JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 89, No. 5, 438–445. 2000

Trichloroethylene Degradation by *Ralstonia* sp. KN1-10A Constitutively Expressing Phenol Hydroxylase: Transformation Products, NADH Limitation, and Product Toxicity

HIROAKI ISHIDA* AND KANJI NAKAMURA

Kurita Water Industries Ltd., 7-1 Wakamiya, Morinosato, Atsugi-city, Kanagawa 243-0124, Japan

Received 6 October 1999/Accepted 8 February 2000

Ralstonia sp. KN1-10A, which was constructed by inserting the _tac_ promoter upstream of the phenol hydroxylase (PH) gene in the chromosomal DNA of the wild-type strain, _Ralstonia_ sp. KN1, is a useful recombinant strain for eliminating trichloroethylene (TCE) from contaminated sites because it exhibits constitutive TCE oxidation activity. During TCE degradation by _Ralstonia_ sp. KN1-10A, noxious chlorinated compounds, such as dichloroacetic acid, trichloroacetic acid, 2,2,2-trichloroethanol, and chloral, were not detected, and more than 95% of chlorine in TCE was released as chloride ions. Among the possible TCE transformation products, only carbon monoxide was detected, and its conversion percentage was 7 mol%. The addition of formate, which _Ralstonia_ sp. KN1-10A could use as an exogenous electron donor, did not enhance the TCE degradation performance, suggesting that NADH depletion did not limit the degradation. The phenol degradation activity of _Ralstonia_ sp. KN1-10A that previously degraded TCE was not markedly lower than that of cells not exposed to TCE, suggesting that _Ralstonia_ sp. KN1-10A was not susceptible to product toxicity associated with TCE degradation. Furthermore, to clarify the mechanisms underlying TCE degradation by PH from _Ralstonia_ sp. KN1, this enzyme was compared with another enzyme, a hybrid aromatic ring dioxygenase exhibiting a high TCE degradation activity in _Escherichia coli_ and _Pseudomonas_ sp. The initial TCE degradation rate of _Ralstonia_ sp. KN1 (pKTP100), which produced PH, was 1/50 lower than that of _Ralstonia_ sp. KN1 (pKTF200), which produced the hybrid aromatic ring dioxygenase. However, because of its lower product toxicity, the strain producing PH could degrade 2.3 times more TCE than that generated by the strain producing the hybrid aromatic ring dioxygenase.

[**Key words:** _Ralstonia_ sp., trichloroethylene, genetically engineered microorganism, phenol hydroxylase, metabolite, NADH, product toxicity]

The widespread use of trichloroethylene (TCE) as an organic solvent and a degreaser has resulted in extensive contamination of soil and groundwater. Because TCE is a suspected carcinogen, much effort has been made to physicochemically or biologically remove the compound from polluted sites (1). Compared to physicochemical treatments, biologic treatments are considered more effective and economical for remediation of a large site polluted with relatively low TCE concentrations.

There are no reports concerning bacteria using TCE as the sole carbon and energy source. However, a variety of aerobic microorganisms have been reported to degrade TCE cometabolically by oxygenases induced by specific substrates. The inducer substrates include methane (2–4), propane (5), toluene (6–8), phenol (9–11), isopropylbenzene (12), and ammonia (13). Recently, TCE itself has been shown to be an inducer of TCE-degrading oxygenases of some bacteria, although it cannot support their growth (14–16).

In aerobic *in situ* bioremediation of TCE, substrates such as methane or phenol must be added into TCE-contaminated aquifers for growth of degraders and induction of oxygenases (17). Despite the fact that the addition of inducer substrates is indispensable for cometabolic TCE degradation, excessive addition may reduce the removal efficiency because the substrate acts as a competitive inhibitor against TCE degradation, by competing with TCE for the active site of the enzyme (18). Although some methods, such as feeding the substrate in pulse (19) or separating the reactor into enzyme induction and TCE removal sections (20), have been proposed to prevent the competitive inhibition, these have failed to markedly increase removal efficiency. To overcome this obstacle, genetically engineered microorganisms (GEMs) that express the TCE-degrading oxygenase but lack the inducer causing competitive inhibition have been developed and reported (10, 11, 21–27).

Strain KN1 was tentatively identified as *Pseudomonas putida* based on the results of standard biochemical and morphologic tests. However, after further investigation, we determined that the strain belonged to the genus *Ralstonia* in light of its 16S rDNA sequence (28). This strain, isolated from an experimental reactor fed with phenol, is a phenol-utilizing microorganism. The phenol hydroxylase (PH) gene of this strain is induced by phenol to degrade TCE. Using homologous recombination, Nakamura and Ishida (29) constructed *Ralstonia* sp. KN1-10A by inserting the *tac* promoter upstream of the PH gene in the chromosomal DNA of *Ralstonia* sp. KN1. The recombinant strain exhibits constitutive TCE degradation activity, which is under the control of the *tac* promoter. Because the PH gene and *tac* promoter are encoded in the chromosome rather than in plasmids, *Ralstonia* sp. KN1-10A can stably maintain these elements during cultivation in a general growth medium without any selective pressure (10, 29). Therefore, we expect that *Ralstonia* sp. KN1-10A will be a useful tool for the remediation of TCE-contaminated sites.

A TCE-degrading microorganism applicable to practical TCE treatment must have a high TCE transforma-

* Corresponding author.

tion capacity (Tc), which is expressed as the maximum mass of TCE degraded by a given mass of cells (30), and produce no harmful metabolites. The Tc of TCE degraders primarily depends on two factors: NADH limitation and product toxicity (18, 30–32). In the case of the first factor, TCE transformation diminishes with decreasing NADH concentration in a cell, because the reaction requires NADH as the reducing energy source (33, 34). In the second factor, enzyme inactivation and cell death occur due to a reactive intermediate produced during TCE degradation (14, 35–38).

In the present study, we describe the transformation products, NADH limitation, and product toxicity resulting from TCE degradation by *Ralstonia* sp. KN1-10A. Furthermore, to clarify the mechanisms underlying TCE degradation by PH from *Ralstonia* sp. KN1, this enzyme was compared with another TCE-degrading enzyme, a hybrid aromatic ring dioxygenase. The hybrid aromatic ring dioxygenase is composed of TodC1 (the large subunit of toluene terminal dioxygenase in *Pseudomonas putida* F1) (39), BphA2 (the small subunit of biphenyl terminal dioxygenase in *Pseudomonas pseudoalcaligenes* KF707), BphA3 (ferredoxin in KF707), and BphA4 (ferredoxin reductase in KF707) (40). Furukawa *et al.* reported that *Escherichia coli* and *Pseudomonas* strains expressing the hybrid aromatic ring dioxygenase exhibited a significant ability to degrade TCE (24, 41).

