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Trichloroethylene and Chloroform Degradation by a Recombinant Pseudomonad Expressing Soluble Methane Monooxygenase from *Methylosinus trichosporium* OB3b

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Soluble methane monooxygenase (sMMO) from *Methylosinus trichosporium* OB3b can degrade many halogenated aliphatic compounds that are found in contaminated soil and groundwater. This enzyme oxidizes the most frequently detected pollutant, trichloroethylene (TCE), at least 50 times faster than other enzymes. However, slow growth of the strain, strong competition between TCE and methane for sMMO, and repression of the *smmo* locus by low concentrations of copper ions limit the use of this bacterium. To overcome these obstacles, the 5.5-kb *smmo* locus of *M. trichosporium* OB3b was cloned into a wide-host-range vector (to form pSMMO20), and this plasmid was electroporated into five *Pseudomonas* strains. The best TCE degradation results were obtained with *Pseudomonas putida* F1/pSMMO20. The plasmid was maintained stably, and all five of the sMMO proteins (α, β, and γ hydroxylase proteins, reductase, and component B) were observed clearly by both sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblotting. TCE degradation rates were quantified for *P. putida* F1/pSMMO20 with a gas chromatograph (V_{max} = 5 nmol per min per mg of protein), and the recombinant strain mineralized 55% of the TCE (10 μM) as indicated by measuring chloride ion concentrations with a chloride ion-specific electrode. The maximum TCE degradation rate obtained with the recombinant strain was lower than that of *M. trichosporium* OB3b but greater than other TCE-degrading recombinants and most well-studied pseudomonads. In addition, this recombinant strain mineralizes chloroform (a specific substrate for sMMO), grows much faster than *M. trichosporium* OB3b, and degrades TCE without competitive inhibition from the growth substrate.

Trichloroethylene (TCE) is an Environmental Protection Agency-priority pollutant (37) and threatens or contaminates the water supply of many communities (49). Several microorganisms are known to degrade TCE aerobically: methanotrophs (1, 2, 21, 24, 29) (including *Methylosinus trichosporium* OB3b [36, 47] and *Methylococcus capsulatus* (Bath) [18]), pseudomonads (33) (including *Pseudomonas mendocina* KR1 [53], *Pseudomonas putida* F1 [51], *Pseudomonas cepacia* G4 [13] and its constitutive mutant *P. cepacia* G4 PR1 [45]) and recombinant *Escherichia coli* strains (JM109/pDTG601 [55] carrying the toluene dioxygenase [TDO] genes of *P. putida* F1 and HB101/pMY402 [53] containing the toluene monooxygenase genes of *P. mendocina* KR1). In addition, strains expressing ammonia monooxygenase (3) and propane monooxygenase (50) degrade TCE.

For practical TCE remediation, a number of conditions should be considered, including the extent of degradation, the ease of cultivation, the growth rate of the strains, the stability of the degradation enzymes, and regulation of the genes responsible for TCE oxidation. The most important factor, however, is the rate of TCE degradation, and the soluble methane monooxygenase (sMMO) from the type II methanotroph *M. trichosporium* OB3b has the highest rate of TCE oxidation (682 nmol of TCE per min per mg of protein in vitro [14]). Using whole cells of *M. trichosporium* OB3b, Brusseau et al. (5) and Oldenhuis et al. (35) reported a maximum degradation rate of 456 to 600 nmol of TCE per min per mg of protein in the presence of 20 to 25 mM formate as a source of reducing equivalents for the TCE degradation reaction (assuming 0.48 g of protein per g of cells [dry weight] [5]). Without formate, the degradation rate is about 40 nmol of TCE per min per mg of protein (47). In contrast, TCE degradation rates by whole, resting cells of other strains are 1 to 2 nmol of TCE per min per mg of protein (11, 14), except for *P. cepacia* G4, which degrades TCE at 8 nmol of TCE per min per mg of protein (13). These rates are at least 50 times lower than those seen with *M. trichosporium* OB3b resting cells supplemented with formate. The sMMO from *M. trichosporium* OB3b also has a broad substrate range; it can oxidize a wide range of chlorinated hydrocarbons that are, in many cases, resistant to degradation in nature (36) (e.g., hydrochlorofluorocarbons [10], chloroform, and dichloroethane).

Although sMMO degrades TCE rapidly, TCE degradation with *M. trichosporium* OB3b is difficult. This fastidious organism grows relatively slowly: its maximum specific growth rate is less than 0.1/h (38). This limits the speed with which sMMO-containing biomass can be generated. In addition, transcription of the *smmo* locus is repressed by low concentrations of copper ions (0.25 μM) (47); hence, sMMO may not be expressed in the natural environment, and it will be difficult to use *M. trichosporium* OB3b for in situ bioremediation (6, 12, 39). Furthermore, sMMO is competitively inhibited by the substrates methane and TCE (35). Methane gas is required to induce sMMO in *M. trichosporium* OB3b and to supply carbon and energy for cells which continuously degrade TCE.

To overcome these obstacles, the complete *smmo* locus of *M. trichosporium* OB3b was cloned into *E. coli* and five *Pseudomonas* strains with a wide-host-range vector. One of the recombinants, *P. putida* F1/pSMMO20, was found to express active sMMO and was capable of mineralizing chloroform (a specific substrate of sMMO in *M. trichosporium* OB3b [38])

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and TCE. It also degrades TCE faster than other well-studied pseudomonads. For this recombinant system, copper repression of the *smmo* locus was eliminated by placing the structural genes under the control of the chemically inducible *tac* promoter. Furthermore, sMMO produced by the recombinant cell is not subject to competitive inhibition and can be used solely for TCE degradation, because the inducer of sMMO expression in the wild-type strain, methane, is no longer necessary (the recombinant uses other carbon and energy sources).

To form a bacterium which degrades TCE with sMMO and grows faster than *M. trichosporium* OB3b, emphasis was placed on cloning *smmo* into *Pseudomonas* strains. Pseudomonads have relatively high growth rates and are some of the most versatile catabolic organisms (28). In addition, the pseudomonads which degrade TCE have the ability to accommodate TCE and the intermediates of TCE oxidation (some, like chloral hydrate, may be very toxic to cells [1, 34]). The GC content of pseudomonads (41) (especially *P. putida*) is also similar to that of methanotrophs (20).

The DNA sequence of the *smmo* locus of *M. trichosporium* OB3b was determined by Cardy et al. (7, 8) and found to be very similar to that of *M. capsulatus* (Bath). The 5.3-kb *smmo* locus is composed of six genes, *mmoXYBZC* (which code for the α, β, component B, γ, and reductase proteins, respectively) and *orfY* (the product and function of *orfY* are not known). The hydroxylase, $(\alpha\beta\gamma)_2$, contains an oxygen-bridged, dinuclear iron cluster ([Fe-OX-Fe], where X is a proton, a carboxylate group, or an alkyl group) (17, 26), the reductase is a [2Fe-2S] flavoprotein, and component B mediates control of electron transfer (15).

