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Constitutive Trichloroethylene Degradation Led by *tac* Promoter Chromosomally Integrated Upstream of Phenol Hydroxylase Genes of *Ralstonia* sp. KN1 and Its Nucleotide Sequence Analysis

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***Ralstonia* sp. KN1-10A is a strain capable of degrading trichloroethylene (TCE) constitutively due to the *tac* promoter (*Ptac*) integrated upstream of the phenol hydroxylase genes (*phy*) in its chromosome. The expression of *Ptac* was analyzed using *luxAB* of *Vibrio harveyi* as a reporter. After determining the nucleotide sequence of *phyABCDE* required for TCE degradation, a *luxAB*-encoding fragment was integrated downstream of *phyE* by homologous recombination in strain KN1-10A, obtaining strain KN1-10A-LX. In the same manner, the *luxAB*-encoding fragment was integrated into the chromosome of the wild-type strain, KN1. The resultant strain KN1-LX was used to analyze the gene expression caused by phenol induction. The expression induced by *Ptac* was compared to that by phenol induction. Although the level of *luxAB* expression led by *Ptac* was almost equal to that induced by phenol, the TCE degradation rate by the *Ptac*-carrying KN1-10A-LX was markedly slower than that by the phenol-induced KN1-LX. These results suggest that an important gene for TCE degradation was not transcribed by *Ptac* in KN1-10A-LX. The nucleotide sequence analysis showed the existence of a small gene, *phyZ*, upstream of *phyA*, and *Ptac* was found to be integrated into the middle of *phyZ* in KN1-10A-LX. The effect of *phyZ* on TCE degradation was examined by using recombinant strains expressing *phyABCDE* with or without *phyZ* in a plasmid. The coexistence of *phyZ* markedly accelerated TCE degradation. Through an exhaustive expression analysis, it was demonstrated that the chromosomal integration of *Ptac* was a very attractive method for high and stable production of phenol hydroxylase for TCE degradation.**

[**Key words:** trichloroethylene, phenol hydroxylase, *Ralstonia* sp., constitutive degradation]

Trichloroethylene (TCE) is a common soil and groundwater contaminant in industrialized countries because of its long usage as a solvent. Although the toxicity of TCE is not high (1), an anaerobically produced product, vinyl chloride, is a potent human carcinogen (2). Therefore, TCE-contaminated soil or ground water should be cleaned up.

Many enzymes, including methane monooxygenase (3), propane monooxygenase (4), ammonia monooxygenase (5), toluene dioxygenase (6), toluene monooxygenase (7, 8), phenol hydroxylase (9, 11), and isopropylbenzene dioxygenase (12) are known to decompose TCE aerobically. Because all of these enzymes are inducible, it is essential to use inducer substrates when using wild strains to encode these enzymes for bioaugmentation. The induction process to obtain high enzyme activity is occasionally complicated due to the rapid loss of enzymatic activity after exhaustion of the inducer substrate. In addition, inducer substrates are not always suitable for bacterial cultivation. Accordingly, the cultivation and induction processes must be separated, making the whole process more complex. In order to overcome these difficulties, many strains which express the TCE-degrading genes constitutively have been developed (10, 13–15).