MATERIALS AND METHODS

Strains and growth conditions *E. coli* DH5 (Toyobo, Osaka) was used to construct plasmids, and *E. coli* S17-1 (42) was used for the transconjugation of plasmids to *Ralstonia* sp. KN1. All strains were cultivated in L-broth (LB) medium (43), which was supplemented with appropriate antibiotics as required. Chloramphenicol was used at 50 μg/ml, and tetracycline was added at 10 μg/ml. *Ralstonia* and *E. coli* strains were incubated at 30°C and 37°C, respectively.

Construction of *Ralstonia* strains KN1 (pKTP100) and KN1 (pKTF200) The *trc* promoter expression vector pRTL100 was constructed according to standard DNA recombination methods (44). Plasmid pTrc99A (Pharmacia Biotech, Uppsala, Sweden) was digested with *Nco*I, treated with S1 nuclease (Toyobo), and then self-ligated to remove the ATG start codon that might cause synthesis of an extra protein. To replace the ampicillin resistance (Ap^r) gene with the tetracycline resistance (Tc^r) gene, the resulting plasmid was digested with *Bsp*HI, blunted with T4 DNA polymerase (Toyobo), and ligated with a 1.4-kb fragment containing the Tc^r gene; this fragment was excised from pBR322 with an *Eco*RI/*Ava*I restriction digest, and was then blunted. For later convenience, the *Eco*RI-*Hin*dIII fragment, containing the multicloning site (MCS) of the resulting plasmid, was replaced with the *Eco*RI-*Hin*dIII MCS fragment from pNEB193 (New England Biolabs, Beverly, MA, USA), yielding pKT100. In addition, the *Bsa*AI-*Alw*NI fragment harboring the replication origin in pKT100 was replaced with the blunt-ended *Pst*I-*Pvu*II fragment containing the replication origin of the broad-host-range vector pKT240 (45), producing pRTL100. We confirmed that pRTL100 could be cloned into *Ralstonia* sp. KN1 by conjugation via *E. coli* S17-1. Genes inserted at the multicloning site downstream of the *trc* promoter in pRTL100 require induction by isopropyl-β-D-thiogalactopyranoside (IPTG) for their expression because the plasmid contains *lacI*^{q}.

Plasmid pRTL100 was digested with *Sma*I and ligated with a 6.0-kb *Bam*HI fragment that was excised from pNEM101 (10) and blunted with T4 DNA polymerase, yielding pKTP100. Plasmid pKTP100 carries the PH gene and the catechol 2,3-dioxygenase gene from the chromosomal DNA of *Ralstonia* sp. KN1 (Fig. 1). We did not delete the gene encoding catechol 2,3-dioxygenase, which has been reported not to affect TCE degradation by aromatic oxygenases such as toluene 2-monooxygenase (46) and toluene dioxygenase (6), because the gene was useful as a marker gene during construction of the objective plasmid. Similar to pKTP100, plasmid pKTF200 (Fig. 1) was constructed by inserting a blunt-ended 5.0-kb *Sac*I-*Ppu*MI fragment from pJHF101 (40) into the *Sma*I site of pRTL100. The *Sac*I-*Ppu*MI fragment contains a hybrid gene cluster (*todC1*::*bphA2A3A4*), comprising *todC1* (the gene encoding the large subunit of toluene terminal dioxygenase in *P. putida* F1) (39), *bphA2* (the gene encoding the small subunit of biphenyl terminal dioxygenase in *P. pseudoalcaligenes* KF707), *bphA3* (the gene encoding ferredoxin in KF707), and *bphA4* (the gene encoding ferredoxin reductase in KF707) (47). pKTP100 and pKTF200 were first introduced into *E. coli* S17-1 by electroporation, then the cells harboring pKTP100 or pKTF200 were mated with *Ralstonia* sp. KN1 on membrane filters on LB agar medium overnight. The objective transconjugants, *Ralstonia* sp. KN1 (pKTP100) and KN1 (pKTF200), were screened on agar plates containing the basal salts medium (BSM) (48) supplemented with 20 mM lactate and 10 μg/ml tetracycline.

TCE degradation assay *Ralstonia* sp. KN1-10A was cultivated in LB medium overnight to an OD_{600} of 6.0 to 7.0. *Ralstonia* sp. KN1 (pKTP100) and KN1 (pKTF200) were cultivated in LB medium to an OD_{600} of 0.5 to 1.0 and were then further incubated for 2 h in the presence of 5 mM IPTG. Cells were harvested by centrifugation (8000 × *g*, 10 min, 4°C), washed three times with BSM, and resuspended to a final OD_{600} of 2.0 in BSM. A 10-ml (except where noted) aliquot of the cell suspension was transferred to a 157-ml glass vial, which was sealed with a Teflon-coated butyl-rubber septum and an aluminum crimp seal. To obtain the desired final TCE concentration, an aliquot of TCE-saturated water was added to the cell suspension by injection through the septum with a gas-tight syringe. The vials were maintained at 30°C and shaken at 200 rpm. For TCE analysis, samples (100 μl) of the gas phase in the vials were periodically removed and injected into a GC-9A gas chromatograph (Shimadzu, Kyoto) equipped with a flame

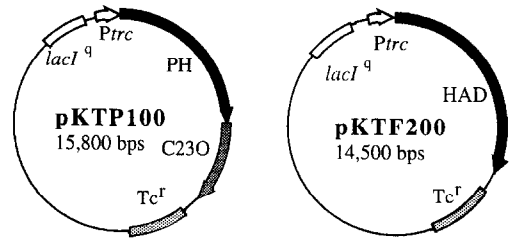

FIG. 1. Structure of pKTP100 and pKTF200. *Ptrc*, *trc* promoter; PH, phenol hydroxylase gene; C23O, catechol 2,3-dioxygenase gene; Tc^r, tetracycline resistance gene; HAD, hybrid aromatic ring dioxygenase gene.

ionization detector and a 10% Silicone DC-550 column. TCE concentrations in this study were assumed as if being completely dissolved in the aqueous phase.