The α and β proteins of the hydroxylase have been produced by use of an in vitro *E. coli* transcription-translation system by Cardy et al. (8), but they did not examine the activity of these proteins. For *M. capsulatus* (Bath), only the component B and reductase of its similar sMMO have been functionally expressed in *E. coli* (52). Therefore, this is the first report of whole, active sMMO expressed in a foreign strain and one of the first reports of expression of a methanotrophic protein.

MATERIALS AND METHODS

Plasmids, bacterial strains, and routine culture conditions. Plasmids pDVC202 (*orfY*$^+$ *mmoC*$^+$) and pDVC210 (*mmoX*$^+$ *Y*$^+$*B*$^+$*Z*$^+$) (7, 8) were kindly provided by J. C. Murrell at the University of Warwick, Coventry, United Kingdom, and the wide-host-range plasmid pMMB277 (31) was obtained from M. Bagdasarian at Michigan State University. *E. coli* XL1-Blue {*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r)]} was used for cloning in *E. coli*, was purchased from Stratagene, La Jolla, Calif., and was cultivated in LB (40) containing 10 μg of tetracycline per ml. *M. trichosporium* OB3b was obtained from R. S. Hanson at the Gray Freshwater Institute, University of Minnesota. This methanotroph was cultivated in HNMS (Higgins nitrate minimal salt) medium without copper sulfate, and methane gas (Liquid Carbonic, Chicago, Ill.) was supplied twice a day as described by Park et al. (38). *P. putida* KT2440 (4) ($r^- m^+$) was obtained from M. Bagdasarian and was grown in LB medium. *P. cepacia* G4 (13) and *P. cepacia* G4 PR1 (45) were provided by M. S. Shields at the University of West Florida and were grown in LB alone and LB with 25 μg of kanamycin per ml, respectively. *P. mendocina* KR1 (54) was obtained from K.-M. Yen at Amgen Inc., Thousand Oaks, Calif., *P. putida* F1 (51) was provided by D. Gibson at the University of Iowa, Iowa City, and *Pseudomonas oleovorans* (43) was purchased from the American Type Culture Collection (culture 29347). LB was used for the cultivation of these strains. All *E. coli* strains were grown at 37°C, and the pseudomonads were cultivated at 30°C by agitating 20 ml of medium in a 250-ml Erlenmeyer flask in rotary shakers at 250 rpm (series 25; New Brunswick Scientific Co. Inc., Edison, N.J.).

For the induction of the TDO of *P. putida* F1, M9 minimal medium (40) with toluene (0.4%, vol/vol) was used. M9-glucose (1%) medium was used for the cultivation of recombinant pseudomonads to measure TCE degradation. The concentrations of chloramphenicol were 50, 75, 300, and 500 μg/ml for *P. cepacia* G4, *P. cepacia* G4 PR1, *P. mendocina* KR1, and the two strains of *P. putida* containing plasmid pSMMO20.

DNA manipulation and electroporation. DNA restriction digestions, ligation, dephosphorylation, phosphorylation, and modification reactions (such as excision of the 3′ overhang of DNA) were conducted with enzymes purchased from United States Biochemical Corp. (Cleveland, Ohio), New England Biolabs (Beverly, Mass.), GIBCO BRL (Gaithersburg, Md.), and Boehringer Mannheim Biochemicals (Indianapolis, Ind.). For the purification of DNA fragments resulting from restriction digestions, 0.75% low-melting-point agarose (FMC Bioproducts, Rockland, Maine) and β-agarase (New England Biolabs) were used according to the instructions provided by the manufacturers. The *Eco*RI-*Sma*I adapter (5′-AATTCCC GGG, 5′-CCCGGG) used for constructing pSMMO20 (Fig. 1) was purchased from New England Biolabs and was phosphorylated with T4 polynucleotide kinase (New England Biolabs). Construction of the plasmid containing the *smmo* locus (pSMMO20) is described in Results.

To transform plasmids into the various pseudomonads and *E. coli*, electroporation was performed with 0.1-cm cuvettes with a Gene Pulser/Pulse Controller purchased from Bio-Rad Laboratories (Hercules, Calif.). The voltages and the time constants were varied from 5 to 15 kV/cm and from 5 to 25 ms, respectively. The best electroporation conditions for each strain are summarized in Table 1. Electrocompetent cells were prepared according to the method of Smith and Iglewski (46). Plasmids for electroporation were obtained by the cesium chloride-ethidium bromide gradient ultracentrifugation method of Sambrook et al. (42) using a model Optima TLX ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.).

Screening recombinant *E. coli* and *Pseudomonas* strains containing the *smmo* locus. For plasmid constructions in *E. coli*, XL1-Blue harboring pBluescript-derived plasmids with inserts were selected as white colonies on MacConkey agar plates (Difco Laboratories, Detroit, Mich.) containing 50 μg of ampicillin and 10 μg of tetracycline per ml (to select for plasmid-bearing cells). For the pseudomonads, colony lift hybridization was used to screen cells containing the *smmo*-bearing plasmids. A 2.1-kb *Eco*RI segment of the *smmo* locus (component B gene probe) (48) obtained from R. S. Hanson was labeled with digoxigenin, using the Genius labeling kit and protocol of Boehringer Mannheim Biochemicals. For the colony lift, hybridization of bound DNA with the labeled probe, and the detection of hybridized DNA protocols, the Genius kit instructions were followed, with the exception that the baked membranes were incubated in TEN buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl [pH 8]) instead of the prehybridization solution prior to the removal of cell debris on the membranes. The agar plates used for the colony lift were kept at room temperature to redevelop colonies. From the colonies determined to have the plasmids with the *smmo* locus, 10 to 20 colonies were picked randomly and were examined by horizontal gel electrophoresis (42) after digestion with *Eco*RI,