We isolated a phenol-utilizing bacterium, strain KN1, from a phenol-fed reactor (16). Strain KN1 degraded TCE after phenol induction. Although strain KN1 was originally identified as *Pseudomonas putida* based on its biochemical characteristics and the presence of polar flagella, our recent 16S rRNA analysis (DDBJ accession no. AB031995) showed that the strain belonged to the genus *Ralstonia*. In order to develop a recombinant for TCE degradation, firstly, we cloned a DNA fragment that encodes the phenol hydroxylase genes from the chromosomal DNA of this strain, *Ralstonia* sp. KN1 (11). The DNA fragment was inserted into the multiple cloning sites (MCS) of pRCL100 (11) which encoded *Ptrc*, *lacI*^{q}, a chloramphenicol resistance gene, and the replicon from RSF1010. The resultant plasmid, pNEM101, was transferred into *Ralstonia* sp. KN1. *Ralstonia* sp. KN1 (pNEM101) degraded TCE in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG). Then, *lacI*^{q} was eliminated from pNEM101 to construct a new recombinant plasmid, pNEM201, which was constitutive for the gene expression led by *Ptrc* in *Ralstonia* sp. KN1. *Ralstonia* sp. KN1 (pNEM201) constitutively decomposed TCE. However, the recombinant strain easily lost pNEM201 during cultivation in the absence of chloramphenicol. Thirdly, in order to develop a stable strain for TCE degradation, we integrated *Ptac* in front of the phenol-hydroxylase-gene-encoding region in the chromosome (17). The resultant strain, *Ralstonia* sp. KN1-10A, decomposed TCE constitutively, and the inserted DNA fragment was stably maintained. However, *Ralstonia* sp. KN1-10A did not degrade TCE as rapidly as the wild-strain did after phenol induction. Although primary analysis on this event was conducted in a previous study (17), the results were unclear because of inadequate nucleotide sequence data.

In this report we describe in detail the analysis of the

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TCE degradation by *Ralstonia* sp. KN1-10A and the nucleotide sequences of the phenol hydroxylase genes responsible for TCE degradation.

MATERIALS AND METHOD

Bacterial strains, plasmids, and growth conditions The bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* and *Ralstonia* sp. strains were cultivated in L broth medium containing (per liter of distilled water) 10 g of tryptone (Difco, Detroit, MI, USA), 5 g of yeast extract (Difco), and 5 g of NaCl. The pH was adjusted to 7.0 with NaOH. When necessary, the medium was supplemented with ampicillin (Ap, 100 μg/ml), chloramphenicol (Cm, 50 μg/ml), or kanamycin (Km, 50 μg/ml). *E. coli* strains were cultivated at 37°C, and *Ralstonia* sp. strains at 30°C.

Recombinant DNA techniques Standard recombinant techniques used in this research were carried out according to published protocols (18). *E. coli* DH5 (19) was used for plasmid construction, and *E. coli* S17-1 (20) for conjugal transfer of the plasmid to a recipient strain. Restriction digestions were performed with restriction endonucleases purchased from Toyobo Co. (Tokyo). Blunt ends were made with T4 DNA polymerase supplied by Toyobo Co. Ligation was carried out using Ligation High of Toyobo Co.

Nucleotide sequence determination and analysis The nucleotide sequence of the 8.7-kb *Eco*RI-*Sal*I fragment encoding the phenol hydroxylase genes was determined by the dideoxy method of Sanger *et al.* (23) facilitated by the use of an ABI 377 sequencer (Perkin-Elmer, Foster City, CA, USA). The sequence data were compared with those in the GenBank and Swiss Prot databases using the BLASTX and BLASTP 2.06 programs, respectively, to find similar putative amino acid sequences.

PCR synthesis of *luxAB* The *luxAB* genes of *Vibrio harveyi* B392 were PCR-synthesized using the 2.2-kb *Sal*I-*Pvu*II fragment encoding *luxAB* (24) as the template. PCR was performed using *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) and the Gene Amp PCR System 2400 (Perkin-Elmer). A primer pair of 5'-ggGGTAC CAACAAATAAGGAAATGTTATG (underline; *Kpn*I site) and 5'-ggGAGCTCACGTTACGAGTGGTATTTGA C (underline; *Sac*I site) was used to synthesize only the necessary region for *luxAB*. The synthesized fragment was digested with *Kpn*I and *Sac*I, and then cloned into pUC19. The cloned fragment was excised with *Kpn*I and *Sac*I, and the ends were made blunt with T4 DNA polymerase.