Analysis of TCE metabolites After TCE was degraded by *Ralstonia* sp. KN1-10A, the concentrations of TCE metabolites remaining in the vials were measured. The analyzed compounds included chloride ions, glyoxylate, formate, dichloroacetic acid, trichloroacetic acid, chloral, 2,2,2-trichloroethanol, and carbon monoxide. With the exception of CO, the concentrations of the metabolites in the liquid phase were analyzed after cells had been removed by filtration. Chloride ion levels were determined using a 2020I ion chromatograph (Dionex Corp., Sunnyvale, CA, USA) equipped with an AS-4A column. When TCE degradation experiments were conducted for chloride concentration monitoring, cells were suspended in 40 mM phosphate buffer (pH 7.0) instead of BSM because BSM interfered with collection of chloride data. Glyoxylate and formate concentrations were determined by a LC-6A high-performance liquid chromatograph (HPLC; Shimadzu) equipped with a Shim-pack SCR-101H column. Chloral and 2,2,2-trichloroethanol concentrations were measured by gas chromatography-mass spectrometry (GC-MS). Dichloroacetic acid and trichloroacetic acid concentrations were determined by the same GC-MS after methyl esteration with trimethyl-silyl-diazomethane (GL Sciences, Tokyo). The GC-MS was performed using a 5970B GC-MS apparatus (Hewlett-Packard, Palo Alto, CA, USA) equipped with a DB-1 column. For monitoring production of CO during TCE degradation, 78.5 ml instead of 10 ml of cell suspension was transferred to the 157-ml glass vials to increase the concentration of CO accumulated in the gas phase and facilitate the analysis. For analysis of CO concentration, a 100-μl sample of headspace gas was withdrawn and injected into a GC-9A gas chromatograph (Shimadzu) equipped with a methanizer (GL Sciences), a flame ionization detector, and an Active Carbon 60/80 column. CO concentrations in this study were assumed as if being completely dissolved in the aqueous phase.

Biodegradation tests of potential TCE metabolites *Ralstonia* sp. KN1-10A cells grown overnight in LB medium were harvested by centrifugation, washed three times with BSM, and resuspended in BSM to an appropriate OD_{600}. After adding the appropriate amounts of substrates, shaking of suspensions at 200 rpm (at 30°C) and periodic measurement of substrate concentrations were started. The substrates being tested were used at the following concentrations: glyoxylate, 1000 mg/*l*; formate, 1000 mg/*l*; dichloroacetic acid, 100 mg/*l*; trichloroacetic acid, 100 mg/*l*; chloral, 100 mg/*l*; 2,2,2-trichloroethanol, 100 mg/*l*; and carbon monoxide, 1 mg/*l*. Analytical methods for determining the concentration of residual substrates were used as described previously. To distinguish biodegradation from abiotic degradation, the same experiments were conducted using autoclaved cell suspensions.

Analysis of phenol degradation activity Phenol was added to cell suspensions to a final concentration of 20 mg/*l*. The suspensions were maintained at 30°C and shaken at 200 rpm for several hours. Aliquots of the suspension were periodically removed and passed through 0.22-μm membrane filters. Phenol concentrations of the filtrates were determined using a LC-6A HPLC (Shimadzu) with a UV detector and a YMC-pack ODS-A column. The phenol degradation activity was calculated based on the time course of the phenol concentrations.

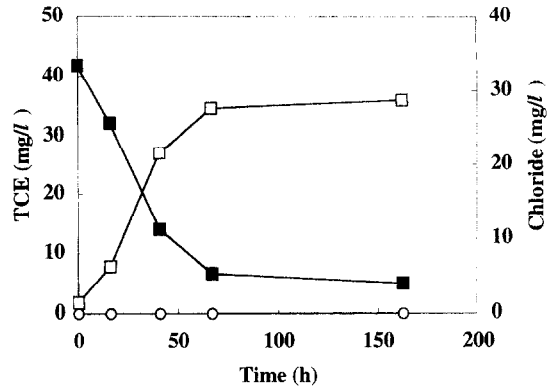

FIG. 2. Chloride production during TCE degradation by *Ralstonia* sp. KN1-10A. Symbols: ■, TCE concentrations; □, Cl^- (cells with TCE); △, Cl^- (cells without TCE).

RESULTS

TCE transformation products When *Ralstonia* sp. KN1-10A grown in LB medium was subjected to a TCE degradation test, it degraded TCE without induction by phenol (Fig. 2) as reported previously (49). In the present experiment, degradation from the initial TCE concentration of 42 mg/*l* to <7 mg/*l* occurred within 67 h. In cell suspensions to which TCE had been added, chloride concentration increased as the TCE concentration decreased, whereas chloride release was not observed in the cell suspension lacking TCE (Fig. 2). These results suggest that the chloride release was due to TCE biodegradation. More than 95% of chlorine in TCE was released as chloride ions within 163 h, indicating that strain KN1-10A almost completely dechlorinated TCE. In addition, carbon monoxide was produced in a pattern similar to that for chloride release during the TCE degradation (Fig. 3). Approximately 7.0 mol% of the added TCE was converted to CO within 160 h. The percentage of TCE transformed to CO did not depend on the amount of TCE degraded or the reaction time, suggesting the absence of further interactions, such as biodegradation or adsorption, between the produced CO and the cells. Other potential metabolites of TCE degradation by oxygenases, such as cytochrome P-450 (50), methane monooxygenase (3, 33, 51), and toluene 2-monooxygenase (34), include glyoxylate, formate, dichloroacetic

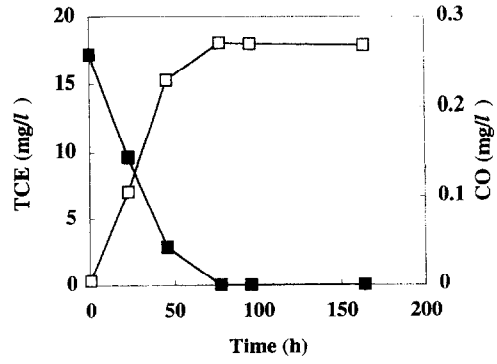

FIG. 3. CO production during TCE degradation by *Ralstonia* sp. KN1-10A. Symbols: ■, TCE concentrations; □, CO concentrations.

TABLE 1. Product formation during TCE degradation by *Ralstonia* sp. KN1-10A

Product	mol% of TCE degraded
Carbon monoxide	7.0
Formate	<6.0
Glyoxylate	<5.3
Dichloroacetic acid	<0.3
Trichloroacetic acid	<0.03
Chloral	<0.03
2,2-Trichloroethanol	<0.12

acid, trichloroacetic acid, chloral, and 2,2,2-trichloroethanol. Although we analyzed filtrates of cell suspensions that had degraded 23.6 mg/*l* TCE for the presence of these possible metabolites, none was detected. In addition, in an effort to find other TCE transformation products, we performed GC-MS to analyze headspace samples and hexane extracts from liquid samples of cells incubated with TCE. However, we could not find any additional peaks derived from TCE (data not shown). The percentages of the products, except that of CO, transformed from TCE by *Ralstonia* sp. KN1-10A (Table 1) reflect the detection limit of each compound.

