Optimization of Trichloroethylene Degradation Using Soluble Methane Monooxygenase of *Methylosinus trichosporium* OB3b Expressed in Recombinant Bacteria

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By complementing cell-free extracts of *Pseudomonas putida* F1/pSMMO20 with purified soluble methane monooxygenase (sMMO) components of *Methylosinus trichosporium* OB3b, the low cloned-gene sMMO activity in the recombinant strain was found to be due to incomplete activity of the hydroxylase component. To address this incomplete activity, additional sMMO-expressing strains were formed by transferring *mmo*-containing pSMMO20 and pSMMO50 into various bacterial species including pseudomonads and α-2 subdivision strains such as methanotrophs, methylotrophs, *Agrobacterium tumefaciens* A114, and *Rhizobium meliloti* 102F34 (11 new strains screened); sMMO activity was detected in the last two strains. To increase plasmid segregational stability, the *hok/sok* locus originally from *Escherichia coli* plasmid R1 was inserted downstream of the *mmo* locus of pSMMO20 (resulting in pSMMO40) and found to enhance plasmid stability in *P. putida* F1 and *R. meliloti* 102F34 (first report of *hok/sok* in *Rhizobium*). To further increase sMMO activity, a modified Whittenbury minimal medium was selected from various minimal and complex media based on trichloroethylene (TCE) degradation and growth rates and was improved by removing the sMMO-inhibiting metal ions [Cu(II), Ni(II), and Zn(II)] and chloramphenicol from the medium and by supplementing with an iron source (3.6 μM of ferrous ammonium sulfate). Using chemostat-grown *P. putida* F1/pSMMO40, it was found that sMMO activity was higher for cells grown at higher dilution rates. These optimization efforts resulted in a twofold increase in the extent of TCE degradation and more consistent sMMO activity. © 1996 John Wiley & Sons, Inc.

Key words: trichloroethylene • methane monooxygenase • *Methylosinus trichosporium*

INTRODUCTION

Soluble methane monooxygenase (sMMO) from the type II methanotroph (obligate methylotroph) *Methylosinus trichosporium* OB3b can cometabolically degrade trichloroethylene (TCE), an Environmental Protection Agency (EPA) priority pollutant, with the highest rate among biological systems [510 nmol/(min mg protein) in the presence of 20 m*M* formate as a reducing equivalent (source of NADH)[33]] and has a wide spectrum of substrates including benzene,[7] chloroform,[34] hydrochlorofluorocarbons,[9] and vinyl chloride.[12] This enzyme also has potential applications for converting waste methane into methanol, synthesizing homochiral epoxides, and probing the biochemical fundamentals of the methane oxidation reaction. The sMMO expression in the wild-type strain is known to be regulated by the concentration of copper ions [sMMO is expressed at copper concentrations lower than 0.86 μmol/g dry cell weight,[3] and particulate methane monooxygenase (pMMO) is expressed at higher concentrations[32]] and induced by methane[36] (resulting in competitive inhibition between TCE and methane for sMMO). In addition, *M. trichosporium* OB3b grows slowly ($\mu_{max} < 0.1$/h[35]). To overcome these intrinsic obstacles of the wild-type strain which prevent efficient TCE degradation, the *mmo* locus from *M. trichosporium* OB3b was cloned previously into *Pseudomonas putida* F1 (*P. putida* F1/pSMMO20) and active sMMO expression was observed.[27] In this recombinant system, copper repression, competitive inhibition, and slow growth of cells were eliminated.

However, low and inconsistent sMMO activity was encountered with *P. putida* Fl/pSMMO20. The TCE degradation rate was 5 nmol/(min mg protein) in the absence of reducing equivalents [cf. 40 nmol TCE/(min mg protein) for *M. trichosporium* OB3b in the absence of formate[44]], and this rate was observed in only 15% of the cultures. These most active cultures degraded 35% of 20 μM TCE in 5 h, whereas the remaining 85% of the cultures showed about 15% degradation[27] (the negative controls showed less then 6% decrease in 5 h).

To investigate the inconsistent sMMO activity, cell-free extracts of *P. putida* F1/pSMMO20 were comple-

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mented with sMMO components purified from *M. trichosporium* OB3b to determine which sMMO component is responsible for the low and inconsistent activity. In addition, the *mmo* locus was cloned into α-2 strains (classification of purple eubacteria based on 16S rRNA sequence data by Woese et al.[49]) that are phylogenetically close to *M. trichosporium* OB3b[45] to obtain additional sMMO-expressing recombinant strains (three additional pseudomonads were also tried). The *mmo*-harboring plasmid was stabilized using the *hok/sok* locus (a post-segregational-killing locus) which is known to increase segregational plasmid stability by killing plasmid-free cells of many gram-negative bacteria.[19] In addition, a minimal medium was optimized for a higher recombinant sMMO activity by removing sMMO-inhibiting metals and chloramphenicol[26] and by supplementing with an iron source. The effect of specific growth rates on sMMO activity of the recombinant strain was also studied using chemostat cultures.

MATERIALS AND METHODS

Bacterial Strains and Routine Growth Conditions

Bacterial strains and plasmids used in this study are summarized in Table I. For cultivating the methylotrophs (*Methylophilus methylotrophus* AS1, *Methylobacterium extorquens* AM1, *Methylobacterium organophilum* XX, and *Pseudomonas* sp. M27), MacLennan minimal medium (MM)[30] supplemented with 0.5% (v/v) methanol (Fisher, Tustin, CA) or 0.05% (w/v) methylamine (Aldrich, Milwaukee, WI) was routinely used [although these strains can grow in Luria–Bertani (LB)[37] medium as well]. The methanotrophs (*Methylocystis parvus* OBBP and *Methylocystis pyriformis* #14) were grown in Higgins nitrate minimal salt medium (HNMS)[14] containing 0.5% (v/v) methanol or supplemented twice daily with a 1:5 (v/v) methane–air mixture. The cultivation temperature for the methylotrophs and methanotrophs was 30°C with the exception that *M. methylotrophus* AS1 was grown at 37°C.

