American Mineralogist, Volume 83, pages 1573–1582, 1998

Involvement of genes of the two-step protein secretion pathway in the transport of the manganese-oxidizing factor across the outer membrane of *Pseudomonas putida* strain GB-I

G.J. Brouwers,* J.P.M. de Vrind, P.L.A.M. Corstjens, and E.W. de Vrind-de Jong

Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, P.O. Box 9502, 2300 R.A. Leiden, The Netherlands

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 *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 *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 *xpc* gene family of the related species, *Pseudomonas putida* WCS358 and *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 *pilD/xcpA* 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 *Pseudomonas putida* strain GB-1.

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 Kieper 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 *Pseudomonas putida* strain GB-1.

Like all Gram-negative bacteria, *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-

* E-mail: Brouwers@chem.leidenuniv.nl

0003–004X/98/1112–1573$05.00

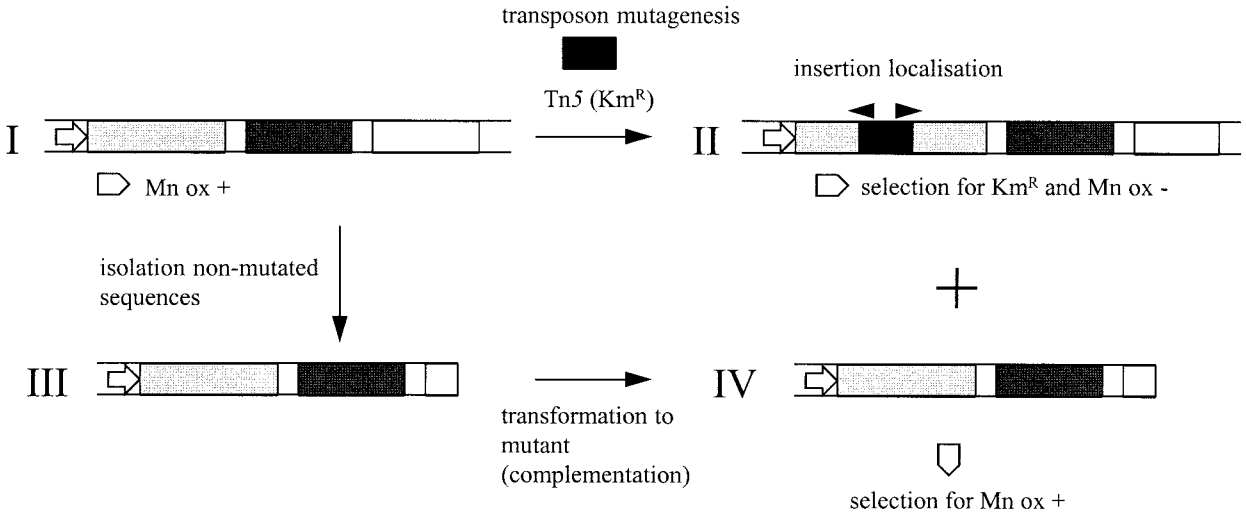

FIGURE 1. Approach for the identification of genes involved in Mn^{2+} oxidation. Open arrow = promoter, light gray = gene A, medium gray = gene B, white = gene C, black = Tn*5*. Closed arrowheads indicate the location of the sequence primers. Km^R: kanamicin resistance. I: Mn^{2+} oxidation depends on hypothetical operon containing genes A, B, and C. Expression of gene A leads to manganese oxidizing activity (Mn ox+, brown colonies). II: Transposon (Tn*5*) insertion, selection on Km^R and no manganese oxidizing activity (Mn ox−, white colonies). Sequence analysis to locate site of transposon insertion using Tn*5* 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).

taining media, the cells deposit manganese oxide around the outer membrane and form brown colonies (Okazaki et al. 1997). 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 portholes 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.