Cloning of homologous region and *luxAB* into pMOK180 The organization of the phenol hydroxylase genes (*phy*) is shown in Fig. 1. The schematic diagram for cloning the homologous region and *luxAB* is shown in Fig. 2. In order to construct a new cloning vector, the 1.5-kb *Eco*T22I-*Nco*I fragment of pTrc99A was eliminated. The remaining fragment was then ligated with a *Pac*I linker, d(pTTAATTAA), after making the ends blunt with T4 DNA polymerase. This plasmid was digested with *Hin*dIII, and the ends were made blunt with T4 DNA polymerase so that it could be ligated with another *Pac*I linker, d(pGTTAATTAAC). The resulting plasmid, pKNA82, was used to clone the homologous region and *luxAB*. First, the 3.1-kb *Kpn*I-*Sal*I fragment encoding *phyDEFG* was inserted into the MCS of pKNA82. Second, the PCR-synthesized *luxAB*-encoding fragment was cloned into the *Eco*T22 site of the resulting plasmid after making the ends blunt with T4 DNA polymerase. Finally, the 5.2-kb *Kpn*I-*Sal*I fragment containing *luxAB* was excised using *Pac*I, and inserted into the *Pac*I site of pMOK180, obtaining the plasmid pMOK1801.

Construction of *Ralstonia* sp. KN1-LX and KN1-10A-LX The plasmid pMOK1801 was introduced into *E. coli* S17-1, and the resulting strain, *E. coli* S17-1 (pMOK1801), was cultivated at an inoculum concentration of 1% (v/v) of a one-day-old culture in L broth medium containing Km with shaking for 17 h at 37°C. *Ralstonia* sp. KN1 was cultivated in the same manner but at 30°C, without Km. One milliliter of each culture was centrifuged at 8000 × *g* at 4°C and the resultant pellet was suspended in 0.5 ml of mineral salts basal medium (MSB) (25). This procedure was repeated, and the two suspensions were mixed together, centrifuged, and finally suspended in 100 μl of MSB. The cell mixture was dropped onto a 0.22-μm-pore-size nitrocellulose filter (Millipore Co., Bedford, MA, USA), which was placed on an L broth agar plate, and incubated for 10 h at 30°C. The cells on the filter were suspended in 1 ml of MSB, and diluted samples were spread on MSB agar

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
E. coli		
DH5	*endA1 hsdR supE44 recA thi-1 relA1 gyrA96*	(19)
S17-1	*thi pro recA hsdR* chr::RP4-2(Tc^r::Mu Km^r::Tn7)	(20)
Ralstonia sp.		
KN1	Prototroph	(11)
KN1-10A	*phyZ*::Tc^r and *Ptac*	(17)
KN1-LX	*phyF*::*luxAB*	This study
KN1-10A-LX	*phyZ*::Tc^r and *Ptac*, *phyF*::*luxAB*	This study
KN1-ΔPH	Δ*phyRZABCDE*	This study
Plasmids		
pUC19	Cloning vector, Ap^r	(21)
pTrc99A	*Ptrc* expression vector, Ap^r	(22)
pKNA82	Cloning vector, Ap^r	This study
pMOK180	Homologous recombination vector, Km^r	(17)
pRCL100	*Ptrc* expression vector, *lacI^q*, Cm^r	(11)
Clones in pRCL100		
pNIN103	containing 4.4-kb *Sac*I-*Eco*T22I fragment encoding *phyABCDE*	This study
pNIN104	containing 4.7-kb *Bam*HI-*Xba*I fragment encoding *phyZABCDE*	This study