Agrobacterium tumefaciens A114 (Ti-plasmidless) and *Rhizobium meliloti* 102F34 were cultivated at 30°C in Luria broth (LB) and tryptone yeast extract (TY) medium,[5] respectively. The *Pseudomonas* strains were grown in LB medium at 30°C. Antibiotics were added to the media at the concentrations shown in Table I when the strains contained plasmids.

Plasmid Construction and DNA Manipulation

To construct the *mmo*-containing, Inc P-1 plasmid pSMMO50 (pSMMO20 and 40 are Inc Q-derivatives) for expression of sMMO in methylotrophs and methanotrophs, the *Kpn* I–*Bam*H I fragment of pBE200[27] was inserted into *Kpn* I–*Bgl* II-digested pHX200V-47-m1 [*Hind* III, *Xba* I, and *Bam*HI sites of the multiple-cloning-site of pHX200V-47[53] were removed in the process of truncating the promoter region (P_{moxF})[22]]. Ligated DNA was extracted with a mixture of chloroform/phenol/*iso*-amyl alcohol (Sigma, St. Louis, MO), precipitated with ethanol, and electroporated into *Escherichia coli* JM109 (Stratagene, San Diego, CA) as described below. The plasmid from a tetracycline (20 μg/mL)-resistant colony on a LB plate was isolated using a plasmid minipreparation,[37] and pSMMO50 was confirmed using *Kpn* I, *Bgl* II, *Pst* I, and *Hind* III. This new plasmid contains the *mmo* locus under the control of methanol dehydrogenase promoter (P_{moxF}) from *M. organophilum* XX (Fig. 1).

To construct pSMMO40 (*hok/sok*-containing pSMMO20) for increased plasmid segregational stability, the 1.6-kb *Bam*H I fragment of pKG1022[19] was isolated from a low-melting-point agarose gel[27] and was ligated into the *Bam*H I site of pSMMO20. After electroporation into *E. coli* XL1-Blue (Stratagene) and selection of a chloramphenicol (100 μg/mL) and kanamycin (25 μg/mL) resistant colony, the orientation of *hok/sok* in pSMMO40 was confirmed with *Eco*R I and *Bam*H I (Fig. 2).

Plasmid stability was determined using sequential-batch cultures (cells inoculated into fresh medium every 12 h to maintain primarily exponential growth) in which the percentage of plasmid-bearing cells was determined by a colony-lift hybridization using a digoxigenin-labeled, 2.1-kb *Eco*R I fragment of *mmo* locus as described previously.[27] Full induction of *mmo* was achieved by adding 1 m*M* IPTG (*iso*-propyl-β-D-1-thiogalactopyranoside, dioxane free, Fisher) to the antibiotic-free growth medium (LB medium for *A. tumefaciens* A114 and *P. putida* F1 and TY medium for *R. meliloti* 102F34).

Construction of Recombinant Strains Containing pSMMO20, pSMMO40, and pSMMO50

The plasmids were transferred into bacterial cells using electroporation except for *Pseudomonas* sp. M27. The pSMMO20 and pSMMO50 were isolated from *E. coli* XL1-Blue/pSMMO20 and *E. coli* JM109/pSMMO50, respectively, using a CsCl-gradient method,[27] whereas pSMMO40 was isolated from *E. coli* XL1-Blue/pSMMO40 with a plasmid minipreparation method. The plasmids were electroporated into *E. coli, Pseudomonas, Agrobacterium,* and *Rhizobium* strains at 15 kV/cm (using a 0.1-cm electroporation cuvette), 25 μF, and 200 Ω (5 ms time constant) with a Gene Pulser/Pulse Controller (Bio-Rad Laboratories, Hercules, CA). For electroporation of the methylotrophs and methanotrophs, voltages were varied between 12.5 and 17.5 kV/cm, and the time constant was fixed at 10 ms (25 μF $\times$ 400 Ω). Electro-competent cells were prepared using 10% glycerol[10] (*E. coli* strains) or 300 m*M* sucrose solu-

Table I. Bacterial strains and plasmids used in this study.

Strains/plasmids	Characteristics	Antibiotics[a] (μg/mL)	Phylogenetic group[b]	Source (Ref.)
Methylotrophs				
Methylophilus methylotrophus AS1	Type I methylotroph	Tet (2)	β-3	Hanson (45)
Methylobacterium extorquens AM1	Type II methylotroph	Tet (4)	α-2	Hanson (45)
Methylobacterium organophilum XX	Type II methylotroph	Tet (15)	α-2	Hanson (45)
Pseudomonas sp. M27	Type II methylotroph	Tet (10)	α-2 (?)	Hanson (43)
Methanotrophs				
Methylocystis parvus OBBP	Type II methanotroph	Tet (15)	α-2	Hanson (45)
Methylocystis pyriformis #14	Type II methanotroph	Tet (5)	α-2 (?)	Hanson (43)
Other α-2 strains				
Agrobacterium tumefaciens A114	Ti-plasmidless	Cm (80), Kan (40)	α-2	Nester (47)
Rhizobium meliloti 102F34	Nod$^+$, Fix$^+$	Cm (30), Kan (60)	α-2	Sadowski (4)
Pseudomonads				
P. cepacia DBO1	Wild type	Cm (50)	β-2	G. J. Zylstra
P. fluorescens	Wild type	Cm (500)	γ-3	J. D. Lipscomb
P. fragi	Wild type	Cm (50)	γ (?)	ATCC4973
P. putida F1	Toluene dioxygenase$^+$	Cm (500), Kan (25)	γ-3	Gibson (46)
Lab strains				
Escherichia coli:	General expression hosts		γ-1	
XL 1-Blue		Tet (15)		Stratagene
HB101		Cm (100)		H. C. Lim
JM109		Kan (25)		Stratagene
Plasmids				
pBE200	pBluescript II KS (−) Ω*smmo*	Amp^R		This lab (27)
pSMMO20	pMMB277 Ω*smmo*	Cm^R		This lab (27)
pSMMO40 and 41	pSMMO20 Ω*hok/sok*	Cm^R, Kan^R		This study
pHX200V-47-m1	Deletion derivative of pHX200V-47	Tet^R		Hanson (53)
pSMMO50	pHX200V-47-m1 Ω*smmo*	Tet^R		This study
pKG1022	pGEM3 Ω*hok/sok* Ω*aphA*	Kan^R, Amp^R		Gerdes (19)
pRK2013	*tra*$^+$ ColE1 replicon (helper plasmid)	Kan^R		Lipscomb (17)