In TCE degradation by *Ralstonia* sp. KN1-10A, our failure to observe typical TCE metabolites other than chloride and CO might be due to their further decomposition by other enzymes in the strain. To elucidate this phenomenon, utilization tests of the possible metabolites were performed by incubating strain KN1-10A with each substrate (Table 2). Strain KN1-10A could completely degrade glyoxylate, formate, and chloral, but not CO, dichloroacetic acid, trichloroacetic acid, or 2,2,2-trichloroethanol. In the utilization test for chloral, nearly the same number of moles of trichloroacetic acid accumulated as that of chloral was degraded. This result suggests that strain KN1-10A oxidizes chloral to trichloroacetic acid, which cannot be further degraded by the strain. Taken together, these results suggest that glyoxylate and formate were not detected as products of TCE degradation because these were further degraded by the strain.

NADH limitation Formate acts as a reductant supply for microorganisms able to use it, because formate hydroxylase can regenerate NAD(P)H from NAD(P) during formate oxidation. Because the enzyme directly oxidizes formate to H_2O and CO_2, this substrate is not used for growth. Several studies on methanotrophic TCE degraders, which can utilize formate, revealed that the addition of formate can enhance TCE degradation through replenishment of NADH by formate oxidation (18, 31, 32, 52). As described earlier in the present report, *Ralstonia* sp. KN1-10A could oxidize formate.

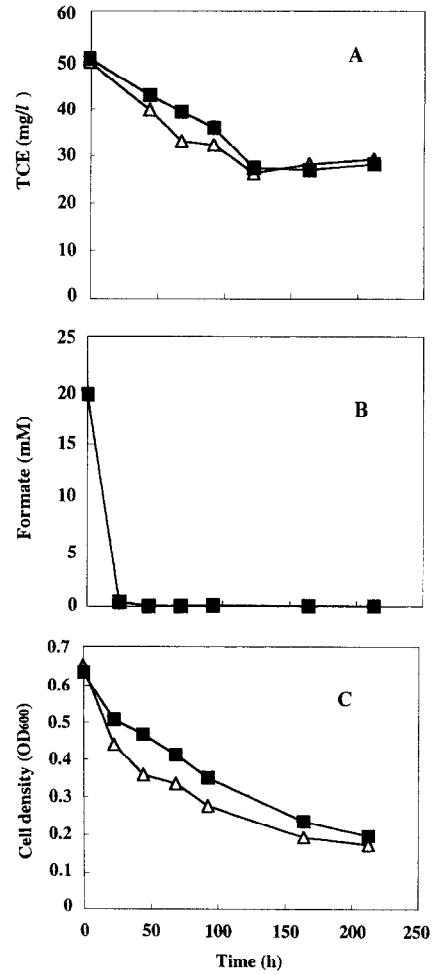

FIG. 4. Effect of addition of 20 mM formate on TCE degradation by *Ralstonia* sp. KN1-10A. (A) TCE concentration, (B) formate concentration, and (C) cell density. Symbols: ■, formate added; △, formate not added.

To evaluate the effects of adding formate during TCE degradation by *Ralstonia* sp. KN1-10A, the resting cells were incubated with TCE in the presence of 20 mM formate, and the degradation performance was compared with that in the absence of formate. Despite the complete consumption of formate within 24 h, addition of this substrate had no effect on TCE degradation (Fig. 4). Accordingly, NADH depletion unlikely limited TCE degradation by strain KN1-10A.

Product toxicity We investigated whether the decrease in the oxidation rate of TCE by *Ralstonia* sp. KN1-10A is due to reduction in PH activity caused by

TABLE 2. Degradation of potential TCE byproducts by *Ralstonia* sp. KN1-10A

Substrate	Initial substrate conc. (mg/*l*)	Initial cell density OD_{600} (−)	Incubation time (h)	% substrate remaining	
				not autoclaved	autoclaved
Carbon monoxide	1	2.0	164	99	98
Glyoxylate	1000	2.0[a]	16	0	105
Formate	1000	2.0[a]	16	0	105
Dichloroacetic acid	100	0.5	144	102	101
Trichloroacetic acid	100	0.5	144	100	105
Chloral	100	0.5	144	2	99
2,2,2-Trichloroethanol	100	0.5	144	88	85

[a] The cell density of the autoclaved sample was 1.0.

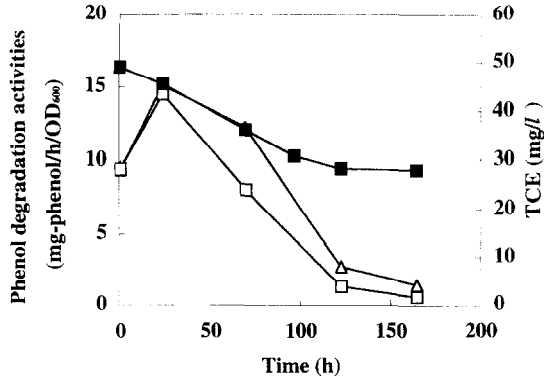

FIG. 5. Time course of phenol degradation activity of *Ralstonia* sp. KN1-10A during TCE degradation. Symbols: ■, TCE concentrations; □, phenol degradation activity (cells with TCE); △, phenol degradation activity (cells without TCE).

product toxicity associated with TCE degradation. We periodically measured the TCE concentration and phenol degradation activity in replicate vials containing cells with or without TCE. In samples containing TCE, its concentration decreased from 49.0 mg/*l* (initial concentration) to 30.9 mg/*l* within 97 h without a marked decline in the degradation rate (Fig. 5). The TCE degradation rate started decreasing at around 100 h, then reached nearly zero at around 120 h. The phenol degradation activity of cells incubated with TCE at 24 h was higher than the initial value, then the activity declined with time (Fig. 5). At the beginning of the experiment, the cells might have had some reversible damage due to washing or centrifugation. In cells incubated without TCE, the phenol degradation activity exhibited nearly the same time course as that for cells incubated with TCE. The phenol degradation activity at 24 h did not differ among samples, and at 70 h the phenol degradation activity of cells incubated with TCE was 66% of that for cells incubated without TCE. In the case of TCE degradation by other oxygenases severely affected by product toxicity, enzyme activity rapidly decreases and is completely lost within several hours (6, 38). In contrast, we found that TCE degradation by *Ralstonia* sp. KN1-10A likely did not cause marked product toxicity and subsequent reduced PH activity.