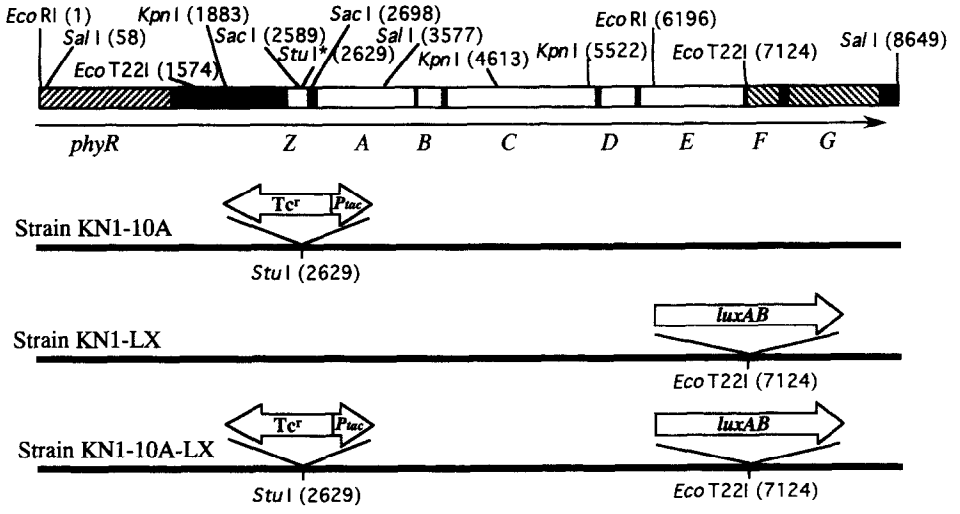

FIG. 1. Organization of *phy* genes and the constructed derivatives. (*StuI** site is not unique) Phenol hydroxylase genes are indicated by the open boxes. Other ORFs are indicated by the shaded boxes. The arrow shows the direction of transcription. Three derivatives were constructed by homologous recombination. The tetracycline resistance (Tc^r) gene was from pBR322. *Ptac* was from *tac* Promoter GenBlock of Pharmacia Biotech. *luxAB* was from *Vibrio harveyi* B392.

plates supplemented with 20 mM lactate, 40 μg/ml of X-Gal (5-chloro-4-bromo-3-indolyl-β-D-galactopyranoside), and 100 μg/ml of Km. The plates were incubated for 3 d at 30°C and Km^r blue colonies were isolated. These colonies would be single-crossover strains in which the entire pMOK1801 was integrated. An isolated single-crossover strain was cultivated on L broth for one day, and then the culture was diluted and spread on L broth agar plates containing 40 μg/ml of X-Gal to obtain a double-crossover strain. Approximately 1% of the colonies were white because pMOK180 had been lost from their chromosomes. In some cases *luxAB* was lost along with the