[a]Concentrations of antibiotics added to growth media for plasmid-containing strains (tetracycline for pSMMO50, chloramphenicol for pSMMO20, and both kanamycin and chloramphenicol for pSMMO40-containing cells). Ω: insertion.
[b]From refs. 43, 45, and 49–51.

tion[39] (non-*E. coli* strains). After electroporation, 40 μL of cells was incubated at 37°C (*E. coli* strains and *M. methylotrophus* AS1) or 30°C (other strains) in 1 mL of antibiotic-free TY (*R. meliloti*), LB (*E. coli* and *Pseudomonas* strains), MM with 0.5% (v/v) methanol (methylotrophs), or HNMS with 0.5% (v/v) methanol (methanotrophs) for 8–10 h (methylotrophs and methanotrophs) or 4–5 h (other strains).

Incubated cell suspensions (100 μL) were plated on agar (1.5%, w/v) media (the same as incubation media) containing appropriate antibiotics (Table I). After the colonies grew to 2–3 mm diameter (3–7 days for methylotrophs, about 2 weeks for methanotrophs, and 2–3 days for other bacteria), colonies were inoculated into liquid media with antibiotic(s), and a plasmid minipreparation[37] was conducted. For plasmid mini-preparations from methylotrophic cells, an acetone-alkaline-lysis method[28] was used. Since electro-competent cells of *Pseudomonas* sp. M27 were not satisfactory for electroporation due to the pellet growth of this strain (the pellet retained ionic species and caused arcing during electroporation), tri-parental filter mating[38] with *E. coli* HB101/pSMMO50 (donor), *Pseudomonas* sp. M27 (recipient), and *E. coli* HB101/pRK2013 (helper) was used to obtain recombinant *Pseudomonas* sp. M27 containing pSMMO50.

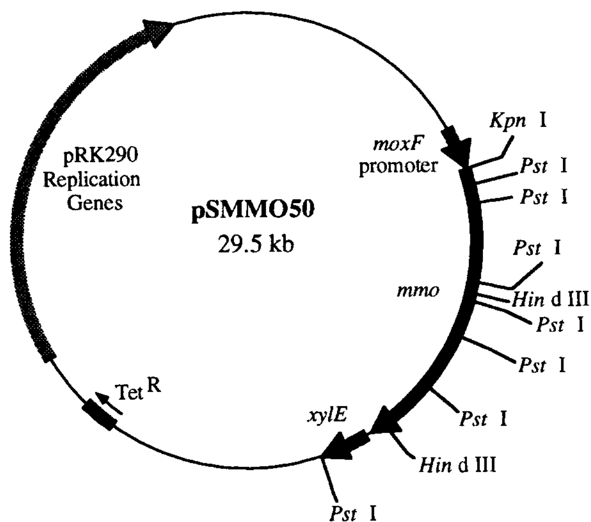

Figure 1. Restriction map of pSMMO50. The *mmo* locus from pBE200 was inserted downstream of the *moxF* promoter of pHX200V-47-ml.

Propene Oxidation Assay Using Cell-Free Extracts and Purified-sMMO Components

To prepare cell-free extracts of *P. putida* Fl/pSMMO20, one loop of a −85°C glycerol (15%, v/v) stock culture was streaked on LB agar containing 500 μg/mL of chloramphenicol. A colony grown at 30°C for about 30 h was inoculated into 20 mL of LB containing chloramphenicol and cultivated for 12–15 h at 30°C in a rotary (250 rpm) shaker (series 25, New Brunswick Scientific, New Brunswick, NJ). This culture (10 mL) was inoculated into 800 mL of M9[37]-glucose (1%, w/v) containing 500 μg/mL of chloramphenicol in a Fernbach flask (Fisher). This flask was shaken at 30°C in a rotary shaker, and 1 m*M* of dioxane-free IPTG was added when the cell density (A_{600}) reached 0.5–0.8 (usually about 12 h of cultivation). The cultivation broth was induced for 5 h, harvested, and washed twice with 100 m*M* sodium phosphate buffer (pH 7.0), and the cell paste was stored at −85°C.

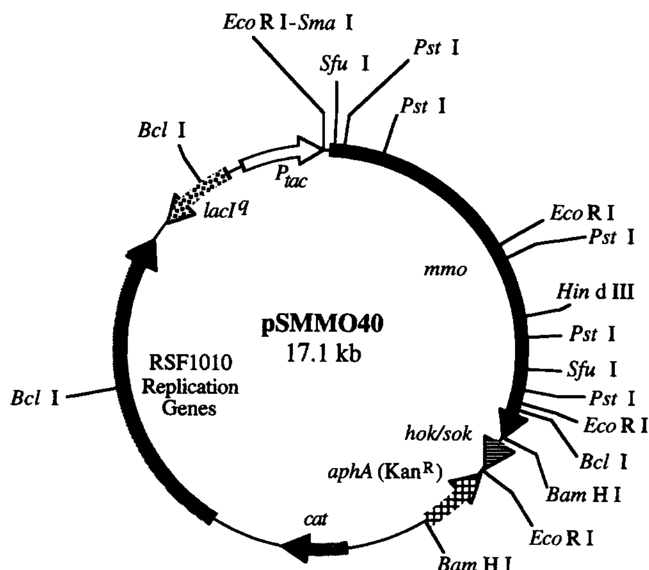

Figure 2. Restriction map of pSMMO40. The *Bam*H I fragment of pKG1022 (*hok/sok* with *aphA*) was inserted downstream of the *mmo* locus of pSMMO20.