Comparison of TCE-degrading properties of PH and a hybrid aromatic ring dioxygenase We compared the TCE degradation ability of *Ralstonia* sp. KN1 (pKTP100), which expresses PH, with that of *Ralstonia* sp. KN1 (pKTF200), which expresses a hybrid aromatic ring dioxygenase. This hybrid enzyme exhibits a notable TCE degradation potential in *E. coli* and *Pseudomonas* sp. *Ralstonia* sp. KN1 (pKTF200) rapidly degraded TCE immediately after addition of the substrate (Fig. 6). However, the degradation rate rapidly declined within a few hours and had almost reached zero within 5 h. In contrast, although the initial TCE degradation rate of *Ralstonia* sp. KN1 (pKTP100) was almost 1/50 that of *Ralstonia* sp. KN1 (pKTF200), the strain containing PH continued to degrade TCE for about 100 h without any marked decrease in the degradation rate. Accordingly, TCE concentrations in samples with *Ralstonia* sp. KN1 (pKTP100) were lower than those with *Ralstonia* sp. KN1 (pKTF200) after 50 h. Finally, the total amount of TCE degraded within 700 h by the recombinant strain expressing PH was 2.3 times more than that degraded by

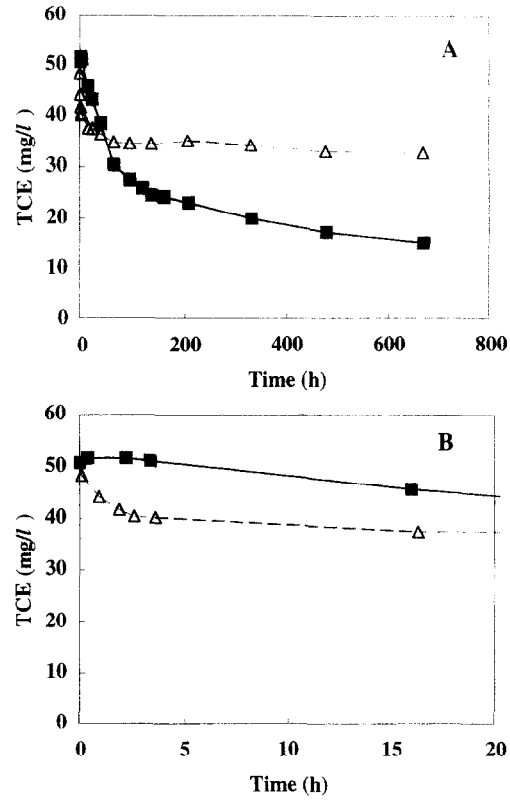

FIG. 6. (A) TCE degradation by *Ralstonia* sp. KN1(pKTP100) and *Ralstonia* sp. KN1(pKTF200). (B) Compressed timescale of the TCE degradation. Symbols: ■, *Ralstonia* sp. KN1(pKTP100); △, *Ralstonia* sp. KN1(pKTF200).

the strain expressing the hybrid aromatic ring dioxygenase. These results show that even in the same host strain (*Ralstonia* sp. KN1), the characteristics of the TCE degradation highly depend on the enzyme that oxidizes TCE.

DISCUSSION

Several studies on the decomposition of TCE by various oxygenases have shown that TCE is initially converted to TCE epoxide or chloral, and the rate at which each intermediate is formed is enzyme-dependent. TCE oxidation by cytochrome P-450 (50) or methane monooxygenase (33, 51, 53) resulted in the formation of chloral via chlorine migration, but chloral was not generated during TCE degradation by toluene 2-monooxygenase from *Burkholderia cepacia* G4 (34). We found that neither chloral nor its oxidation product, trichloroacetic acid, were products of TCE degradation by *Ralstonia* sp. KN1-10A. Therefore, PH likely converts TCE primarily to TCE epoxide rather than to chloral. Because chloral and trichloroacetic acid are potential health hazards, the absence of their production during TCE degradation is a distinct advantage in regard to the use of strain KN1-10A for bioremediation.

Past studies have indicated that TCE epoxide is spontaneously degraded to formate, glyoxylate, CO, and dichloroacetic acid, and the formation rate for each compound varies depending on the experimental conditions. In the case of *in vitro* TCE oxidation by cytochrome P-450, Miller and Guengerich proposed that when TCE

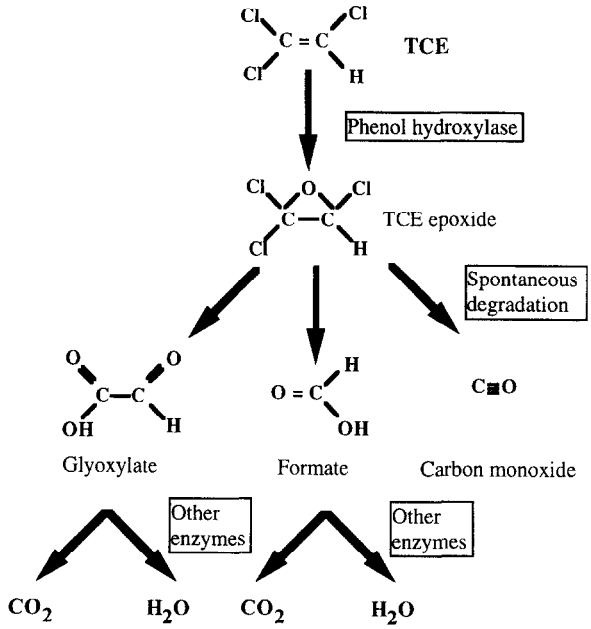

FIG. 7. Proposed pathway of TCE degradation by *Ralstonia* sp. KN1-10A.

epoxide is converted to CO, the other carbon atom in a degraded TCE molecule is utilized to form formate (50). Applying their hypothesis to TCE degradation by PH of *Ralstonia* sp. KN1-10A, CO production of 7.0 mol% of TCE suggests that formate might be produced at the same percentage. The remaining components (>90%) of the degraded TCE can be assumed to have been converted to glyoxylate because dichloroacetic acid, which could not be further degraded by the strain, was not detected. Consequently, we propose that glyoxylate, formate, and CO are the transformation products from TCE degradation by PH of strain KN1-10A (Fig. 7). However, neither formate nor glyoxylate was detected in cell suspensions of strain KN1-10A during TCE degradation. This result can be explained by the fact that strain KN1-10A could degrade both compounds. Further studies, such as *in vitro* experiments using purified PH and stoichiometric analysis using ^{14}C-labeled TCE, are required to confirm the metabolic pathway.

According to the putative TCE degradation pathway of *Ralstonia* sp. KN1-10A, TCE is assumed to be converted mainly to glyoxylate. Because strain KN1-10A can grow on glyoxylate as the sole carbon and energy source (data not shown), the microorganism may regenerate a part of the NADH lost during TCE oxidation by utilizing the glyoxylate generated from TCE during that process. Theoretically, oxidation of one molecule of TCE by an oxygenase results in consumption of one molecule of NADH, whereas mineralization of one molecule of glyoxylate yields two molecules of NADH, as shown in The University of Minnesota Biocatalysis/Biodegradation Database (http://www.labmed.umn.edu/umbbd/index.html) (54). A comparison of these two values indicates that strain KN1-10A can continue to degrade TCE without NADH limitation. Actually, formate addition, which is assumed to increase the NADH content in the cell, did not enhance the TCE degradation potential of the strain.