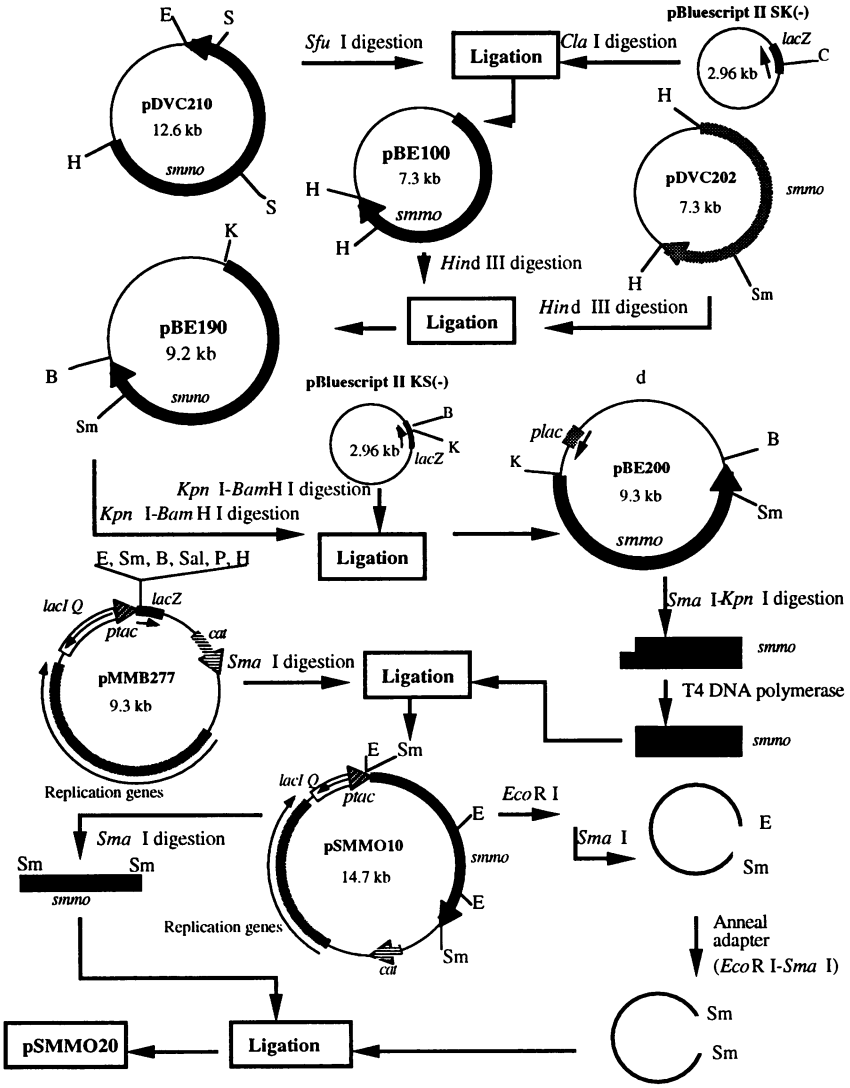

FIG. 1. Schematic diagram for cloning the *smmo* locus into the wide-host-range vector pMMB277. Restriction sites are *Bam*HI (B), *Cla*I (C), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Pst*I (P), *Sfu*I (S), *Sal*I (Sal), and *Sma*I (Sm).

*Sma*I, and *Hin*dIII restriction enzymes to verify the presence of pSMMO20.

SDS-PAGE and Western blot analysis. To prepare total cellular protein samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 20 ml of cells adjusted to an A_{600} of 0.3 with 0.1 M phosphate buffer (pH 7.0) was centrifuged at 4°C for 10 min at 5,000 × *g* (model J2-21; Beckman Instruments, Inc.) and resuspended in 0.3 ml of TE (10 mM Tris, 1 mM EDTA [pH 7.4]). To the resuspended cells, 0.3 ml of 2× sample buffer (10% SDS, 125 mM Tris [pH 6.8], 15% sucrose, 10% β-mercaptoethanol, 1 mM EDTA, 0.05% bromophenol blue, 1 mM phenylmethylsulfonyl fluoride) was added, and the vortexed mixture was boiled for 3 min and then stored at −20°C. A discontinuous polyacrylamide gel (25) (12%) was prepared on a P2X vertical gel electrophoresis unit (Owl Scientific Plastics, Cambridge, Mass.), and 10 to 15 μl of cell protein sample was loaded into each well. After electrophoresis, the gel was stained with Coomassie blue.

For Western blot (immunoblot) analysis, the proteins were separated with a Mini-Protein II Dual Slab Cell (Bio-Rad Laboratories) and the proteins in the polyacrylamide gel were transferred to 0.45-μm-pore-size nitrocellulose membranes (Schleicher and Schuell, Keene, N.H.) with a Trans-Blot Cell (Bio-Rad Laboratories). The sMMO proteins were detected with antisera obtained from G. Georgiou at the University of Texas (antibody against hydroxylase of sMMO) and R. S. Hanson (antibodies against component B and reductase). The secondary antibody and detection kit (ProtoBlot Western blot AP system) were purchased from Promega Corp., Madison, Wis.

Determination of total cell protein. Cells were lysed with a Sonic Dismembrator model 300 (Fisher Scientific, Tustin, Calif.). The total cell protein was analyzed with the Total Protein kit (Lowry micro method) purchased from Sigma Chemical Co., St. Louis, Mo.

TCE and chloroform gas chromatograph (GC) assay. To avoid the slow growth observed (30 to 40 h) to reach an A_{600} of 1 when the recombinant pseudomonad colonies were directly inoculated into M9-glucose medium from LB agar plates, the cells were grown in two stages. One to two days after streaking

TABLE 1. Electroporation of pSMMO20 into *Pseudomonas* strains and degradation of TCE by cells grown in M9-glucose medium supplemented with 1 mM IPTG

Strain	Voltage (kV/cm)	Time constant (ms)	No. of transformants/μg of DNA	TCE degradation[a]
P. putida KT2440	15	4.4	10,500	−
P. putida F1	12.5	9.1	150	+
P. cepacia G4	15	4.6	19	ND
P. cepacia G4 PR1	12.5	9.0	571	ND
P. mendocina KR1	15	4.7	10,300	−
P. oleovorans	5–15	4.5–23.2	0	NA

[a] +, degradation; −, no degradation; ND, not determined because of plasmid instability; NA, not applicable.

cells onto LB agar plates containing chloramphenicol (at the same concentration as described for LB broth), 10 to 20 shake flask cultures were made by inoculating a recombinant pseudomonad colony into 20 ml of LB supplemented with chloramphenicol and incubating the cultures for 10 to 15 h. For each of these precultures, 0.5 ml of the LB seed culture was washed twice with M9 medium (to avoid transferring copper ions), the washed cells were transferred to 50 ml of M9-glucose (10 g/liter) medium, and the cells were cultured for 7 to 8 h. To avoid any possibility of inhibition of the sMMO activity by copper ions, twice-purified (reverse-osmosis-treated and distilled) water was used for the preparation of the minimal medium. This two-stage growth was deemed prudent, since the *Pseudomonas* strains used as hosts can tolerate high concentrations of chloramphenicol (without harboring plasmids which carry the chloramphenicol acetyltransferase gene). Hence, to avoid the possibility that non-plasmid-bearing cells might take over the culture, the total culture time was decreased by using this two-stage growth strategy (however, plasmid instability was not encountered with *P. putida* F1/pSMMO20).