For the propene oxidation assay of sMMO,[13,14] 5 g of frozen cell paste was thawed, sonicated, and used to prepare cell-free extracts as described previously.[13] Cell-free extract (150 μL) was added to a 3-mL conical-glass vial sealed with a Teflon-faced silicon septum (Wheaton, Millville, NJ). To check for enhanced sMMO activity, sMMO components purified from *M. trichosporium* OB3b (provided by John D. Lipscomb of the University of Minnesota, Minneapolis, MN) were added to cell-free extracts of *P. putida* Fl/pSMMO20 (about 10 nmol of the hydroxylase, 20 nmol of component B, and 10 nmol of the reductase). Gas chromatography was used to detect propene oxide as described previously[13,14] with the exception that a capillary column (DB-1, 0.25 mm × 30 m, 1 μm film thickness) purchased from Alltech (Deerfield, IL) was used. Hydrogen (1.5 mL/min) and nitrogen were used as the carrier and make-up gases, respectively, and the split ratio was 12.5. The temperatures of injector, oven, and detector were 175°C, 75°C, and 200°C, respectively.

Optimization of Media and Extent of TCE Degradation

Various complex and minimal media (Table II) were examined to select a cultivation medium for higher extents of TCE degradation and cell growth rates of *P. putida* Fl/pSMMO40, *A. tumefaciens* A114/pSMMO40, and *R. meliloti* 102F34/pSMMO40. Glycerol stock cultures stored at −85°C were streaked on LB or TY (*R. meliloti* 102F34/pSMMO40) agar plates containing two antibiotics (chloramphenicol and kanamycin) at concentrations shown in Table I. Plates were incubated at 30°C for 2–3 days, and one colony of each strain was inoculated into 20 mL of LB or TY medium with two antibiotics in a 250-mL Erlenmeyer flask and incubated at 30°C in a rotary shaker (250 rpm) until cell density (A_{600}) reached 0.8–1.5 (12–24 h). Culture broth (0.5 mL) was inoculated into 40 mL of complex and minimal media listed in Table II in a 250-mL Erlenmeyer flask, and cell density (A_{600}) was measured every 1.5 h with a spectrophotometer (Spectronic 20D, Milton Roy, Rochester, NY). Specific growth rates of each strain were calculated from four to five data points in the mid-exponential phase.

To evaluate the inhibitory effects of Cu^{2+}, Ni^{2+}, Zn^{2+}, and chloramphenicol in vivo, *P. putida* Fl/pSMMO40 and *R. meliloti* 102F34/pSMMO40 were grown in the modified Whittenbury minimal medium [phosphate concentrations (0.33 g/L of Na_2HPO_4 and 0.26 g/L of KH_2PO_4) in the original Whittenbury minimal me-

Table II. Growth and TCE degradation by resting cells (A_{600} = 2) grown in various complex and minimal media.[a]

Media[a]	Ref.	*P. putida* F1/pSMMO40		*A. tumefaciens*/pSMMO40		*R. meliloti*/pSMMO40	
		μ_{max} (h^{-1})	TCE[b] deg. (%)	μ_{max} (h^{-1})	TCE deg. (%)	μ_{max} (h^{-1})	TCE deg. (%)
LB	37	0.54	10	0.37	12	ND	20
TY	5	ND	25	ND	8	0.38	13
M9 minimal	37	0.35	16	0.21	18	0.18	12
Hutner's minimal	17	0.26	22	0.25	19	0.11	13
Modified Wb minimal	—	0.21	25	0.34	20	0.34	23
Vitamin minimal	6	0.16	24	0.15	13	0.22	15
Glutamate minimal	47	No growth	No growth	0.14	17	0.15	22
Modified M9 minimal	20	ND	18	ND	20	0.15	5
AB minimal	8	0.41	9	0.21	16	ND	13

ND: not determined.
[a]When not specified, the carbon source and nitrogen source were 10 g/L of glucose and 1 g/L of ammonium chloride, respectively.
[b]Percentages of initial TCE concentration (20 μM) after 5 h of resting-cell incubation.

dium[25,41] were increased to be the same as those of HNMS (0.86 g/L of Na_2HPO_4 and 0.53 g/L of KH_2PO_4) for higher buffering capacity] with different metal concentrations, and the extents of TCE degradation with resting cells (A_{600} = 2) were compared.

For the metal ions, the original concentrations of $CuSO_4 \cdot 5H_2O$ (0.2 mg/L), $NiCl_2 \cdot 6H_2O$ (0.01 mg/L), and $ZnSO_4 \cdot 7H_2O$ (0.4 mg/L) of the modified Whittenbury medium (Cu^+ was not included in this medium) were varied uniformly and simultaneously [e.g., inhibition with 1× concentrations of the metals (the original concentrations), 2× (twice of the original concentrations), etc.]. To find the best iron source and concentration for higher extents of TCE degradation, various iron compounds (see the results section) with the iron content fixed at 1.8 μM were added to the modified Whittenbury minimal medium lacking the original iron source [0.5 mg/L (1.8 μM) of $FeSO_4 \cdot 7H_2O$].