In recent years, the introduction of molecular genetical 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

TABLE 1. Bacterial strains and plasmids

Strain and Plasmid		Relevant characteristics	References
Pseudomonas putida	GB-1	Wild type, Ap^r	Corstens et al. (1992)
Pseudomonas putida	GB-1-002	Spontaneous mutant of GB-1-001, Ap^r, Sm^r	This study
Pseudomonas putida	GB-1-008 and GB-1-009	Tn*5* excretion mutants of GB-1-002, Ap^r, Sm^r, Km^r	This study
Escherichia coli	DH5α	F−, *supE*44, Δ*lac*U169, (Φ80/*acZ*M15), *hsdR*17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1	Sambrook et al. (1989)
Escherichia coli	HB101	F, *supE*44, *hsdS*20, (r_B m_B), *recA*13, *ara*-14, *proA*2, *lacY*1, *galK*2*rpsL*20, *xyl*-5, *mtl*-1	Sambrook et al. (1989)
Plasmids	pBR322	*ori* ColE1, Ap^r, Tc^r, narrow host range	Bolivar et al. (1977)
	pBR322::Tn*5*	*ori* ColE1, Ap^r, Tc^r, Kn^r, narrow host range	Goosen
	pLAFR3	cosmid cloning vector, Tc^r	Staskawich et al. (1987)
	pRK2013	*ori* ColE1, Km^r, Tra^+ Mob^+	Figurski and Helinski (1979)
	pUC19	*ori* ColE1, Ap^r, *lacI* 80d*lacZ,* narrow host range	Yanish-perron et al. (1985)
	pPLH5	14.0 kb *Eco*RI fragment (Km^r, Tn*5*) from GB-1-008 cloned in pUC19	This study
	pPLH6	18.0 kb *Eco*RI fragment (Km^r, Tn*5*) from GB-1-009 cloned in pUC19	This study
	pPLH116A+B	*Eco*RI/*Bam*HI fragments from pPLH6 cloned in pUC19	This study

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 Corstjens 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::Tn*5* 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 *Leptothrix 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 μ*M* $MnCl_2$.

P. putida GB-1 is resistant to ampicillin up to a concentration of 1 mg/mL. Growth is inhibited by streptomycin. On streptomycin-containing media, streptomycin-resistant colonies were spontaneously generated with a frequency of about 5×10^{-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 m*M N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES) buffer pH 7.5. Eventually, cells were resuspended in an original volume of 1 m*M* 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 μ*M*. Every 30 min samples of 100 μL 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 microtiterplate. The absorbance was measured at 620 nm with a Titertek Multiskan. $KMnO_4$ was used as a standard. In the LBB assay, 240 μ*M* of $KMnO_4$ is equivalent to 600 μ*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::Tn*5* (Bio-Rad Gene Pulser, 2.5 kV, 200 Ω, 25 μF, 10^9 cells in 100 μL 10 m*M* HEPES pH 7.5 with 1 m*M* $MgCl_2$). Tn*5* contains a Km-resistance gene which was used for selection. The electroporated 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). *Eco*RI 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). Tn*5*-containing *Eco*RI fragments were hybridized with digoxygenin (DIG) labeled pBR322::Tn*5*. The DIG label was detected with specific antibodies and visualized autoradiographically with CSPD (Boehringer Mannheim).

Cloning and nucleotide sequence analysis of genomic Tn*5*-flanking regions of the transposon mutants

Complete *Eco*RI digests of the genomic DNA of the Tn*5*-carrying mutants GB-1-008 and GB-1-009 were cloned in vector pUC19 and transformed to *E. coli* DH5α. Because Tn*5* does not have an *Eco*RI restriction site, recombinant clones containing the *Eco*RI restriction fragments in which Tn*5* had integrated could be selected for on Km. This resulted in the plasmids pPLH5 and pPLH6, harboring the Tn*5*-containing *Eco*RI fragments of GB-1-008 and GB-1-009, respectively. In a subsequent step, the *Eco*RI fragment of pPLH6 was subcloned using the unique *Bam*HI site of Tn*5* (cf. Fig. 4). A 4.5 kilobase (kb) *Bam*HI-*Eco*RI and a 13.5 kb *Bam*HI fragment (3.1 kb Tn*5* + 1.4 kb *Pseudomonas* DNA and 2.7 kb Tn*5* + 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 Tn*5* 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 Tn*5*. A similar subcloning and sequencing approach was unsuccessful with pPLH5. Restriction mapping of pPLH5 (cf. Fig. 4) showed that Tn*5* 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 *Sau*3A. Fragments of approximately 25–30 kb were isolated using a sucrose gradient (Sambrook et al. 1989) and ligated in a *Bam*HI-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 7×10^4) were stored as a genomic library. The total length of the inserts of the 1920 cosmids corresponds 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 10^8 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::Tn*5*. 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 $MnCl_2$-containing media (Fig. 2) were subcultured. In

