit of type IV pil that adhere the bacterial cells to substrates. PilA is synthesized as a prepilin with a highly conserved N-terminal signal peptide, which is cleaved by the signal peptidase PilD/XcpA (Strom et al. 1993). The mature protein contains a conserved phenylalanine at position 1 and a conserved glutamate at position 5. The phenylalanine residue is methylated by PilD/XcpA, which thus has a dual function, and recognition of the glutamate residue was shown to be required for methylation (cf. Bitter et al. 1998). A hydrophobic region at the N-terminal end of the mature protein appears to be involved in subunit-subunit interactions (Dalrymle and Mattick 1997). Most of these sequence features are conserved in the pseudopilins. Moreover, the pseudopilins and prepilin are both processed by the pilD/xcpA product, although the xcp and pil gene clusters are located in different regions of the genome. The close relationship between the MTB-GSP machinery and type IV pilus biogenesis is further illustrated by extensive sequence similarities among many of the other Xcp and Pil proteins (Bally et al. 1992).

The MTB-GSP pathway has mainly been studied in pathogenic bacteria like P. aeruginosa, which are known to secrete various exoenzymes. Non-pathogenic P. fluorescens and P. putida strains were shown to harbor xcp genes (de Groot et al. 1991), but these genes did not appear to be operative under the culture conditions employed (de Groot et al. 1996). In this study we showed that transposon insertions in pilD/xcpA and xcpT homologues preclude extracellular Mn$^{2+}$ oxidation in P. putida GB-1-002, demonstrating that the MBT-GSP operates in the transport of the Mn$^{2+}$-oxidizing factor.

At the moment, the genomic region flanking the xcpT gene of P. putida GB-1-002 has not been characterized. Consequently, we cannot compare the genomic organization of the xcp operon with that of P. putida WCS358 (de Groot et al. 1996). The organization of both pil operons (containing pilD/xcpA) proved to be very similar (Fig. 5). Upstream pilD/xcpA two genes homologous to pilC and pilA of P. aeruginosa were localized. The pilA products of both the P. putida strains contained an isoleucine instead of the conserved phenylalanine residue adjacent to the signal peptidase cleavage site (Fig. 7B). PilD was shown to tolerate diverse amino acid substitutions at this position without loss of function (Hobbs and Mattick 1993). In both P. putida strains, pilB, present in P. aeruginosa, is lacking in the gene cluster. Whether this gene is absent or present at a different chromosomal position remains to be investigated (cf. de Groot et al. 1994). Downstream the pilD/xcpA gene two additional ORF were found. The potential product of orfX, so called in analogy with homologous ORF in P. putida WCS358 (de Groot et al. 1994) and N. gonorrhoeae (Freitag et al. 1995), contained a predicted nucleotide binding site (Fig. 6). The presence of an ATP-binding domain suggests a function for this protein, but unequivocal evidence for functional expression of the gene was not demonstrated in any of the these organisms (cf. Freitag et al. 1995). A possible function of orfY, which was homologous to a corresponding ORF in N. gonorrhoeae, also remains unclear.

As far as known, P. putida GB-1-002 does not form pili in laboratory cultures. Nevertheless, pilD, and conceivably other genes in the same operon, appeared to be functionally expressed in the organism, as indicated by its requirement for protein secretion. Possibly, expression of additional genes required for pil biogenesis is induced only under special growth conditions. Another possibility is that next to pilD/XcpA, other pil products are involved in the MTB-GSP of P. putida GB-1. Recently, it has been shown that PilA also plays a role in protein secretion in P. aeruginosa (Lu et al. 1997).

The xcp and pil operons appeared to be located in different chromosomal regions in P. putida GB-1-002, as Southern analysis of the mutant DNA revealed transposon-carrying restriction fragments of different lengths. Mutant GB-1-009 appeared to contain a double insertion, and although the chance on such an event is extremely low, it has been reported before (Cornelis et al. 1992). The transposon insertion in the locus that was analyzed adequately explains the non-oxidizing phenotype. However, we cannot completely exclude that the second transposon insertion also affected Mn$^{2+}$ oxidation in strain GB-1-009 in a so far unknown manner.
The different chromosomal positions of the xcpT and pilD/xcpA genes were further illustrated by the fact that wild-type DNA fragments complementing one of the mutants did not complement the other mutation and vice versa. Not all of the wild-type DNA fragments hybridizing with the Tn5-containing mutant DNA were able to complement the corresponding mutation. Non-complementing fragments may not have contained the complete gene required for restoration of the Mn^{2+}-oxidizing phenotype. Alternatively, the non-oxidizing phenotypes may not only have resulted from the insertions in the Tn5-containing genes themselves, but also from a polar effect on the expression of genes downstream in the same operon. In such a case, hybridizing fragments lacking the latter genes would not be capable of restoring the oxidizing phenotype. This question will be resolved in future studies analyzing the minimum length of fragments required for mutant complementation. In spite of this uncertainty, it is clear that genes in at least two operons involved in the secretion of proteins mediate the transport of the Mn^{2+}-oxidizing factor of P. putida GB-1-002.

The nature and function of the oxidizing outer membrane-enzyme remains to be clarified. Genetic analysis of the Mn^{2+}-oxidizing processes in a marine Bacillus (van Waasbergen et al. 1996) and in L. discophora (Corstjens et al. 1997) revealed the involvement of multicopper oxidases in Mn^{2+} oxidation. In this respect it may be significant that the metal complexator o-phenanthroline strongly inhibits Mn^{2+} oxidation in P. putida GB-1, apparently by interfering with the enzyme itself (Okazaki et al. 1997). The functions of the Mn^{2+}-oxidizing processes in all oxidizing species are still a matter of speculation. As preliminary evidence suggested that an electron transport chain is involved in Mn^{2+} oxidation in P. putida MnB1 (cf. Tebo et al. 1997) and in P. putida GB-1-002 (our unpublished observations), Mn^{2+} oxidation might supply energy for growth through electron transport-linked ATP generation. However, many alternative functions have been proposed (cf. Tebo et al. 1997), one of them referring to a role of the produced oxide as an electron acceptor during respiration under anaerobic conditions (de Vrind et al. 1986). As microbial Mn^{2+} oxidation is such a widespread phenomenon in nature, it is hard to imagine that the oxidizing organisms evolved these apparently extensive biochemical mechanisms without benefiting from the process.

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