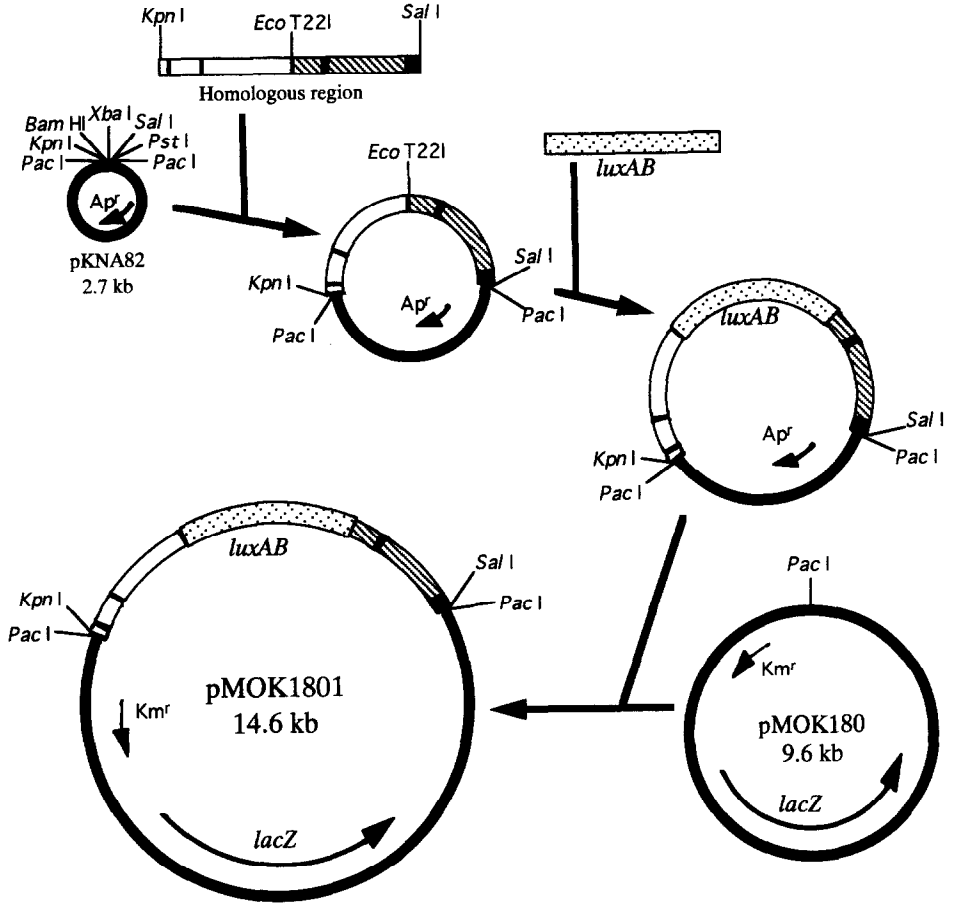

FIG. 2. Construction of plasmid pMOK1801 for homologous recombination.

rest of the plasmid. A double-crossover strain, *Ralstonia* sp. KN1-LX (Fig. 1) containing *luxAB*, was isolated from among these white colonies. In the same manner, *Ralstonia* sp. KN1-10A-LX (Fig. 1) was obtained. The integration of *luxAB* in these two strains was confirmed by PCR using a primer pair (5'-ATTCATCTCGGCCGGCG ACGCACAGAACAG-3', 5'-AAGGTCTTGCAGGCGAG CACAAACCCACTC-3') that was designed to amplify the region containing the integrated site.

Construction of pNIN103 and pNIN104 The 4.4-kb *Sac*I (nt 2698)-*Eco*T22I (nt 7124) fragment encoding *phyABCDE* was cloned into the MCS (*Sac*I-*Pst*I) of pRCL100 to construct pNIN103. To construct pNIN104, the 0.23-kb *phyZ*-containing fragment (corresponding to nt 2467-2699 in Fig. 1) was synthesized by PCR with the primer pair, 5'-gggGGATCCGAAGGAGCAGCGCC ATGATCAACC-3' (underline; *Bam*HI site) and 5'-gggTCTAGATCAGTCGTCCTGATCGGGCGC-3' (underline; *Xba*I site). The synthesized fragment was digested with *Bam*HI and *Xba*I, and then ligated into the MCS of pKNA82 shown in Fig. 2. The 0.16-kb *Bam*HI-*Stu*I (nt 2629) fragment in this cloned DNA was excised and ligated with the 4.5-kb *Stu*I-*Eco*T22I fragment, which had the *Eco*T22I end blunted and then converted to an *Xba*I site with an *Xba*I linker, p(dCTCTAGAG). The resulting 4.7-kb *Bam*HI-*Xba*I fragment, encoding *phyZABCDE*, was cloned into the MCS (*Bam*HI-*Xba*I) of pRCL100 to construct pNIN104. Plasmids pNIN103 and pNIN104 were each transformed into strain KN1-ΔPH, whose *Sma*I (nt 470)-*Eco*T22I (nt 7124) region, encoding *phyRZABCDE*, was eliminated from the chromosome by homologous recombination using the *Eco*RI (nt 1)-*Sma*I (nt 470) and the *Eco*T22I (nt 7124)-*Sma*I (nt 7569) fragments as the homologous regions.