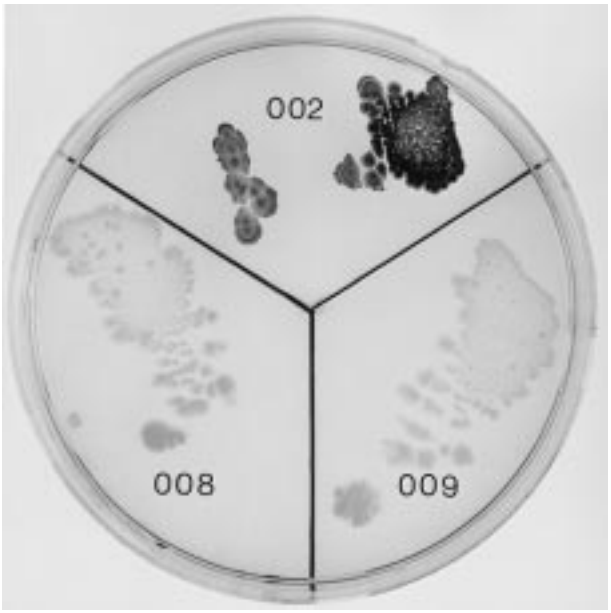

FIGURE 2. Plate assay for the detection of Mn^{2+}-oxidizing activity. *P. putida* GB-1-002 and mutants GB-1-008 and GB-1-009 were plated on LD medium containing 100 m*M* $MnCl_2$ and incubated at room temperature. GB-1-002 is encrusted by Mn oxide, whereas GB-1-008 and GB-1-009 remain white.

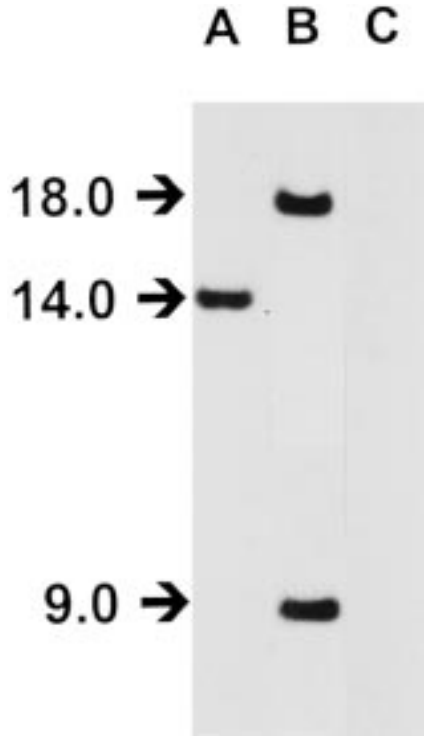

FIGURE 3. Southern blot analysis of the *Eco*RI-digested chromosomal DNA of *P. putida* mutants GB-1-008 and GB-1-009. pBR322::Tn5 was used as a probe. Lane A: GB-1-008; B: GB-1-009; C: GB-1-002. Arrows indicate the molecular weight markers in kb.

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 *Eco*RI digests of genomic DNA of GB-1-008 and GB-1-009 were hybridized with DIG-labeled pBR322::Tn*5*. The size of the Tn*5*-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 Tn*5* does not contain an *Eco*RI 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 cosmids by triparental mating. One of the cosmids restored Mn^{2+}-oxidizing activity as indicated by Mn-oxide formation on plate. This cosmid did not complement the mutation in GB-1-009. With the pPLH6 probe ten positive clones were detected of which six restored the Mn^{2+}-oxidizing activity in GB-1-009. None of them restored the activity in GB-1-008.