To induce sMMO expression, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the M9-glucose broth when the cell density reached an A_{600} of 0.5. After 5 to 6 h of induction, cells were harvested and washed twice with 0.1 M potassium phosphate buffer (pH 7.0) and the cell density was adjusted to an A_{600} of 2.0 with the same phosphate buffer. Five milliliters of the washed cell suspension was transferred to a 60-ml serum bottle which was sealed by crimping an aluminum cap over a Teflon-faced, silicon septum (Wheaton, Millville, N.J.). TCE dissolved in *N,N*-dimethyl formamide (0.1 to 2.0 M stock solution) or chloroform dissolved in water (50.5 mM stock solution) in vials that contained no headspace gas were added to the vials with a gas-tight syringe (Hamilton, Reno, Nev.). TCE, *N,N*-dimethyl formamide, and chloroform were purchased from Fisher Scientific. Triplicate vials were used for each datum point, and the vials were incubated at 30°C, with inversion and shaking at 250 rpm. The disappearance of TCE is reported relative to time zero no-cell controls which were always used to establish a baseline for TCE disappearance and which consisted of phosphate buffer and TCE in a crimped vial (similar baseline values were obtained from heat-killed *P. putida* F1 or heat-killed *P. putida* F1/pSMMO20 cells with phosphate buffer and TCE).

For the GC assay of TCE and chloroform, 5 μl of headspace gas was injected into a Varian 3600 GC equipped with an electron capture detector (Varian Associates, Sunnyvale, Calif.) at specific time intervals. The column was a 0.1% AT-1000 on Graphpac GC, 80/100 (Alltech Associates, Inc., Deerfield, Ill.). The carrier gas was nitrogen (30 ml/min for TCE and 20 ml/min for chloroform). The column, injector, and detector temperatures for TCE analysis were 150, 170, and 190°C, respectively. For chloroform, the temperatures were uniformly 20°C lower than those for TCE. TCE concentrations used in this study (except where noted) assume all the added TCE remains in the liquid phase and ignore volatilization (for ease of comparison with the results of other studies); hence, the actual TCE concentration seen by the cells is considerably lower, since TCE partitions into the gas and liquid phases according to Henry's law (13).

Measurement of chloride ion concentrations. A model 13-620-519 chloride ion-selective electrode and model 13-620-47 reference silver chloride electrode (Fisher Scientific) were used with a pH/Ion 350 meter (Corning Incorporated, Corning, N.Y.) to detect chloride ions generated during TCE and chloroform mineralization reactions (32). To reduce the background concentration of chloride ions, the cells were washed twice with 0.1 M potassium phosphate buffer (pH 7.0) before TCE or chloroform was added to the 60-ml sealed serum bottle as described above for the GC assay. After 5 h of incubation (after the GC assay was finished), triplicates were analyzed for TCE mineralization by removal of the aluminum crimp tops and analysis of the well-stirred liquid. For chloroform, the time course of mineralization was analyzed by measuring the chloride ion concentration of triplicate vials that were sacrificed at specific times. The amounts of chloride ions produced during TCE or chloroform degradation were normalized by the amount of chloride ions produced by glucose-grown *P. putida* F1 host cells with either TCE or chloroform in the phosphate buffer or normalized by the endogenous chloride ions produced by *P. putida* F1/pSMMO20 in phosphate buffer without TCE (for both negative controls, 10 to 15 μM chloride ions were generated routinely).

RESULTS

Cloning the *smmo* locus into a wide-host-range vector (Fig. 1). The *smmo* locus of *M. trichosporium* OB3b was obtained as two partially overlapping DNA fragments in plasmids pDVC202(7) and pDVC210(8). To recreate the *smmo* locus, the 4.4-kb *Sfu*I fragment of pDVC210 was ligated into a *Cla*I site of pBluescript II SK(−). The resulting plasmid, pBE100, was digested with *Hin*dIII, and the 6.1-kb fragment was ligated with the 3.1-kb *Hin*dIII fragment of pDVC202. This resulting plasmid (pBE190) contained the complete *smmo* locus in the orientation opposite from that of the *lac* promoter. To revert it, the *Kpn*I-*Bam*HI fragment was cloned into pBluescript II KS(−) to form the 9.3-kb plasmid, pBE200, which contains the *colE1* origin of replication, so that it cannot replicate in *Pseudomonas* strains. To clone the *smmo* locus into a wide-host-range plasmid that could be electroporated into *Pseudomonas* strains, the *Kpn*I-*Sma*I fragment was excised from pBE200, made blunt with T4 DNA polymerase, and ligated into the *Sma*I site of the wide-host-range vector pMMB277. The resulting plasmid, pSMMO10, contained a fused reading frame of *lacZ*α and *mmoX*, so that a 63-kDa fusion protein of LacZα and the α protein of the hydroxylase of sMMO was formed along with the wild-type α protein (58 kDa). To remove the fusion protein, pSMMO10 was digested with *Sma*I, and the pMMB277 vector portion was isolated and then digested with *Eco*RI to alter the reading frame between the *lacZ*α ribosome binding site and the ribosome binding site of *mmoX*. The *Eco*RI site was made blunt with an *Eco*RI-*Sma*I adapter, and the *Sma*I segment of *smmo* was religated with the vector (four bases were removed from the original sequence and six bases were added from the adapter; hence, the net

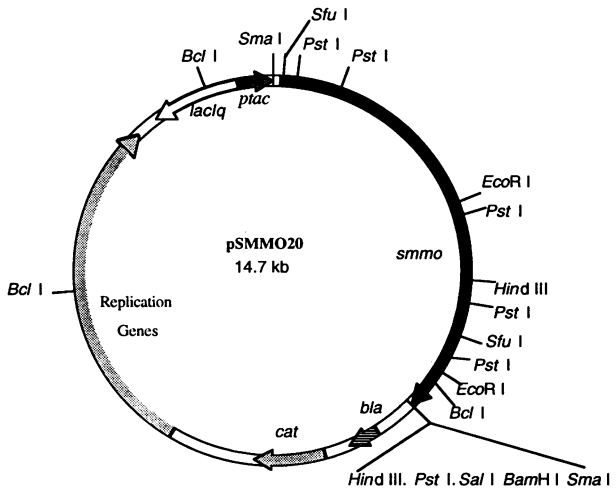

FIG. 2. Restriction map of pSMMO20.