TCE degradation *Ralstonia* sp. KN1-10A and KN1-10A-LX were cultivated at an inoculum concentration of 1% (v/v) of a one-day-old culture in L broth medium with shaking for 17 h at 30°C. Cells were harvested by centrifugation at 8000×*g* at 4°C, and the resultant pellet was washed with modified MSB (MMSB; concentrations of minerals added to the phosphate buffer were decreased to 2% of original concentrations, because of the inhibitory effect on TCE degradation). This procedure was repeated, and finally the cell concentration was adjusted to an optical density of 1.0 at 600 nm (OD_{600}). The appropriate cell suspension (10 ml) was added to a glass vial (total volume, 155 ml). The vial was sealed with a Teflon-coated butyl-rubber septum with an aluminum crimp seal after adding an appropriate volume of TCE-saturated water to obtain a final concentration of approximately 250 μM TCE. TCE concentrations were calculated on the basis of the amount of liquid in the vial, without considering the partitioning of TCE into the vapor phase. The vial was incubated at 30°C on a rotary shaker at 250 rpm. One hundred microliters of the gas phase sample was removed from the vial periodically, and injected to a Shimadzu gas chromatograph 9A (Shimadzu Co., Kyoto) equipped with a flame ionization detector and fitted with a Silicone DC-550 packed column (GL Sciences Inc., Tokyo) operated at 100°C. The values of the TCE concentrations plotted in the figures are the means of duplicate measurements. At least two sets of the experiments were conducted to check the reproducibility of the data.

When *Ralstonia* sp. KN1-LX was used for the TCE degradation experiment, the strain was cultivated, harvested, washed, and suspended in MMSB as described above. Then phenol was added to the suspension at a concentration of 2 mM. The suspension was cultivated for 5 h, and then harvested, washed, and suspended into MMSB (OD_{600}=1.0). The prepared cell suspension was used for the TCE degradation experiment as explained above. *Ralstonia* sp. KN1-ΔPH (pNIN103) and *Ralstonia* sp. KN1-ΔPH (pNIN104) were cultivated in L broth supplemented with C_m. When the OD_{600} reached 0.6, IPTG was added to a final concentration of 5 mM and cell cultivation was continued for 2 h. Cells were harvested, washed, and suspended in MMSB (OD_{600}=1.0) as previously described.

Light emission measurement *Ralstonia* sp. KN1-10A-LX was cultivated at an inoculum concentration of 1% (v/v) of a one-day-old culture in L broth medium with shaking for 17 h at 30°C. A one percent volume of the culture was inoculated into fresh L broth medium, and luminescence was measured periodically as described below. *Ralstonia* sp. KN1-LX was grown for 17 h in the same manner. Cells were then harvested by centrifugation at 8000×*g* at 4°C, and the resultant pellet was suspended in MMSB. This procedure was repeated, and finally the cells were suspended in MMSB at OD_{600}=1.0. Then 250 μM phenol was added to the culture, and luminescence was measured at predetermined periods.

Luminescence was measured using a TD-20e luminometer (Turner Designs, Sunnyvale, CA, USA). A bacterial sample of 20 μl was diluted to 1 ml with L broth medium and incubated at room temperature for 5 min as recommended elsewhere (26). Ten microliters of 10% *n*-decyl aldehyde (in ethanol) was placed in a 8×50 mm test tube in the luminometer, and 200 μl of the incubated sample was injected into the test tube. The delay time and integrate time were set at 5 and 10 s, respectively. Light emission was expressed as light unit (LU) per cell concentration (OD_{600}). Phenol concentrations were measured using a Shimadzu liquid chromatograph 6A (Shimadzu Co., Kyoto) fitted with YMC-Pack ODS-A (YMC Co., Kyoto). Light emission and phenol concentration values plotted in the figures are the means of duplicate measurements. Three sets of the experiments were conducted to check the reproducibility of the data.

Nucleotide sequence accession number The nucleotide sequence reported here has been submitted to the DDBJ, EMBL and GenBank, and assigned the accession number, AB031995.