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-1-008 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 Tn*5* in the non-oxidizing mutants

Plasmids pPLH5 and pPLH6 were analyzed to map and characterize the locations of Tn*5* in mutants GB-1-008

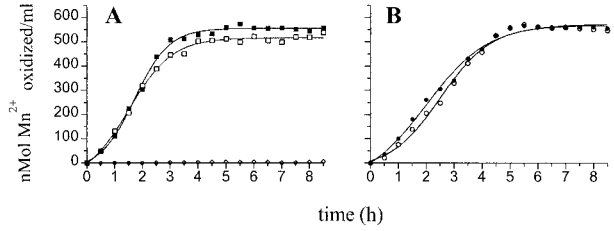

FIGURE 4. Mn^{2+}-oxidizing activity in the early stationary growth phase. At time point zero 600 μM $MnCl_2$ was added. For further experimental details see experimental procedures. (**A**) cells of *P. putida* GB-1-002 (closed squares), cells of mutant GB-1-009 (diamonds), cell lysate of mutant GB-1-009 (open squares). (**B**) cells of complemented mutant GB-1-008 (filled circles) and mutant GB-1-009 (open circles).

and GB-1-009, respectively. Restriction analysis of the *Eco*RI insert of pPLH5 showed that Tn*5* 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. Tn*5* 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*