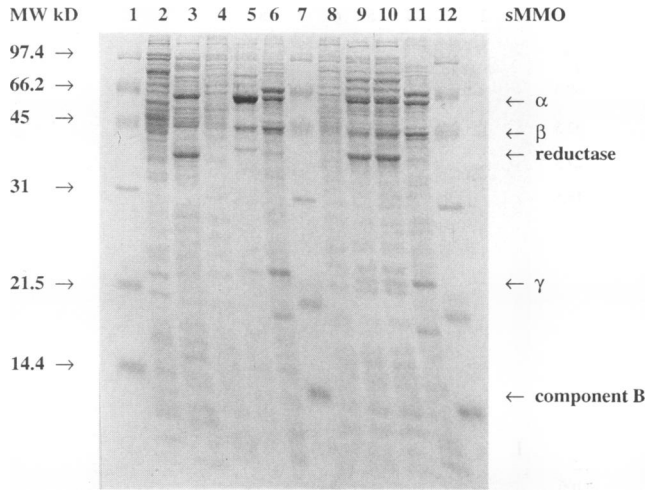

FIG. 3. SDS-PAGE of total cellular protein of sMMO-expressing recombinant pseudomonads containing pSMMO20. All five sMMO proteins (indicated by arrows on the right) were seen clearly in the recombinants. Copper sulfate (10 μM) was added to the growth medium for all except *M. trichosporium* OB3b (lanes 6 and 11) and efficient TCE-degrading *P. putida* F1/pSMMO20 (lane 9). Samples (10 to 15 μl) prepared as described in Materials and Methods were loaded into each well. Lanes 1, 7, and 12, molecular standards (MW); lane 2, *P. mendocina* KR1; lane 3, *P. mendocina* KR1/pSMMO20; lane 4, *P. putida* KT2440; lane 5, *P. putida* KT2440/pSMMO20; lane 6, *M. trichosporium* OB3b; lane 8, *P. putida* F1 grown on glucose; lane 9, *P. putida* F1/pSMMO20 (efficient TCE degradation, no copper ion in the growth medium); lane 10, *P. putida* F1/pSMMO20 (nonefficient TCE degradation); lane 11, *M. trichosporium* OB3b. Molecular standards (in kilodaltons) phosphorylase *b* (97.4), bovine serum albumin (66.2), ovalbumin (45.0), carbonic anhydrase (31.0), soybean trypsin inhibitor (21.5), and lysozyme (14.4) are indicated on the left.

result was a two-base addition). The resulting 14.7-kb plasmid, pSMMO20, is shown in Fig. 2 and contains the IPTG-inducible *tac* promoter upstream of the *smmo* locus. The whole *smmo* locus can be removed by a single *Sma*I digestion, and the DNA restriction pattern of pSMMO20 has been checked by horizontal gel electrophoresis after being cleaved with 11 restriction enzymes (*Bcl*I, *Sma*I, *Sfu*I, *Pst*I, *Eco*RI, *Hin*dIII, *Bam*HI, *Not*I, *Kpn*I, *Nde*I, and *Cla*I).

Electroporation of pSMMO20 into various *Pseudomonas* strains. Since pSMMO20 is a derivative of the wide-host-range vector, pMMB277, it can replicate in various *Pseudomonas* species. To obtain transformants, pSMMO20 isolated from *E. coli* XL1-Blue by the cesium chloride-ethidium bromide gradient method was electroporated into various pseudomonads, with the results and electroporation parameters shown in Table 1. To screen transformants, colony lift hybridization with a digoxigenin-labeled sMMO component B probe was used. The efficiency of transformation varied among the strains, and *P. oleovorans* transformants were not found. Plasmid pSMMO20 in *P. mendocina* KR1, *P. putida* F1, and *P. putida* KT2440 was relatively stable (no segregational instability was encountered during the shake flask experiments), while *P. cepacia* G4/pSMMO20 and *P. cepacia* G4 PR1/pSMMO20 were extremely unstable (segregational instability was observed after 10 to 15 h of growth in the LB preculture).

Inconsistency of TCE degradation by *P. putida* F1/pSMMO20. Although *P. putida* F1/pSMMO20 indubitably degrades TCE using sMMO (as described below), only 10 to 20% of the minimal medium shake flask cultures inoculated with this recombinant developed a cell population which degraded TCE efficiently. Nonefficient shake flask cultures degraded only 15% of the TCE in 5 h (at 20 μM), whereas the efficient TCE-degrading cultures degraded 35% of the TCE in 5 h. Hence, IPTG-induced *P. putida* F1/pSMMO20 cultures always degraded significantly more TCE than the small amount that was depleted in the negative controls (glucose-grown *P. putida* F1, heat-killed *P. putida* F1, and cell-free phosphate buffer). Similarly, efficient chloroform-degrading cultures arose in 10% of the cultures. For the TCE and chloroform degradation data presented in this paper, results are shown only for the efficient TCE-degrading cultures.

The discrepancy in TCE degradation results between seemingly identical shake flasks has not been adequately resolved. It was found that a single LB culture gives rise to both efficient and nonefficient minimal medium cultures. Furthermore, both efficient and nonefficient phenotypes were not stable upon subcultivation and attempts to isolate an efficient TCE-degrading colony failed. The plasmid appears to be maintained stably in the nonefficient TCE-degrading cultures (pSMMO20 observed upon isolation and visualization with horizontal electrophoresis), and sMMO is clearly synthesized in the nonefficient TCE-degrading cultures, as evidenced by SDS-PAGE (Fig. 3) and Western blots (Fig. 4); therefore, the problem seems to be one of inconsistent sMMO activity in *P. putida* F1/pSMMO20.

SDS-PAGE and Western blot analysis of sMMO polypeptides produced in *Pseudomonas* strains. By SDS-PAGE, total protein analysis of the sMMO-expressing pseudomonads, all five of the sMMO proteins were clearly seen to be produced by *P. putida* F1/pSMMO20 and *P. putida* KT2440/pSMMO20 (Fig. 3). For these recombinant pseudomonads, the reductase band is significantly stronger than that of *M. trichosporium* OB3b and the other four sMMO proteins were synthesized at levels comparable to levels in *M. trichosporium* OB3b grown on methane.

In the Western immunoblot assay (Fig. 4), all five proteins of sMMO were easily detected for *P. putida* KT2440/pSMMO20 and *P. putida* F1/pSMMO20. For *P. putida* F1/pSMMO20, these five sMMO proteins were clearly produced for both cells which degraded TCE efficiently and those that did not degrade TCE as well. In addition, the SDS-PAGE gel (Fig. 3) shows there were no differences in sMMO expression for efficient *P.*