To evaluate extents of TCE degradation, cells were grown in various media, and IPTG (1 m*M*) was added to flasks when cell density reached A_{600} values of 0.3–0.8. After 5 h of IPTG induction, cells were harvested, washed twice with 100 m*M* sodium phosphate buffer (pH 7.0), and the cell density was adjusted to be A_{600} = 2.0 for TCE degradation experiments with resting cells as described previously.[27]

Chemostat Culture of *P. putida* F1/pSMMO40

A glycerol (15%, v/v) stock culture of *P. putida* F1/pSMMO40 stored at −85°C was streaked on LB agar containing 500 μg/mL of chloramphenicol and 25 μg/mL of kanamycin. A colony grown at 30°C for about 30 h was inoculated into 50 mL of LB containing kanamycin (no chloramphenicol) and cultivated for 12–15 h at 30°C in a rotary (250 rpm) shaker. This culture (40 mL) was used to inoculate the fermentor, which contained 1.1 L of the optimized Whittenbury minimal medium [10 g/L of glucose, 1 g/L of NH_4Cl, 1 g/L of $MgSO_4 \cdot 7H_2O$, 0.2 g/L of $CaCl_2 \cdot 2H_2O$, 0.86 g/L of Na_2HPO_4, 0.53 g/L of KH_2PO_4, 0.5 mg/L of Na_2MoO_4, 0.02 mg/L of $MnCl_2 \cdot 4H_2O$, 0.01 mg/L of H_3BO_3, 0.05 mg/L of $CoCl_2 \cdot 6H_2O$, 1.41 mg/L of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, and 0.25 mg/L of ethylenediaminetetraacetic acid (EDTA) disodium salt] supplemented with 25 μg/mL of kanamycin and sterilized by filtration (0.2-μm Mini Capsule Filter, Gelman Science). The IPTG (1 mM) was added to the fermentor medium after the batch-growth stage.

The fermentor (Bioflo III, New Brunswick Scientific) was agitated at 500 rpm and aerated with 1.5 L air/min while the temperature was controlled at 30°C, the pH was maintained at 6.8 with 10% (v/v) ammonium hydroxide (Fisher), and foaming was controlled with 10% (v/v) antifoam 286 (Sigma). Dissolved oxygen (DO) was maintained above 70% saturation at these conditions. Fermentation parameters (temperature, pH, DO, and rpm) were controlled using the Advanced Fermentation Software (New Brunswick Scientific) with data acquisition via a IBM-type personal computer. Steady states at each dilution rate were obtained after three to five residence times (t_R) and were maintained for 1 to 2 t_R to take three samples for the TCE degradation experiments. To confirm the presence of the plasmid, a plasmid minipreparation was performed at the end of each dilution rate and steady state.

RESULTS

sMMO Activity of Cell-Free Extracts of *P. putida* F1/pSMMO20

To reveal which component of sMMO is responsible for the low and inconsistent sMMO activity of *P. putida* F1/pSMMO20 which was observed previously,[27] sMMO activities of cell-free extracts complemented with purified sMMO components of *M. trichosporium* OB3b were examined using the propene-oxidation assay. As

shown in Table III, addition of component *B* or the reductase did not show any noticeable impact on the sMMO activity of the cell-free extracts, but addition of the hydroxylase to cell-free extracts of *P. putida* F1/pSMMO20 enhanced sMMO activity.

The enhanced sMMO activity of cell-free extracts plus the hydroxylase could be due to the presence of hydrogen peroxide in the cell-free extracts which might be produced by *P. putida* F1/pSMMO20 and lead to oxidation of propene by the hydroxylase via the hydrogen peroxide shunt.[1] Because component *B* is known to strongly inhibit the H_2O_2-hydroxylase reaction (80% of the H_2O_2-activated hydroxylase activity was inhibited by adding component *B* at 2 mol component B/mol hydroxylase[18]), the possibility of this H_2O_2-shunt reaction was eliminated by showing that addition of component B did not decrease the sMMO activity of the cell-free extract plus the hydroxylase (Table III). Therefore, the low sMMO activity (and perhaps the inconsistency as well) of *P. putida* F1/pSMMO20 is due to incomplete activity of the hydroxylase component.

Construction of Recombinant Strains Harboring pSMMO20 or pSMMO50

Electroporation of pSMMO20 into *Pseudomonas, Agrobacterium,* and *Rhizobium* strains was successful. However, pSMMO20 (a derivative of RSF1010-based Inc Q plasmid) was not maintained in methylotrophs (probably due to an inability to replicate in these strains). Therefore, plasmid pSMMO50 (a pRK290-originated Inc P-1 derivative) was constructed (Fig. 1) using a broad-host-range plasmid (pHX200V-47-m1 (this plasmid is known to replicate in methylotrophic strains)[53] and was used for transferring the *mmo* locus into methylotrophs and methanotrophs by electroporation. Plasmid pSMMO50 has the *mmo* locus downstream of the *mox*F promotoer (Fig. 1). The *xyl*E encoding catechol 2,3-dioxygenase (a reporter gene in pHX200V-47-m1) was not removed because it is located downstream of the *mmo* locus so that transcription should not be affected by this extra structural gene.

Table III. Complementation of cell-free extracts of *P. putida* F1/pSMMO20 with purified sMMO components from *M. trichosporium* OB3b.

sMMO source	Relative activity[a]
H + B + R[b]	1.0
Cell-free extract	0[c]
Cell-free extract + H	0.09
Cell-free extract + B	0
Cell-free extract + R	0
Cell-free extract + H + B	0.13
Cell-free extract + H + R	0.14

[a]Activity was measured after 18 min of incubation. Relative activity of 1 is 324 nmol propene oxide/(min mg hydroxylase).

[b]Amounts of purified sMMO components: 10 nmol of H (hydroxylase), 20 nmol of B (component B), and 10 nmol of R (reductase).

[c]Propene oxide (oxidation product of propene by recombinant sMMO) was observed for longer incubation times (90 min).