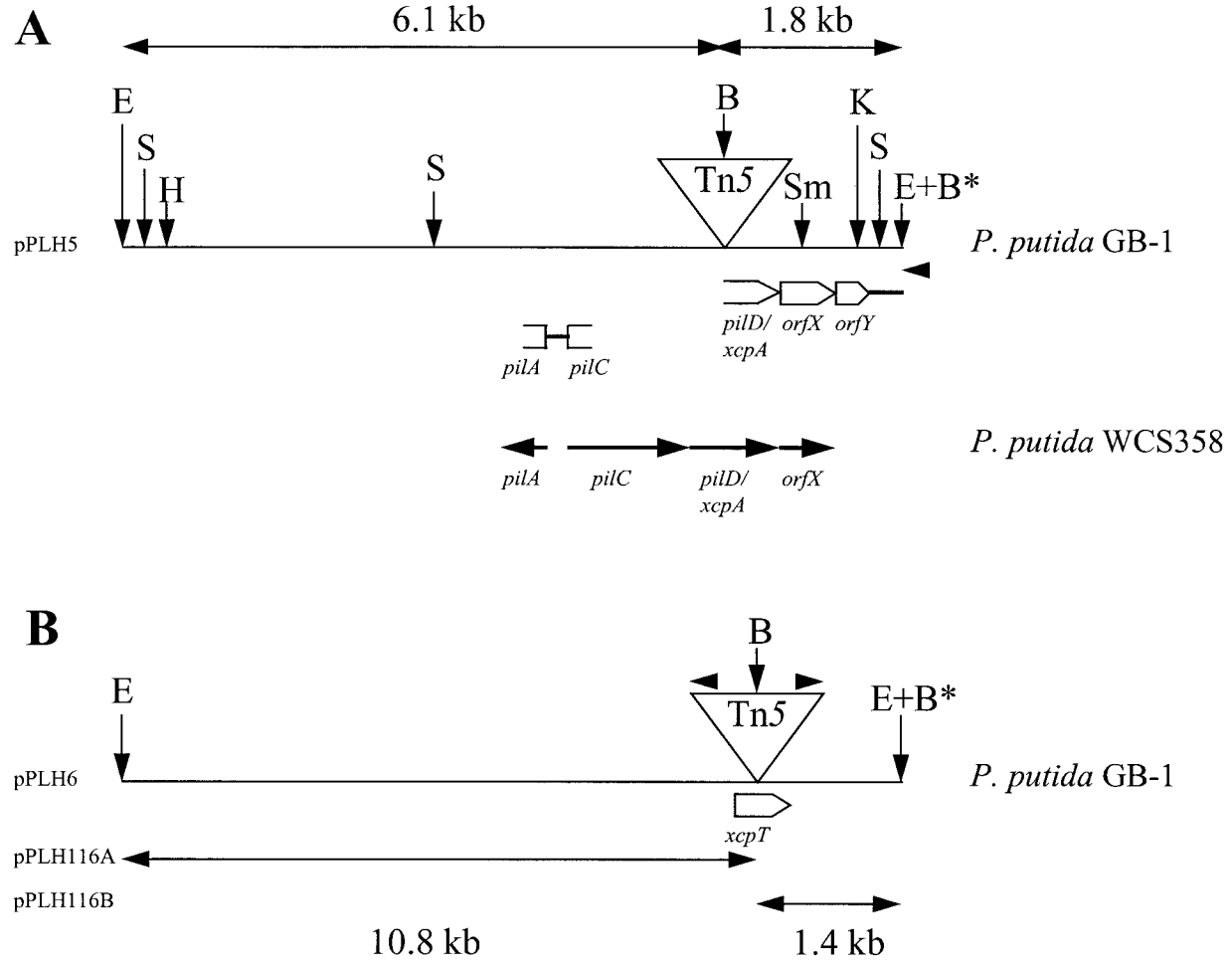

FIGURE 5. Map of the Tn*5* containing *Eco*RI fragment from *P. putida* mutant GB-1-008 (**A**) and GB-1-009 (**B**). The triangle indicates the site of transposon insertion. Open arrows indicate predicted gene location and orientation. Closed arrows show the genes of the *pil* operon of *P. putida* WCS358. Closed arrowheads indicate the location of the reverse pUC19(A) and the Tn*5* (B) sequence primer. Small vertical arrows indicate restriction enzyme sites. Abbreviations restriction enzymes; E, *Eco*RI; S, *Sal*I; H, *Hin*DIII; Sm, *Sma*I; K, *Kpn*I; B, *Bam*HI. The *Bam*HI restriction site in the MCS of pUC19 is indicated by B*.

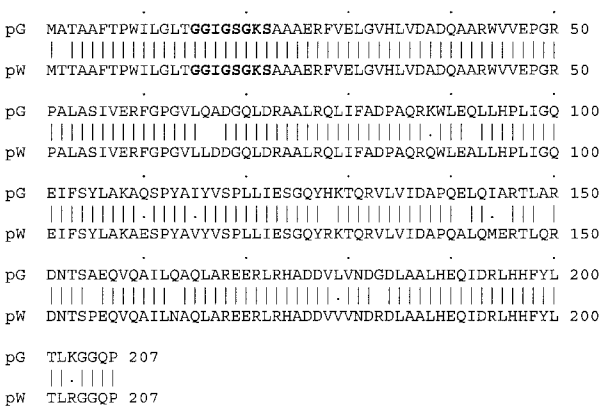

FIGURE 6. Alignment of potential OrfX proteins from *P. putida* GB-1-002 (pG) and *P. putida* WCS358 (pW). Identical (I) and similar (.) amino acids are indicated. The predicted nucleotide-binding site or Walker box A is shown in bold.

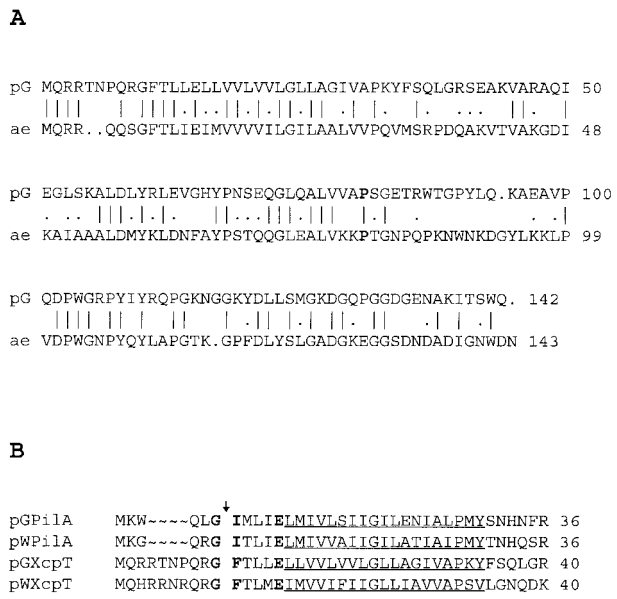

FIGURE 7. (**A**) Alignment of potential XcpT proteins from *P. putida* GB-1-002 (pG) and *P. aeruginosa* (ae). The conserved proline is shown in bold. Identical (I) and similar (.) amino acids are indicated. (**B**) Alignment of the conserved N-terminal region of PilA and XcpT proteins from *P. putida* GB-1-002 (pG) and *P. putida* WCS358 (pW). The prepilin peptidase cleavage site is indicated by an arrow. Critical residues required for cleavage and methylation are shown in bold. The hydrophobic domain is underlined.

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 Tn*5*-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. 7B). The orientations of *pilA* and *pilC* relative to *pilD/xcpA*, and the length of the intergenomic 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 Tn*5* 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 Tn*5* 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 phenylanaline 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

al. 1997). 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 pili 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. Bleves 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 MBT-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 pili 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 Tn*5*-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 Tn*5*-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.