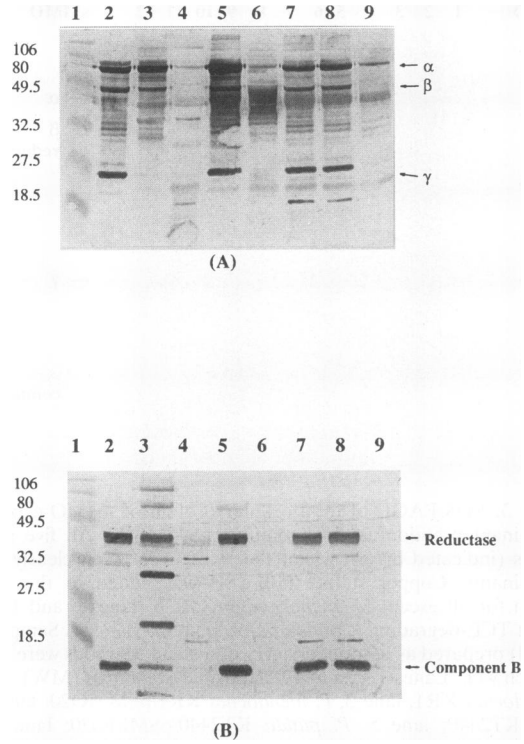

FIG. 4. Western blot analysis of the sMMO proteins produced in recombinant pseudomonads by an antibody against the hydroxylase of sMMO (α, β, and γ proteins [arrows] (A) and antiserum mixture against component B and the reductase (B). All five bands of sMMO protein were observed clearly in this Western analysis. Samples were the same as for Fig. 3, except that 5-μl aliquots were loaded into each well. Lane 1, molecular standards; lane 2, *M. trichosporium* OB3b; lane 3, *P. mendocina* KR1/pSMMO20; lane 4, *P. mendocina* KR1; lane 5, *P. putida* KT2440/pSMMO20; lane 6, *P. putida* KT2440; lane 7, *P. putida* F1/pSMMO20 (efficient TCE degradation, no copper ion in the growth medium); lane 8, *P. putida* F1/pSMMO20 (nonefficient TCE degradation); lane 9, *P. putida* F1. Molecular standards (prestained; in kilodaltons) phosphorylase *b* (106), bovine serum albumin (80), ovalbumin (49.5), carbonic anhydrase (32.5), soybean trypsin inhibitor (27.5), and lysozyme (18.5) are indicated on the left.

putida F1/pSMMO20 TCE-degrading cells and nonefficient *P. putida* F1/pSMMO20 cells. For *P. mendocina* KR1/pSMMO20, the γ protein of the hydroxylase was not observed in the Western blot and there were extra, smaller bands detected in the Western analysis for component B and the reductase, which might be breakdown products in *P. mendocina* KR1/pSMMO20. Analysis of sMMO protein production in *P. cepacia* G4/pSMMO20 and *P. cepacia* G4 PR1/pSMMO20 was not possible because of plasmid segregational instability.

The wild-type host, *M. trichosporium* OB3b, expressed sMMO, since it was grown in medium that lacked copper ions (Fig. 3 and 4). In contrast, the recombinant pseudomonads were capable of expressing sMMO in M9-glucose medium containing 10 μM copper ions as copper sulfate, as shown in Fig. 3 and 4. Hence, transcription of the *smmo* locus in the recombinant pseudomonads was controlled by the *tac* promoter and the copper repression of this locus present in *M. trichosporium* OB3b has been overcome. It is important to note that for TCE degradation, sMMO was expressed in *P. putida* F1/pSMMO20 in the absence of copper ions to assure that active enzyme was obtained (sMMO has been reported to be both sensitive and insensitive to copper in vitro [12, 19]).

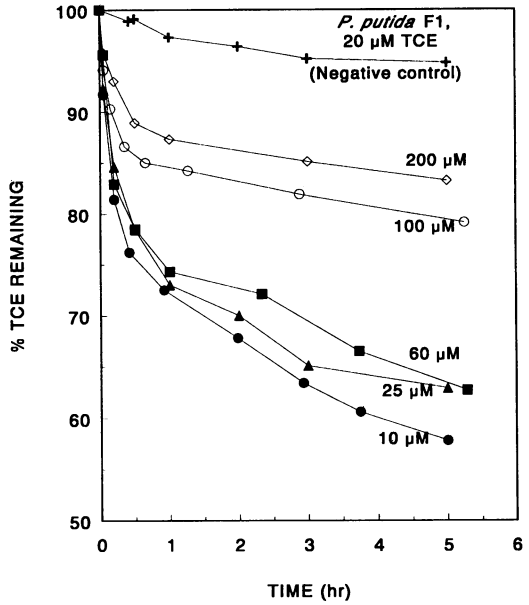

FIG. 5. TCE degradation of *P. putida* F1/pSMMO20 measured with a GC. Cells (including *P. putida* F1) were grown in M9-glucose medium supplemented with 1 mM IPTG. The TDO of *P. putida* F1 was not expressed, so that the TCE concentration did not decrease substantially in the negative control (less than 6%). The TCE concentrations shown assume that all TCE is dissolved in the liquid phase. Actual TCE concentrations in the liquid phase calculated on the basis of Henry's law are 18.5% of those shown.

The amount of sMMO proteins produced by the recombinant was significant even though the copy number of pSMMO20 is low (based on the low copy number of the cloning vector pMMB277 [31] and verified by the low yields seen for pDNA mini-preps). Furthermore, regulation of sMMO transcription from the *tac* promoter was fairly tight, since sMMO synthesis was low in the absence of the inducer IPTG (data not shown). Therefore, the control of the promoter by the *lacI*^{q} repressor and the inducer (IPTG) seemed satisfactory in *P. putida* KT2440/pSMMO20, *P. putida* F1/pSMMO20, and *P. mendocina* KR1/pSMMO20.

TCE and chloroform degradation by *P. putida* F1/pSMMO20. The ability of the pseudomonads to degrade TCE was evaluated with a GC, and a chloride ion-specific electrode was used to gauge the degree of mineralization. Of the three recombinants with stable plasmids, only *P. putida* F1/pSMMO20 degraded TCE (Table 1). For *P. putida* F1/pSMMO20, TCE degradation was measured as the concentration of TCE varied from 10 to 200 μM (it was assumed that TCE remained completely in the aqueous phase) by monitoring the TCE headspace concentration with a GC (Fig. 5). TCE initial degradation rates increased as the TCE concentration increased (0.39, 0.91, 1.25, 2.8, and 4.0 nmol of TCE per min per mg of protein at 10, 25, 60, 100, and 200 μM concentrations of TCE), and a larger percentage of TCE was removed by the resting cells as the concentration of TCE decreased (maximum was 42% TCE degraded after 5 h by resting cells with TCE at 10 μM). For comparison, the extent of TCE degradation in *M. trichosporium* OB3b is 100% in 20 min at 80 μM with no formate (47) (similar results were obtained by this laboratory). Without induction of sMMO (no IPTG control), *P. putida* F1/pSMMO20 was unable to degrade TCE.