The sMMO expression in nonmethylotrophic strains bearing pSMMO20 (*A. tumefaciens* A114, *R. meliloti* 102F34, *Pseudomonas fluorescens,* and *Pseudomonas fragi*) after IPTG induction was confirmed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (five large proteins seen), and Western immuno-blotting was performed to verify that the sMMO proteins were produced in methylotrophs bearing pSMMO50 (5 large proteins detected for *M. methylotrophus* AS1, *M. extorquens* AM1, *M. organophilum* XX, and *Pseudomonas* sp. M27, data not shown).

Plasmid pSMMO20 was segregationally unstable in *Pseudomonas cepacia* DBO1, as in cases of *P. cepacia* G4 and *P. cepacia* G4 PR1 in previous work,[27] so TCE degradation could not be evaluated. In addition, pSMMO50-containing methanotrophs (*M. parvus* OBBP and *M. pyriformis* #14) could not be isolated. Although many colonies (30–50 colonies per plate) of *M. parvus* OBBP grew on tetracycline-containing agar medium after electroporation (they also grew when inoculated into the liquid medium with the antibiotic), a correct DNA digestion pattern was not obtained.

Among the recombinant strains newly constructed, *A. tumefaciens* A114/pSMMO20 and *R. meliloti* 102F34/pSMMO20 were found to express active sMMO. Both strains belong to the α-2 subdivision of purple eubacteria according to the 16S-rRNA-sequence analysis as *M. trichosporium* OB3b does.[45,49] The sMMO activities of these two strains were similar to that of *P. putida* F1/pSMMO20 [about 15–20% degradation of 20 μM TCE in 5 h using resting cells (A_{600} = 2)].

Stability of *hok/sok*-containing Recombinant Plasmids

To determine whether plasmid stability was enhanced by cloning the *hok/sok* locus (plasmid segregational instability was encountered when a 25-L batch culture of *P. putida* F1/pSMMO20 was attempted), pSMMO20- and pSMMO40-containing cells of *P. putida* F1, *A. tumefaciens* A114, and *R. meliloti* 102F34 from sequential-batch cultures were analyzed for the presence of the plasmid. The media did not contain antibiotics and were supplemented with 1 m*M* IPTG; therefore, the *hok/sok* locus was the only plasmid-selection pressure in the presence of a strong metabolic burden (it has been shown that recombinant protein expression causes plasmid loss[52]).

The *hok/sok* locus was most effective in *R. meliloti* 102F34 among the three strains (e.g., 61% of cells contained pSMMO40 whereas pSMMO20 was present in 6% of cells after 17 h of cultivation with induction) but was not significantly effective in *A. tumefaciens* A114 (stability about 1% better for pSMMO40 after 17 h of

cultivation). This killing locus was also effective in *P. putida* F1 (pSMMO40 was present in 61% of the cells but pSMMO20 was present in 9% of the cells after 12 h of cultivation with induction) but was less effective than in *R. meliloti* 102F34 (less than 40% of the *P. putida* F1 cells contained pSMMO40 after 17 h). It has been previously reported that the *hok/sok* system is effective in *P. putida,*[19] and these results clearly confirm this.

In shake-flask experiments, TCE degradation with pSMMO40-containing cells (*hok/sok*$^+$) were slightly higher than cells with pSMMO20 (*hok/sok*$^-$). For example, 31.4 ± 7.0% of 20 μM TCE was degraded in 5 h with resting cells (A_{600} = 2) of *R. meliloti* 102F34/pSMMO40, whereas pSMMO20-containing cells degraded 27.5 ± 2.7% of 20 μM TCE.

Modified Whittenbury Minimal Medium

Pseudomonas putida F1/pSMMO40, *R. meliloti* 102F34/pSMMO40, and *A. tumefaciens* A114/pSMMO40 were cultivated in a number of different media to compare cell growth rates and extents of TCE degradation (Table II). Cells grew in complex media faster than minimal media, as anticipated, but extents of TCE degradation were higher with cells grown in minimal media. The modified Whittenbury minimal medium provided the strongest TCE degradation and an above-average growth rate (Table II), so this medium was chosen for further optimization.

Cu(I), Cu(II), Ni(II), and Zn(II) have been shown to inhibit purified sMMO in vitro by aggregating the hydroxylase and the reductase or by affecting the hydroxylase without protein precipitation.[26] This metal-ion inhibition was confirmed in vivo in this study using *P. putida* F1/pSMMO40 and *R. meliloti* 102F34/pSMMO40. Although the in vivo inhibitory effects of these metal ions were not as drastic as the in vitro inhibition (probably due to lower intracellular concentrations of metal ions as a result of the membrane-transport regulation of charged-metal ions), a reduction in TCE degradation was observed even at 1× concentrations of the inhibitory metal ions (extents of TCE degradation decreased by 4–12%, 18–19%, and 24–38% at 1×, 4×, and 10× concentrations, respectively). These metal ions were removed from the modified Whittenbury minimal medium, and significant changes in cell growth rate were not observed.

Chloramphenicol was also found previously to inhibit purified sMMO.[26] This result was also confirmed in vivo using whole resting cells (A_{600} = 2) of *R. meliloti* 102F34/pSMMO40 and *P. putida* F1/pSMMO40 after growth with both kanamycin and chloramphenicol or just kanamycin. Elimination of chloramphenicol from the culture media increased the extents of TCE degradation by 25% for *P. putida* F1/pSMMO40 and 1% for *R. meliloti* 102F34/pSMMO40. The chloramphenicol concentration of *R. meliloti* 102F34/pSMMO40 culture was 30 μg/mL (compare to 500 μg/mL for *P. putida* F1/pSMMO40) so the smaller improvement in *R. meliloti* 102F34/pSMMO40 was probably due to the lower chloramphenicol concentration that was removed.