References cited

Bally, M., Filloux, A., Akrim, M., Ball, G., Lazdunski, A., and Tommassen, J. (1992) Protein secretion of *Pseudomonas aeruginosa*: characterization of seven xcp genes and processing of secretory apparatus components by prepilin peptidase. Molecular Microbiology, 6, 1121–1131.

Beijerinck, M.W. (1913) Oxydation des mangancarbonates durch bacterien und schimmelpilze. Folia microbiologica: Hollaendische beitrage zur gesamten mickrobiologie, 2, 123–134.

Bitter, W., Koster, M., Latijnhouwers, M., de Cock, H., and Tommassen, J. (1998) Formation of oligomeric rings by XcpQ and PilD, which are involved in protein transport across the outer membrane of *Pseudomonas aeruginosa.* Molecular Microbiology, 27, 209–219.

Bleves, S., Voulhoux, R., Michel, G., Lazdunski, A. Tommassen, J., and Filloux, A. (1998) The secretion apparatus of *Pseudomonas aeruginosa*: identification of a fifth pseudopilin, XcpX (GspK family). Molecular Microbiology, 27, 31–41.

Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., Boyer, H.W., Crosa, J.H., and Falkow, S. (1977) Construction and characterization of new cloning vehicles: II. A multiple cloning system. Gene, 2, 95–113.

Boogerd, F.C. and de Vrind, J.P.M. (1987) Manganese oxidation by *Leptothrix discophora.* Journal of Bacteriology, 169, 489–494.

Cornelis, P., Anjaiah, V., Koedam, N., Delfosse, P., Jaques, P., Thonart, P., and Neirinck, L. (1992) Stability, frequency and multiplicity of transposon insertion in the pyoverdine region in the chromosomes of different fluorescent pseudomonads. Journal of General Microbiology, 138, 1337–1343.

Corstjens, P.L.A.M. and Muyzer, G. (1993) Phylogenetic analysis of the metal-oxidizing bacteria *Leptothrix discophora* and *Sphaerotilus natans* using 16S rDNA sequencing data. Systematic and Applied Microbiology, 16, 219–223.

Corstjens, P.L.A.M., de Vrind, J.P.M., Westbroek, P., and de Vrind-de Jong, E.W. (1992) Enzymatic iron oxidation by *leptothrix discophora*: identification of an iron-oxidizing protein. Applied and Environmental Microbiology, 58, 450–454.

Corstjens, P.L.A.M., de Vrind, J.P.M., Goosen, T., and de Vrind-de Jong, E.W. (1997) Identification and molecular analysis of the *leptothrix discophora* SS-1 mofA gene, a gene putatively encoding a manganese oxidizing protein with copper domains. Geomicrobiology Journal, 14, 91–108.

Dalrymple, B. and Mattick, J.S. (1987) An analysis of the organization and evolution of type 4 fimbrial (MePhe) subunit proteins. Journal of Molecular Evolution, 25, 261–269.

Figurski, D.H. and Helinski, D.R. (1979) Replication of an origin-containing derivative of plasmid function provided in trans. Proceedings of the National Academy of Science, 76, 1648–1652.

Freitag, N.E., Seifert, H.S., and Koomey, M. (1995) Characterization of pilF-pilD pilus-assembly locus of *Neisseria gonorrhoeae.* Molecular Microbiology, 16, 575–586.

Genin, B. and Boucher, C.A. (1994) A superfamily of proteins involved in different secretion pathways in Gram-negative bacteria: modular structure and specificity of the N-terminal domain. Molecular and General Genetics, 243, 112–118.

de Groot, A., Filloux, A., and Tommassen, J. (1991) Conservation of xcp genes, involved in the two-step protein secretion process, in different Pseudomonas species and other gram-negative bacteria. Molecular and General Genetics, 229, 278–284.

de Groot, A., Heijnen, I., De Cock, H., Filloux, A., and Tommassen, J. (1994) Characterization of type IV pilus genes in plant growth-promiting *Pseudomonas putida* WCS358. Journal of Bacteriology, 176, 642–650.

de Groot, A., Krijger, J.J., Filloux, A., and Tommassen (1996) Characterization of type II preotein secretion (xcp) genes in the plant growth-stimulating *Pseudomonas putida*, strain WCS358. Molecular and General Genetics, 250, 491–504.