All cells including *P. putida* F1 without pSMMO20 were

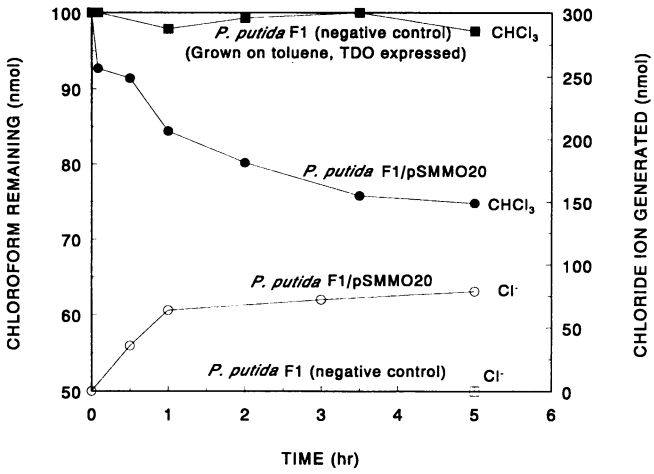

FIG. 6. Chloroform degradation with *P. putida* F1/pSMMO20 and *P. putida* F1. The recombinant *P. putida* F1/pSMMO20 grown in M9-glucose medium supplemented with 1 mM IPTG degraded and mineralized chloroform, while the wild-type host *P. putida* F1 grown on toluene-minimal medium (which induced TDO) did not degrade chloroform. The concentration of chloroform was 20 μM (assuming chloroform remains completely in the liquid phase).

grown on glucose in M9 minimal medium supplemented with 1 mM IPTG. The negative control for these experiments, *P. putida* F1 without the plasmid, did not degrade TCE in 43 different sets of experiments. This result agrees well with that of Jenkins and Dalton (23), who also found *P. putida* F1 NCIB11767 did not produce TDO when grown with glucose. Therefore, TDO was not expressed under these (toluene-free, glucose-minimal medium) conditions. *P. putida* F1, however, degraded TCE when it was grown on toluene to induce TDO (data not shown). As an additional negative control, *P. putida* F1/pSMMO20 cells were cultured without IPTG and checked for the ability to degrade TCE: without IPTG, eight cultures of *P. putida* F1/pSMMO20 were unable to degrade TCE. Furthermore, sMMO bands were clearly seen on SDS-PAGE gels only when IPTG was present to induce the *ptac* promoter (data not shown).

To verify that TCE degradation was catalyzed by sMMO, the ability of *P. putida* F1/pSMMO20 to degrade chloroform when induced with IPTG was also examined. As shown in Fig. 6, *P. putida* F1/pSMMO20 with sMMO induced degraded chloroform (25% of the chloroform was degraded in 5 h at 20 μM). However, the host grown on toluene did not degrade chloroform at all; therefore, TDO could not oxidize chloroform. As a further control, the patterns of total-cell protein production were compared for *P. putida* F1 grown on glucose and on toluene by SDS-PAGE (data not shown). For *P. putida* F1 grown on glucose, TDO bands were not visible; however, for *P. putida* F1 grown on toluene, five distinct bands of the *todC1C2BADE* operon were clearly seen (small and large ISP_{TOL} subunits, $reductase_{TOL}$, 3-methylcatechol 2,3-dioxygenase, and *cis*-toluene dihydrodiol dehydrogenase). On the basis of these four observations (inability of the host to degrade TCE when grown on glucose, inability of the host to degrade chloroform with TDO expressed, SDS-PAGE results suggesting TDO is not formed in the host when it is grown on glucose, and TCE degradation only when *P. putida* F1/pSMMO20 is induced with IPTG), we conclude the sMMO expressed in *P. putida* F1/pSMMO20 is responsible for TCE degradation.

TABLE 2. Generation of chloride ions due to mineralization of TCE by *P. putida* F1/pSMMO20 grown in M9-glucose medium supplemented with 1 mM IPTG

TCE added (nmol)	TCE oxidized (%)[a]	Chloride ions generated (nmol)	Mineralization efficiency (%)[b]
50	42	82	130
125	37	162	117
300	37	262	79
500	21	165	51
1,000	17	366	72

[a] Results after 5 h with resting cells determined by the GC assay (Fig. 5).
[b] Percentage of oxidized TCE that was mineralized.

TCE and chloroform mineralization by *P. putida* F1/pSMMO20. Soluble methane monooxygenase oxidizes TCE to TCE epoxide, which spontaneously forms primarily glyoxylate (5%), carbon monoxide (53%), and formate (35%) (14). The formate and probably carbon monoxide are then converted by the host to carbon dioxide. The overall reaction is as follows (1, 30):

$$C_2Cl_3H + NADH + H^+ + 2O_2 \xrightarrow{\text{sMMO}} 2CO_2 + NAD^+ + 3HCl$$

Therefore, to examine the extent of mineralization of TCE, the concentrations of chloride ions were measured with a chloride ion-specific electrode. The chloride ion measurements shown in Table 2 were obtained by analyzing the chloride ion concentration at the end of the shake flask experiments (Fig. 5) from which TCE degradation rates were determined. At 10 and 25 μM concentrations of TCE, the TCE that was oxidized by sMMO (42 and 37%, respectively, as determined by the GC assay) was completely mineralized. As the initial TCE concentration increased, less of the TCE that reacted with sMMO was mineralized (Table 2). In contrast, *M. trichosporium* OB3b is capable of mineralizing TCE completely at 140 μM in 10 h with 20 mM formate present (36).

At 20 μM, chloroform was also mineralized by *P. putida* F1/pSMMO20, as shown in Fig. 6. As 25 nmol of chloroform was degraded by the recombinant in 5 h (25% of the chloroform in the system as determined by GC analysis), 100% (75 nmol) of the chloride ions was released and detected with the chloride ion-specific electrode; therefore, all of the chloroform which reacted with sMMO was mineralized. In contrast, no chloride ions were generated from chloroform by the host alone (no pSMMO20).

TCE degradation rate by *P. putida* F1/pSMMO20. The actual TCE concentrations in the liquid phase as determined by Henry's law are much lower than those shown in Fig. 5, which assumes all the TCE remains in the liquid. For example, after TCE has been added to make the liquid concentration 200 μM (assuming all the TCE remains in the liquid), at equilibrium, the actual TCE concentration in the liquid phase is 37 μM (for a 5-ml liquid–55-ml gas system). Although the actual liquid-phase TCE concentration is lower than that indicated in Fig. 5, the calculation of the maximum rate is unaffected. The maximum rates of TCE degradation were calculated as 4.9 and 5.3 nmol of TCE per min per mg of protein by both Lineweaver-Burk and Woolf-Augustinsson-Hofstee plots (44) of the initial TCE degradation rates shown in Fig. 5. This V_{max} is based on a protein content of 0.705 mg/ml for IPTG-induced *P. putida* F1/pSMMO20 analyzed at a cell density of 2 (A_{600}). The maximum TCE degradation rate of *P. putida* F1/pSMMO20 is lower than that of *M. trichosporium* OB3b (40 nmol of TCE per min per mg of protein for cells

without formate) (47), but it is significantly higher than those for most other resting cell systems (11, 14).