The concentration of iron in the growth medium was also optimized to enhance TCE degradation since the sMMO–hydroxylase component contains dinuclear iron clusters, and the reductase also requires iron for its [2Fe–2S] center.[15] Ferrous sulfate ($FeSO_4 \cdot 7H_2O$), ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$], EDTA ferric-sodium salt [$(OOCCH_2)_2NCH_2CH_2N(CH_2COO)_2FeNa$], ferric ammonium citrate (compounds of ammonia, iron, and citric acid of unknown structure), and ferric chloride ($FeCl_3$) were added to the modified Whittenbury minimal medium, and the extents of TCE degradation were compared using *R. meliloti* 102F34/pSMMO40. Ferrous ammonium sulfate was better for TCE degradation than the other compounds, and its concentration also affected TCE degradation such that 8.5–30.9% of 20 μM TCE was degraded in 5 h with *R. meliloti* 102F34/pSMMO40 grown in media containing 0–9.0 μM of ferrous ammonium sulfate; 3.6 μM of this iron compound was found to be optimal for sMMO activity (TCE degradation was decreased at lower and higher concentrations). Murdock et al.[31] also showed that excess iron in the growth medium resulted in an increased half-life of recombinant naphthalene dioxygenase (an iron-containing enzyme) activity during indigo biosynthesis. Increased levels of copper ions in the culture medium also enhanced activity of recombinant tyrosinase (a copper-containing enzyme) cloned in *E. coli* from *Streptomyces antibioticus*.[21] To avoid natural oxidation of ferrous iron to ferric ion in the cultivation medium, ferrous ammonium sulfate was added at the moment of IPTG addition to supply fresh ferrous iron. However, no noticeable improvement of TCE degradation was observed (compared to the case of iron addition at inoculation).

In addition, the effects of yeast extract (1–5 g/L) supplemented to the culture medium and dithiothreitol (0–10 mM) added to a resting-cell buffer were examined. Dithiothreitol was reported to enhance TCE degradation with toluene dioxygenase expressed in *P. putida* presumably by protecting cells against oxidative stress[24]; however, TCE degradation did not increase in either experiment.

sMMO Activity and Specific Growth Rate

Pseudomonas putida F1/pSMMO40 was cultivated in a chemostat fermentor containing the optimized Whittenbury minimal medium (no copper, nickel, or zinc) with 1 mM IPTG and 25 μg/mL of kanamycin (no chloramphenicol). As anticipated based on Monod kinetics,[2] the cell density was higher at lower dilution rates. More importantly, sMMO activity was greater at higher dilution rates (Fig. 3): 33% of 20 μM TCE was degraded

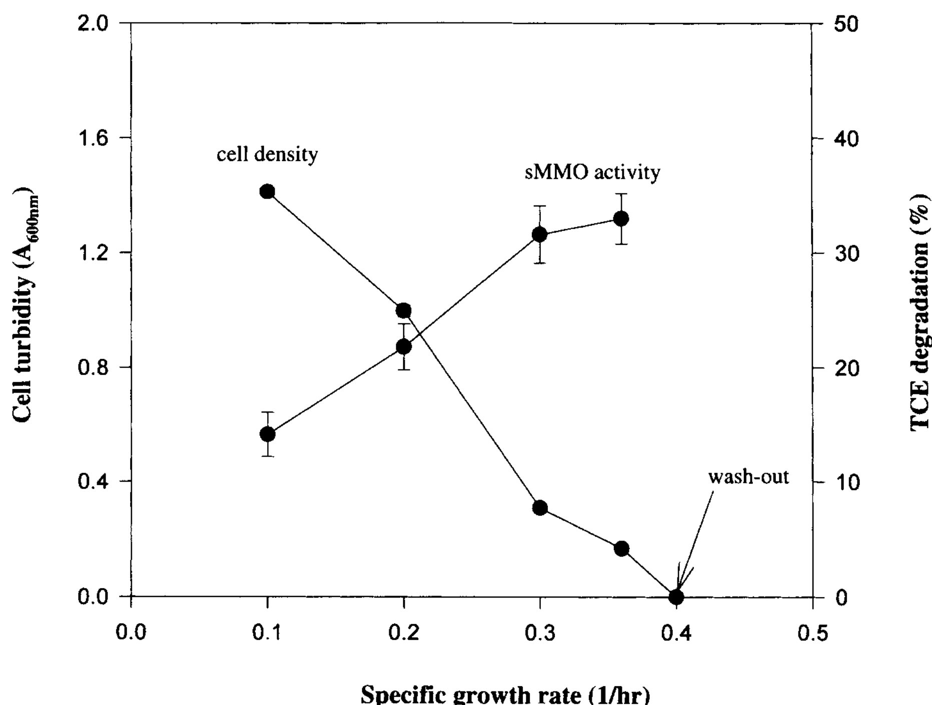

Figure 3. Effect of dilution rates on sMMO activity (assayed by extent of TCE degradation, filled squares) and cell density (filled circles) for *P. putida* Fl/pSMMO40. Data are average of three samples at each dilution rate, and error bars represent one standard deviation. Optimized Whittenbury minimal medium was supplemented with 1 m*M* IPTG and 25 μg/mL of kanamycin (no chloramphenicol) was used for chemostat growth (30°C). To determine the extent of TCE degradation (in 5 h at 25°C), cell broth was taken from the chemostat, adjusted to $A_{600} = 2$, then placed in sealed vials containing TCE.

in 5 h by cells harvested at $D = 0.36\ h^{-1}$ compared to 14% degraded at $D = 0.1\ h^{-1}$ (the cell density was adjusted to $A_{600} = 2$ for all the dilution rates such that a constant cell mass was used to assay TCE degradation). In addition, sMMO activity for TCE degradation was consistent, so cells harvested at certain dilution rates always gave rise to nearly constant TCE degradation results (unlike batch cultures). Therefore, chemostat culture is recommended for consistent recombinant sMMO activity.

Plasmid presence was also examined, and it was found that pSMMO40 in *P. putida* F1 was 100% stable for at least 11–20 generations with kanamycin and induction of *mmo*. Hence, the combination of kanamycin resistance and *hok/sok* provide efficient stabilization of pSMMO40.