RESULTS

Sequence analysis of phenol hydroxylase The nucleotide sequence of the 8.7-kb fragment encoding the phenol hydroxylase genes was determined. Computer analysis identified eight complete open reading frames (ORFs) which were designated *phyZABCDEFG* (Fig. 1). The initial codon of each ORF was predicted to have a putative ribosome-binding site, suggesting that translation of these regions was possible. A similar arrangement has been observed in other multicomponent phenol hydroxylases such as the *dmp* operon from *Pseudomonas* sp. CF600 (27) and the *pox* operon from *R. eutropha* E2 (28). A partial ORF upstream of *phyZ* was identified as a part of the regulatory gene (*phyR*) based on a comparison to similar phenol hydroxylase genes mentioned above. Including the regulatory gene, the gene organization of *phyRZABCDEFG* was identical to

TABLE 2. Comparisons of proteins with similarities to the products of *phy* ORFs

Products of *phy* ORFs	No. of a.a. residues	Expected function	Identity/similarity (%)
PhyZ	72	Phenol hydroxylase component	DmpK (34/49), PoxA(77/86)
PhyA	331	Phenol hydroxylase component	DmpL (53/66), PoxB(85/90)
PhyB	90	Phenol hydroxylase component	DmpM(52/72), PoxC(88/94)
PhyC	504	Phenol hydroxylase component	DmpN(67/82), PoxD(95/97)
PhyD	119	Phenol hydroxylase component	DmpO(43/62), PoxE(85/89)
PhyE	355	Phenol hydroxylase component	DmpP (58/68), PoxF(85/91)
PhyF	115	Ferredoxin-like protein	DmpQ(47/64), PoxG(84/87)
PhyG	310	Catechol 2,3-dioxygenase	DmpB (58/69), —

dmpRKLMNOPQ although the direction of transcription of *dmpR* was opposite. Moreover, the organization and the direction of transcription of *poxRABCDEFG* were identical to those of *phyRZABCDEF*. The products of the *phy* ORFs were compared to the *dmp* and *pox* operon products (27, 28) as shown in Table 2. PhyZABCDEFG was found to exhibit some homology to DmpKLMNOPQB, with 34–58% identity of the amino acids, depending on the gene. Significant homology was found between PhyZABCDEF and PoxABCDEFG, with 77–95% identity.

Expression analysis In the previous report (17), it was shown that the rate of TCE degradation by KN1-10A was lower than that by the phenol-induced wild-type strain, KN1. In order to understand the difference in TCE degradation ability between these two strains in detail, two new strains, KN1-LX and KN1-10A-LX, were constructed. These strains contained *luxAB* from *V. harveyi* B392 (24) integrated to the *Eco*T22I site (nt 7124) in *phyF* (Fig. 1). Thus, the integrated reporter genes, *luxAB*, allowed us to investigate the relationship between the TCE degradation rate and *phy* gene expression led either by phenol induction or by *Ptac*, directly. The depletion of phenol and the corresponding light emission (per optical density at 600 nm) change are shown in Fig. 3. Phenol degradation became apparent after 2 h of cultivation, and was completed (<0.1 μM) 7 h later. The light emission started to increase immediately after the phenol addition and continued to increase until 6 h, with the maximum value of 5100 LU/OD_{600}. The light emission decreased rapidly when phenol was completely oxidized at 7 h. Thus, the phenol-induced promoter promptly responded to the presence of phenol. Figure 4 shows the growth curve of KN1-10A-LX and the corresponding light emission. The light emission decreased just after the inoculation, and then continued to increase with cell density during 21 h of cultivation, indicating constitutive expression of *Ptac*. The final value of light emission was 4700 LU/OD_{600}, which was almost equal to the level detected with the phenol-induced KN1-LX. The results showed that the phenol-induced promoter gave a slightly more rapid production of LuxAB than *Ptac*, although the maximum light emission was approximately equal under the given conditions.