Hobbs, M. and Mattick, J.S. (1993) Common components in the assembly of type 4 fimbriae, DNA transfer systems, filamentous phage and protein-secretion apparatus: a general system for the formation of surface-associated protein complexes. Molecular Microbiology, 10, 233–243.

Ish-Horowicz, D. and Burke, J.F. (1981) Rapid and efficient cosmid vector cloning. Nucleid Acid Research, 9, 2989–2998.

Jackson, D.D. (1901) The precipitation of iron, manganese and aluminium by bacterial action. Journal of the Society of Chemical Industry, 21, 193–197.

Kagami, Y., Ratliff, M., Surber, M., Martinez, A., and Nunn D.N. (1998) Type II protein secretion by *Pseudomonas aeruginosa*: genetic suppression of a conditional mutation in the pilin-like component XcpT by the cytoplasmic component XcpR. Molecular Microbiology, 27, 221–233.

Lu, H.M., Timothy, S., and Lory, S. (1997) Interactions of the components of the general secretion pathway: role of *Pseudomonas aeruginosa* type IV pilin subunits in complex formation and extracellular protein secretion. Molecular Microbiology, 25, 247–259.

Miller, J.H. (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Nealson, K.H., Tebo, B.M., and Rosson, R.A. (1988) Occurrence and mechanisms of microbial oxidation of manganese. Advances in Applied Microbiology, 33, 299–318.

Nunn, D., Bergman, S., and Lory, S. (1990) Products of three accessory genes, pilB, pilC and pilD are required for biogenesis of *Pseudomonas aeruginosa* pili. Journal of Bacteriology, 172, 2911–2919.

Okazaki, M., Sugita, T., Shimizu, M., Ohode, Y., Iwamoto, K., de Vrind-de Jong, E.W., de Vrind, J.P.M., and Corstjens, P.L.A.M. (1997) Partial purification and characterization of manganese oxidizing factors of *Pseudomonas fluorescens* GB-1. Applied and Environmental Microbiology, 63, 4793–4799.

Pugsley, A.P. (1993) The complete general secretory pathway in Gram-negative bacteria. Microbiological Reviews, 57, 50–108.

Sambrook, J., Fritsch, E.F., and Maniatis T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Staskawicz, B., Dahlbeck, N., Keen, N., and Napoli, C. (1987) Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. Journal of Bacteriology, 169, 5789–5794.

Strom, M.S., Nunn, D.N., and Lory, S. (1993) A single bifunctional enzyme, PilD, catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family. Proceedings of the National Academy of Science, 90, 2404–2408.

Sunda, W.G. and Kieper, D.J. (1994) Oxidation of humic substances by manganese oxides yields low-molecular weight organic substrates. Nature, 367, 62–64.

Tebo, B.M. (1991) Manganese^{2+} oxidation in the suboxic zone of the Black Sea. Deep-Sea Research, 38, S883–S905.

Tebo, B.M, Ghiorse W.C., van Waasbergen, L.G., Siering, P.L., and Caspi, R. (1997) Bacterial mediated mineral formation: insights into manganese^{2+} oxidation from molecular genetic and biochemical studies. In Mineralogical Society of America Reviews in Mineralogy, 35, 225–266.

de Vrind, J.P.M., Boogerd, F.C., and de Vrind-de Jong, E.W. (1986) Manganese reduction by a marine *Bacillus* species. Journal of Bacteriology, 167, 30–34.

de Vrind, J.P.M., Brouwers, G.J., Corstjens, P.L.A.M., den Dulk, J., and de Vrind-de Jong, E.W. (1998) The cytochrome *c* maturation operon is involved in manganese oxidation in *Pseudomonas putida* GB-1. Applied and Environmental Microbiology, in press.

van Waasbergen, L.G., Hildebrand, M., and Tebo, B.M. (1996) Identification and characterization of a gene cluster involved in manganese oxidation by spores of the marine *Bacillus* sp. strain SG-1. Journal of Bacteriology, 178, 3517–3530.

Walker, J.E., Saraste, M., Runswick, M.J., and Gay, N.J. (1982) Distantly related sequences in the a and b subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. European Molecular Biology Organisation, 1, 945–951.

Yanisch-perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13mp18 and pUC19 vectors. Gene, 33, 103–119.

Manuscript received February 17, 1998
Manuscript accepted August 5, 1998
Paper handled by Jillian F. Banfield