Product toxicity is one of the factors that limits the degradation of TCE by oxygenases. Previous studies have suggested that oxygenases and/or cells are damaged by reactive acyl chloride intermediate compounds produced during spontaneous degradation of TCE epoxide that has been converted from TCE by the enzyme (33). In TCE degradation by *B. cepacia* G4, one of the most extensively studied TCE degraders, marked product toxicity was not observed (23, 55). However, during the *in vitro* oxidation of TCE with purified toluene 2-monooxygenase from *B. cepacia* G4, TCE-dependent inactivation of the enzyme was observed. Newman and Wackett (34) have proposed that the intracellular environment may protect toluene 2-monooxygenase from inactivation via certain mechanisms, such as through the activity of other enzymes that alter the fate of TCE epoxide. In fact, the markedly higher level of glyoxylate observed during *in vivo* TCE degradation by strain G4 than that during *in vitro* TCE degradation by toluene 2-monooxygenase indicates the different outcomes of TCE epoxide in the two environments (34). Similarly, our results show that high percentages (>90%) of glyoxylate may be obtained during *in vivo* TCE degradation by *Ralstonia* sp. KN1-10A. Resistance of strain KN1-10A against product toxicity associated with TCE degradation may involve the same mechanisms as those of *B. cepacia* G4.

TCE degradation behavior of *Ralstonia* sp. KN1 differed markedly depending on the enzyme used. Although the initial TCE degradation rate of *Ralstonia* sp. KN1(pKTP100), which produced PH, was 1/50 that of *Ralstonia* sp. KN1(pKTF200); the strain with the hybrid aromatic ring dioxygenase, the strain using PH continued to degrade TCE for more than 100 h without a pronounced reduction in the degradation rate. In contrast, the TCE degradation rate of *Ralstonia* sp. KN1(pKTF200) decreased rapidly with time and reached almost zero within 5 h. The TCE transformation capacity (Tc) of *Ralstonia* sp. KN1(pKTP100) was 2.3 times more than that of *Ralstonia* sp. KN1(pKTF200). These results suggest that of these two systems, the combination of *Ralstonia* sp. KN1 and PH is better for constructing a TCE degrader with high Tc. However, *Ralstonia* sp. KN1(pKTP100) was associated with some disadvantages, such as poor plasmid stability, and it required IPTG induction for the expression of TCE degradation activity. Therefore, *Ralstonia* sp. KN1-10A is better than *Ralstonia* sp. KN1(pKTP100) as a practical TCE degrader. Although the Tc (0.05–0.1 g TCE/g cells) of *Ralstonia* sp. KN1-10A was higher than that of mixed methanotrophs (0.036 g TCE/g cells) (31), it was lower than that of a microorganism that used phenol as a substrate during TCE degradation (0.31–0.51 g TCE/g cell) (56) and that of *Methylosinus trichosporium* OB3b (0.29 g TCE/g cell) (37). However, these strains with high Tc for TCE require an inducer substrate, such as phenol or methane, for the expression of TCE degradation activity. For this practical reason, *Ralstonia* sp. KN1-10A is more feasible for TCE treatment of soil and groundwater.

In previously reported TCE degradation tests using *Pseudomonas* sp. expressing the hybrid aromatic ring dioxygenase, the degradation rates rapidly decreased with time and reached almost zero within several hours (41). One of the four components of the hybrid aromatic ring dioxygenase is the large subunit of the terminal dioxygenase of a multicomponent toluene dioxygenase from *Pseudomonas putida* F1 (40). The rapid decrease in the TCE degradation rate was due to product toxicity and

occurred during *in vivo* as well as *in vitro* degradation by toluene dioxygenase of strain F1 (6, 38). Accordingly, we suggest that the rapid decline (within a few hours) in the TCE degradation rate of *Ralstonia* sp. KN1 that expresses the hybrid aromatic ring dioxygenase is due to product toxicity as well as strain F1 expressing toluene dioxygenase. As described earlier in the present report, we propose that the intracellular environment of strain KN1 may protect PH from product toxicity associated with TCE degradation. Since TCE degradation by the toluene dioxygenase uses a TCE degradation pathway different from that of PH (57), such protection in the intracellular environment may not be available in the case of TCE degradation by the hybrid aromatic ring dioxygenase. In addition, we cannot exclude the possibility that the transient accumulation of reactive intermediates at high concentrations during the rapid TCE degradation by the hybrid aromatic ring dioxygenase may cause significant enzyme inactivation.

Our results support the effectiveness of using *Ralstonia* sp. KN1-10A for TCE management of soil and groundwater. This strain can continue to degrade TCE without a rapid decline in the degradation rate, which could be caused by product toxicity or NADH depletion. In addition, *Ralstonia* sp. KN1-10A does not produce any noxious metabolites during the degradation process. Our recent study revealed that the *tac* promoter in strain KN1-10A failed to transcribe *phyZ*, an important gene for TCE degradation that is located upstream of the other genes required for this process. The nucleotide sequence analysis of *Ralstonia* sp. KN1-10A showed that the *tac* promoter was integrated in the middle of *phyZ* (28). Therefore, we expect that a new recombinant strain that has a higher TCE degradation ability than strain KN1-10A can be constructed by inserting the *tac* promoter upstream of *pheZ* of KN1, leading to constitutive expression of all the genes necessary for TCE degradation. In addition to improving the TCE degradation potential of the recombinant *Ralstonia* sp. KN1, we are investigating the behavior of various TCE degraders in soil and groundwater. We have already developed monitoring methods involving the *luxAB* gene that enable us to determine the number of recombinant TCE degraders present in these natural environments (58). The final goal of our efforts is to make the TCE treatment process using recombinant TCE degraders practical for remediation of TCE-contaminated soil and groundwater.

ACKNOWLEDGMENT

We thank Kensuke Furukawa of Kyushu University for providing pJHF10.