Using the actual TCE concentrations (18.5% of those shown in Fig. 5), we calculated the K_m value as 22 μM. For *M. trichosporium* OB3b, the K_m for TCE is 35 μM in vitro (14) and 138 to 145 μM in vivo (5, 35). Therefore, it appears that the recombinant strain may have potential for a greater extent of TCE removal than the methanotroph (perhaps TCE is transported into the pseudomonad more readily).

DISCUSSION

The goal of this research was to construct a recombinant pseudomonad which expresses active sMMO under the control of a chemically inducible promoter, grows faster than *M. trichosporium* OB3b, and degrades TCE at a high rate. The recombinant strain constructed, *P. putida* F1/pSMMO20, grew faster than *M. trichosporium* OB3b (typical *P. putida* F1/pSMMO20 specific growth rate of 0.2 to 0.3 h^{-1} versus less than 0.1 h^{-1} for *M. trichosporium* OB3b) and degraded TCE faster than most other *Pseudomonas* TCE oxidizers as well as the recombinant *E. coli* strains containing either *P. putida* F1 TDO or *P. mendocina* KR1 toluene monooxygenase. However, the TCE degradation rate in the recombinant was considerably lower than that of *M. trichosporium* OB3b. Unlike TCE degradation in either *M. trichosporium* OB3b or *P. putida* F1, there is no competition between TCE and the growth substrate for the degradation enzyme in the recombinant strain; consequently, TCE degradation is efficient (all the sMMO produced is capable of degrading TCE). For complete mineralization, it is necessary to oxidize TCE to carbon dioxide, and on the basis of the results showing the generation of chloride ions, TCE was mineralized to a significant extent with the recombinant *P. putida* F1/pSMMO20, although both the extent of TCE degradation and the extent of mineralization by *M. trichosporium* OB3b are much greater.

The reason why the highest rates of TCE degradation with *P. putida* F1/pSMMO20 were lower than that of wild-type *M. trichosporium* OB3b is not clear. As shown in Fig. 3 and 4, *M. trichosporium* OB3b and *P. putida* F1/pSMMO20 synthesized comparable amounts of sMMO. Similarly, sMMO protein synthesis was not appreciably different in *P. putida* F1/pSMMO20 resting-cell cultures which efficiently degraded TCE (35% degraded in 5 h) compared with cultures which degraded only 15% of the TCE (Fig. 3 and 4). Therefore, both the inconsistency in TCE degradation with *P. putida* F1/pSMMO20 and the lower maximum rate for efficient *P. putida* F1/pSMMO20 cultures than for *M. trichosporium* OB3b may be due to inefficient metal cofactor addition to the sMMO apoenzyme (rather than genetic instability of the *smmo* locus). This seems reasonable, in that sMMO isolated from the wild-type host itself (*M. trichosporium* OB3b) is notorious for having variable activity: the sMMO specific activity varies over a 24-fold range as the ratio of iron per hydroxylase (mol/mol) varies from 1.9 to 4.3 (15).

It has been established that the hydroxylase of sMMO is composed of apoenzyme plus an oxygen-bridged, dinuclear iron cluster, which is relatively rare in natural enzyme systems (17, 26). Also, it has been demonstrated that each sMMO hydroxylase contains four iron atoms (two oxygen-bridged, dinuclear iron clusters per hydroxylase) (15). One possibility is that the recombinant system lacks certain cellular machinery responsible for transferring the necessary cofactor into the active sites of the hydroxylase. Synthesis of the dinuclear cluster is probably spontaneous (22); however, it seems unlikely that this rare cluster is made in adequate amounts by the *P. putida* F1 host (possibly because of inadequate quantities of internal ferrous iron). Therefore, some of the sMMO proteins produced may not be active and this would explain why *P. putida* F1/pSMMO20 degrades TCE more slowly than *M. trichosporium* OB3b and why only 10 to 20% of the *P. putida* F1/pSMMO20 fermentations showed efficient TCE degradation.

For enhanced sMMO activity and consistency of TCE degradation in the recombinant system, cloning the *M. trichosporium* OB3b locus responsible for transferring the oxygen-bridged, dinuclear iron cluster of the hydroxylase (if it exists) along with the *smmo* locus seems worthwhile. A good analogy can be found in the melanin operon (*melC*) of *Streptomyces antibioticus* (9, 27). The *melC* operon is composed of *melC1* and *melC2*. It is believed that *melC1* encodes a 12-kDa protein which transfers the copper ion into the tyrosinase apoenzyme encoded by *melC2*.

Although the reductase of sMMO also contains two prosthetic groups, FAD and a [2Fe-2S] center (15), they are more abundant in biological systems. Hence, West et al. (52) were able to express functional component B and reductase of the related sMMO of *M. capsulatus* (Bath) in *E. coli*.

Another explanation for the TCE degradation rate of the recombinant pseudomonad being lower than that of the wild-type host is that the stoichiometric ratio of each of the sMMO protein components may be inadequate in the pseudomonad; the five proteins of sMMO must form the aggregate $[(\alpha\beta\gamma)_2(B)(\text{reductase})]$ to be active (15, 16). Although the structure of the polycistronic *smmo* locus was the same for the recombinant system as for *M. trichosporium* OB3b, the amounts of the proteins produced in both systems may not be identical. Different ratios of the protein components might cause incorrect aggregation, which results in relatively low activity. For example, Fox et al. (16) have found that for ratios of greater than five component B proteins per hydroxylase, sMMO activity is inhibited. However, both the SDS-PAGE and Western blots reveal that similar amounts of all five sMMO proteins are made by *P. putida* F1/pSMMO20 and *M. trichosporium* OB3b.

P. putida F1, *P. mendocina* KR1, and the two strains of *P. cepacia* were chosen as the hosts of the *smmo* locus because these strains oxidize TCE. This implies TCE oxidation by sMMO in these four strains could be measured without the activity of sMMO being masked by rapid killing of cells or depletion of reducing power. By trying a wider range of hosts, the activity and stability of TCE degradation may be increased.

Another approach to enhance sMMO activity that has not been adequately pursued is to optimize the growth medium. For example, Fox et al. (15) found that adding Fe^{2+} and cysteine to the purification buffer resulted in a significant increase in the in vitro specific activity of the hydroxylase of sMMO. They also found that the iron content in the hydroxylase was important for activity. So, it may be possible to increase the rate of TCE degradation by altering the conditions of cultivation.

With this recombinant that expresses active sMMO, investigations of the impact of copper ions on sMMO activity (in the absence of the effect of copper on transcription) and the role of *orfY* can now be conducted. In addition, *P. putida* F1/pSMMO20 may now be used for site-directed mutagenesis studies of sMMO.

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