DISCUSSION

From the experiments with cell-free extracts of *P. putida* Fl/pSMMO20, it was found that the hydroxylase is responsible for the low sMMO activity of *P. putida* Fl/pSMMO20 which was observed previously.[27] This is reasonable in that active component *B* and reductase of sMMO from *Methylococcus capsulatus* (Bath) have been expressed independently in *E. coli*.[48] Component *B* is a monomeric protein which lacks a prosthetic group or cofactor, and the reductase contains the [2Fe–2S] center and flavin adenine dinucleotide (FAD), which are common in a wide variety of living organisms.[13] The hydroxylase, however, requires a dinuclear iron cluster for catalytic activity, and this iron complex is rare in natural enzyme systems.[16,29] Since the recombinant has been shown to express large amounts of hydroxylase[27] and the content of the dinuclear iron center in the wild-type hydroxylase varies and affects sMMO activity as much as 25-fold,[13] it also seems plausible that the dinuclear iron center is also responsible for the inconsistent activity in the recombinants.

To expedite formation of the dinuclear iron cluster, the intracellular ferrous ion concentration was increased by overproduction of the flavin reductase, which is known to mobilize iron from ferrisiderophores by reducing insoluble Fe(III) to soluble Fe(II).[11,40] The flavin reductase gene (*fre*) of *E. coli* was cloned (using pEE1001[40]) into *E. coli* BL21(DE3)[42] harboring pSMMO20 (unpublished results). A protein band of flavin reductase was observed using SDS–PAGE analysis along with sMMO components expressed simultaneously in *E. coli* BL21(DE3)/pSMMO20/pEE1001, but sMMO activity was not detected. This result indirectly suggests that low activity of the recombinant hydroxylase is unlikely to be linked to insufficient supply of ferrous ions for synthesizing the dinuclear iron centers.

As anticipated from phylogenetic similarity to *M. trichosporium* OB3b (α-2 subdivision), *A. tumefaciens* A114/pSMMO20 and *R. meliloti* 102F34/pSMMO20 expressed active sMMO; however, activity was similar to that of *P. putida* F1/pSMMO20. *Rhizobium meliloti* invades the roots of alfalfa plants and forms nitrogen-fixing nodules[4] (*M. trichosporium* OB3b is also known to fix atmospheric nitrogen[23]). Using this symbiotic property, TCE-degrading *R. meliloti* may be used with plants for phytoremediation of toxic wastes.

Because the *hok/sok* locus enhances plasmid segregational stability in *R. meliloti* 102F34, its constitutive promoters are recognizable by the RNA polymerase of *R. meliloti*, the Hok protein is active, and the membrane potential of *R. meliloti* is susceptible to Hok. Using pSMMO40-containing *P. putida* F1 or *R. meliloti* 102F34, plasmid loss in long-term cultivations with sMMO expression was reduced significantly.

According to chemostat results with varied dilution rates from 0.1 to 0.36 h^{-1} using *P. putida* F1/pSMMO40, TCE degradation increases with specific growth rate (roughly 2.5-fold). This result agrees well with the batch culture observations of Park et al.[35] in which the sMMO activity of *M. trichosporium* OB3b was roughly proportional to the specific growth rate (varied by controlling the temperature and pH of the cultures).

The extent of TCE degradation before the series of optimizations described above was about 15–20% of 20 μM TCE after 5 h of resting-cell incubation ($A_{600} = 2$). The three recombinant strains (*P. putida* F1/pSMMO20, *R. meliloti* 102F34/pSMMO20, and *A. tumefaciens* A114/pSMMO20) showed similar performance. By using a stabilized plasmid (pSMMO40 containing the *hok/sok* locus), an optimized medium (the modified Whittenbury medium with enhanced ferrous ammonium sulfate and without sMMO-inhibiting metal ions and chloramphenicol), and cells grown at a higher dilution rate (0.36 h^{-1}) in a chemostat, TCE degradation was increased to a constant (no inconsistency) 30–40% with the same amount of cells and incubation time.

CONCLUSIONS

Low sMMO activity of *P. putida* F1/pSMMO20 was due to incomplete activity of the recombinant hydroxylase (component *B* and the reductase were much more active). To increase sMMO activity, the *mmo* locus was cloned into additional pseudomonads and other bacterial strains (most of which are classified in the α-2 subdivision of purple eubacteria, which includes *M. trichosporium* OB3b). Two new α-2 strains, *A. tumefaciens* A114/pSMMO20 and *R. meliloti* 102F34/pSMMO20, were found to express sMMO with an activity similar to *P. putida* F1/pSMMO20.

To increase plasmid segregational stability in the sMMO-expressing recombinant strains, the *hok/sok*-killing locus was inserted downstream of the *mmo* locus of pSMMO20. The resulting plasmid pSMMO40 was more stable than pSMMO20 (*hok/sok*$^-$) in *P. putida* F1 and *R. meliloti* 102F34, but not in *A. tumefaciens* A114. It should be noted that adequate expression of *hok/sok* was not verified in *A. tumefaciens* A114.

Modified Whittenbury minimal medium was selected among 9 media as a basal medium for cultivation of sMMO-expressing recombinant strains. Based on in vitro and in vivo sMMO studies, sMMO-inhibiting metals (copper, nickel, and zinc) and chloramphenicol were removed from the medium. To further increase sMMO activity, ferrous ammonium sulfate was chosen as an iron source, and the optimal concentration was determined to be 3.6 μM.

The specific growth rate of *P. putida* F1/pSMMO40 affected sMMO activity; hence, TCE degradation was enhanced at higher growth rates. Using the optimal conditions obtained, TCE degradation by *P. putida* F1/pSMMO40 was increased to 6–8 μM TCE degraded in 5 h using 5 mL of resting cells of $A_{600} = 2$.

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