REFERENCES

1. **Oleszkiewicz, J. A. and Elektorowicz, M.:** Groundwater contamination with trichloroethylene: the problem and some solutions-a review. J. Soil Contam., **2**, 205–228 (1993).
2. **Wilson, J. T. and Wilson, B. H.:** Biotransformation of trichloroethylene in soil. Appl Environ. Microbiol., **49**, 242–243 (1985).
3. **Little, C. D., Palumbo, A. V., Herbes, S. E., Lidstrom, M. E., Tyndall, R. L., and Gilmer, P. J.:** Trichloroethylene biodegradation by a methane oxidizing bacterium. Appl. Environ. Microbiol., **54**, 951–956 (1988).
4. **Uchiyama, H., Nakajima, T., Yagi, O., and Tabuchi, T.:** Aerobic degradation of trichloroethylene by a new type II metanc utilizing bacterium, strain M. Agric. Biol. Chem., **53**, 2903–2907 (1989).
5. **Wackett, L. P., Brusseau, G. A., Householder, S. R., and Hanson, R. S.:** Survey of microbial oxygenases: trichloroethylene degradation by propane-oxidizing bacteria. Appl. Environ. Microbiol., **55**, 2960–2964 (1989).
6. **Wackett, L. P. and Gibson, D. T.:** Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol., **54**, 1703–1708 (1988).
7. **Winter, R. B., Yen, K.-M., and Ensley, B. D.:** Efficient degradation of trichloroethylene by a recombinant *Escherichia coli*. Bio/Technology, **7**, 282–285 (1989).
8. **Nelson, M. J., Montgomery, S. O., Mahaffey, W. R., and Pritchard, P. H.:** Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. Appl. Environ. Microbiol., **53**, 949–954 (1987).
9. **Harker, A. R. and Kim, Y.:** Trichloroethylene degradation by two independent aromatic degrading pathways in *Alcaligenes eutrophus* JMP134. Appl. Environ. Microbiol., **56**, 1179–1181 (1990).
10. **Nakamura, K. and Miyaji, Y.:** Degradation of trichloroethylene by a genetically engineered microorganism containing a phenol hydroxylase gene. Proc. Environ. Eng. Res., **29**, 17–27 (1992). (in Japanese)
11. **Fujita, M., Ike, M., Hioki, J., Kataoka, K., and Takeo, M.:** Trichloroethylene degradation by genetically engineered bacteria carrying cloned phenol catabolic genes. J. Ferment. Bioeng., **79**, 100–106 (1995).
12. **Dabrock, B., Riedel, J., Bertram, J., and Gottschalk, G.:** Isopropylbenzene (cumene)-a new substrate for the isolation of trichloroethene-degrading bacteria. Arch. Microbiol., **158**, 9–13 (1992).
13. **Arciero, D. M., Vannelli, T., Logan, M., and Hooper, A. B.:** Degradation of trichloroethylene by the ammonium-oxidizing bacterium, *Nitrosomonas eutrophus*. Biochem. Biophys. Res. Commun., **159**, 640–643 (1989).
14. **Heald, S. and Jenkins, R. O.:** Trichloroethylene removal and oxidation toxicity mediated by toluene dioxygenase of *Pseudomonas putida*. Appl. Environ. Microbiol., **60**, 4634–4637 (1994).
15. **McClay, K., Streger, S. H., and Steffan, R. J.:** Induction of toluene oxidation activity in *Pseudomonas mendocina* KR1 and *Pseudomonas* sp. strain ENVPC5 by chlorinated solvents and alkanes. Appl. Environ. Microbiol., **61**, 3479–3481 (1995).
16. **Leahy, J. G., Byrne, A. M., and Olsen, R. H.:** Comparison of factors influencing trichloroethylene degradation by toluene-oxidizing bacteria. Appl. Environ. Microbiol., **62**, 825–833 (1996).
17. **Hopkins, G. D., Semprini, L., and McCarty, P. L.:** Microcosm and *in situ* field studies of enhanced biotransformation of trichloroethylene by phenol-utilizing microorganisms. Appl. Environ. Microbiol., **59**, 2277–2285 (1993).
18. **Alvarez-Cohen, L. and McCarty, P. L.:** Product toxicity and cometabolic competitive inhibition modeling of chloroform and trichloroethylene transformation by methanotrophic resting cells. Appl. Environ. Microbiol., **57**, 1031–1037 (1991).
19. **Hopkins, G. D. and McCarty, P. L.:** Field evaluation of *in situ* aerobic cometabolism of trichloroethylene and three dichloroethylene isomers using phenol and toluene as the primary substrates. Environ. Sci. Technol., **29**, 1628–1637 (1995).
20. **Aziz, C. E., Fitch, M. W., Linquist, L. K., Pressman, J. G., Georgiou, G., and Speitel, G. E. J.:** Methanotrophic biodegradation of trichloroethylene in a hollow fiber membrane bioreactor. Environ. Sci. Technol., **29**, 2574–2583 (1995).
21. **Kim, Y., Ayoubi, P., and Harker, A. R.:** Constitutive expression of the cloned phenol hydroxylase gene(s) from *Alcaligenes eutrophus* JMP134 and concomitant trichloroethylene oxidation. Appl. Environ. Microbiol., **62**, 3227–3233 (1996).
22. **Romine, M. F. and Brockman, F. J.:** Recruitment and expression of toluene/trichloroethylene biodegradation genes in bacteria native to deep-subsurface sediments. Appl. Environ. Microbiol., **62**, 2647–2650 (1996).

23. **Shields, M. S. and Reagin, M. J.:** Selection of a *Pseudomonas cepacia* strain constitutive for the degradation of trichloroethylene. Appl. Environ. Microbiol., **58,** 3977–3983 (1992).
24. **Furukawa, K., Hirose, J., Hayashida, S., and Nakamura, K.:** Efficient degradation of trichloroethylene by a hybrid aromatic ring dioxygenase. J. Bacteriol., **176,** 2121–2123 (1994).
25. **Jahng, D. and Wood, T. K.:** Trichloroethylene and chloroform degradation by a recombinant pseudomonad expressing soluble methane monooxygenase from *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol., **60,** 2473–2482 (1994).
26. **Yee, D. C., Maynard, J. A., and Wood, T. K.:** Rhizoremediation of trichloroethylene by a recombinant, root-colonizing *Pseudomonas fluorescens* strain expressing toluene ortho-monooxygenase constitutively. Appl. Environ. Microbiol., **64,** 112 (1998).
27. **Matin, A., Little, C. D., Fraley, C. D., and Keyhan, M.:** Use of starvation promoters to limit growth and select for trichloroethylene and phenol transformation activity in recombinant *Escherichia coli*. Appl. Environ. Microbiol., **61,** 3323–3328 (1995).
28. **Nakamura, K., Ishida, H., and Iizumi, T.:** Constitutive trichloroethylene degradation led by *tac* promoter chromosomally integrated upstream of phenol hydroxylase genes of *Ralstonia* sp. KN1 and its nucleotide sequence analysis. J. Biosci. Bioeng., **89,** 47–54 (2000).
29. **Nakamura, K. and Ishida, H.:** Development of a genetically engineered microorganism for trichloroethylene degradation by homologous recombination and analysis of its gene expression. Proc. Environ. Eng. Res., **33,** 165–175 (1996). (in Japanese)
30. **Alvarez-Cohen, L. and McCarty, P. L.:** A cometabolic biotransformation model for halogenated aliphatic compounds exhibiting product toxicity. Environ. Sci. Technol., **25,** 1381–1386 (1991).
31. **Alvarez-Cohen, L. and McCarty, P. L.:** Effects of toxicity, aeration, and reductant supply on trichloroethylene transformation by a mixed methanotrophic culture. Appl. Environ. Microbiol., **57,** 228–235 (1991).
32. **Chang, H.-L. and Alvarez-Cohen, L.:** Transformation capacities of chlorinated organics by mixed cultures enriched on methane, propane, toluene, or phenol. Biotechnol. Bioeng., **45,** 440–449 (1995).
33. **Fox, B. G., Borneman, J. G., Wackett, L. P., and Lipscomb, J. D.:** Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. Biochemistry, **29,** 6419–6427 (1990).
34. **Newman, L. M. and Wackett, L. P.:** Trichloroethylene oxidation by purified toluene 2-monooxygenase: products, kinetics, and turnover-dependent inactivation. J. Bacteriol., **179,** 90 (1997).
35. **Broholm, K., Jensen, B. K., Christensen, T. H., and Olsen, L.:** Toxicity of 1,1,1-trichloroethane and trichloroethene on a mixed culture of methane-oxidizing bacteria. Appl. Environ. Microbiol., **56,** 2488–2493 (1990).
36. **Henry, S. M. and Grbic-Galic, D.:** Influence of endogenous and exogenous electron donors and trichloroethylene oxidation toxicity on trichloroethylene oxidation by methanotrophic cultures from a groundwater aquifer. Appl. Environ. Microbiol., **57,** 236–244 (1991).
37. **Oldenhuis, R., Oedzes, J. Y., Waarde, J. J. V. D., and Janssen, D. B.:** Kinetics of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene. Appl. Environ. Microbiol., **57,** 7–14 (1991).
38. **Wackett, L. P. and Householder, S. R.:** Toxicity of trichloroethylene to *Pseudomonas putida* F1 is mediated by toluene dioxygenase. Appl. Environ. Microbiol., **55,** 2723–2725 (1989).
39. **Zylstra, G. J. and Gibson, D. T.:** Toluene degradation by *Pseudomonas putida* F1: nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli*. J. Biol. Chem., **264,** 14940–14946 (1989).
40. **Hirose, J., Suyama, A., Hayashida, S., and Furukawa, K.:** Construction of hybrid biphenyl (*bph*) and toluene (*tod*) genes for functional analysis of aromatic ring dioxygenases. Gene, **138,** 27–33 (1994).
41. **Suyama, A., Iwakiri, R., Kimura, N., Nishi, A., Nakamura, K., and Furukawa, K.:** Engineering hybrid pseudomonads capable of utilizing a wide range of aromatic hydrocarbons and of efficient degradation of trichloroethylene. J. Bacteriol., **178,** 4039–4046 (1996).
42. **Simon, R., Priefer, U., and Puhler, A.:** A broad host range mobilization system *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology, **1,** 784–790 (1983).
43. **Maloy, S. R.:** Experimental techniques in bacterial genetics. Jones and Bartlett Publishers, Boston (1990).
44. **Sambrook, J., Fritsch, E. F., and Maniatis, T.:** Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, New York (1989).
45. **Bagdasarian, M., Amann, E., Lurz, R., and Ruckert, B.:** Activity of the hybrid *trp-lac* (*tac*) promoter of *Escherichia coli* in *Pseudomonas putida*. Construction of broad-host-range, controlled-expression vectors. Gene, **26,** 273–282 (1983).
46. **Shields, M. S., Montgomery, S. O., Cuskey, S. M., Chapman, P. J., and Pritchard, P. H.:** Mutants of *Pseudomonas cepacia* G4 defective in catabolism of aromatic compounds and trichloroethylene. Appl. Environ. Microbiol., **57,** 1935–1941 (1991).
47. **Taira, K., Hirose, J., Hayashida, S., and Furukawa, K.:** Analysis of *bph* operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. J. Biol. Chem., **267,** 4844–4853 (1992).
48. **Stanier, R. Y., Palleroni, N. J., and Duodoroff, M.:** The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol., **43,** 159–271 (1966).
49. **Nakamura, K.:** Degradation of trichloroethylene by genetically engineered microorganisms. J. Jpn Soc. Water Environ., **16,** 775–779 (1993). (in Japanese)
50. **Miller, R. E. and Guengerich, F. P.:** Oxidation of trichloroethylene by liver microsomal cytochrome P-450: evidence for chlorine migration in a transition state not involving trichloroethylene oxide. Biochemistry, **21,** 1090–1097 (1982).
51. **Nakajima, T., Uchiyama, H., Yagi, O., and Nakahara, T.:** Novel metabolite of trichloroethylene in a methanotrophic bacterium, *Methylocystis* sp. M, and hypothetical degradation pathway. Biosci. Biotech. Biochem., **56,** 486–489 (1992).
52. **Henry, S. M. and Grbic-Galic, D.:** Inhibition of trichloroethylene oxidation by the transformation intermediate carbon monoxide. Appl. Environ. Microbiol., **57,** 1770–1776 (1991).
53. **Newman, L. M. and Wackett, L. P.:** Fate of 2,2,2-trichloroacetaldehyde (chloral hydrate) produced during trichloroethylene oxidation by methanotrophs. Appl. Environ. Microbiol., **57,** 2399–2402 (1991).
54. **Ellis, L. B. and Wackett, L. P.:** A Microbial Biocatalysis Database. Soc. Ind. Microb. News, **45,** 167–173 (1995).
55. **Folsom, B. R., Chapman, P. J., and Pritchard, P. H.:** Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: kinetics and interactions between substrates. Appl. Environ. Microbiol., **56,** 1279–1285 (1990).
56. **Bielefeldt, A. R., Stensel, H. D., and Strand, S. E.:** Cometabolic degradation of TCE and DCE without intermediate toxicity. J. Environ. Eng., **121,** 791–797 (1995).
57. **Li, S. and Wackett, L. P.:** Trichloroethylene oxidation by toluene dioxygenase. Biochem. Biophys. Res. Commun., **185,** 443–451 (1992).
58. **Ishida, H. and Nakamura, K.:** Monitoring of a genetically engineered microorganism able to degrade trichloroethylene by using luciferase genes. Proc. Environ. Eng. Res., **34,** 41–50 (1997). (in Japanese)