TCE degradation by KN1-LX and KN1-10A-LX TCE degradation experiments were conducted using strains KN1-LX and KN1-10A-LX. Cells of KN1-LX were harvested after 5 h of phenol induction, at which time the light emission reached 5000 LU/OD_{600}. A culture of KN1-10A-LX was prepared after 17 h of cultivation, with a light emission of 3900 LU/OD_{600}. The time courses of TCE degradation by these strains are shown in Fig. 5. Additionally the TCE degradation by KN1-10A was studied to examine the effect of *luxAB* integration on TCE degradation ability. There was no difference in TCE degradation ability between KN-10A and KN1-10A-LX. Thus, the integration of *luxAB* did not have any negative effect on TCE degradation. The time courses of TCE degradation by KN1-LX and KN1-10A-LX were completely different from each other. The phenol-induced KN1-LX rapidly decomposed TCE, and the TCE was almost completely oxidized in 24 h. On the other hand, KN1-10A-LX oxidized TCE slowly and it took almost 100 h for complete degradation. Furthermore, the initial TCE degradation rate by KN1-10A-LX was relatively slow. Thus, KN1-10A-LX decomposed only 20% of the TCE in the first 24 h, while KN1-LX oxidized the entire amount of TCE during the same period of time. As described above, there was no significant difference in the level of light emission between the two strains; however, a remarkable difference was observed

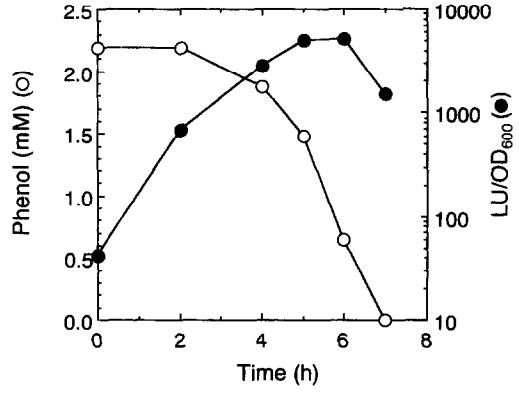

FIG. 3. Change of light emission (●) of KN1-LX induced by phenol (○). Cells were cultivated and harvested as described in Materials and Methods, and suspended in MMSB (OD_{600}=1.0).

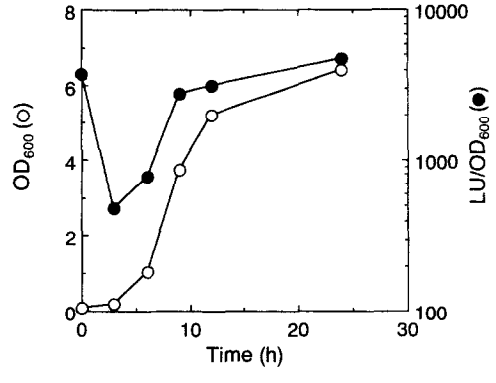

FIG. 4. Change of light emission (●) of KN1-10A-LX with growth shown by OD_{600} (○). Cells were prepared as described in Materials and Methods.

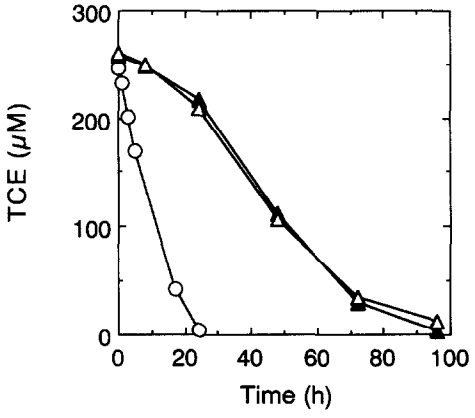

FIG. 5. Degradation of TCE by strains KN1-LX (○), KN1-10A-LX (△), and KN1-10A (▲). Cells were cultivated and harvested as described in Materials and Methods, and suspended in MMSB (OD_{600} =1.0). The TCE concentrations were calculated on the basis of the amount of liquid in the vial, without considering the partitioning of TCE into the vapor phase.

in the TCE degradation rates. Therefore, some factors other than the promoter is responsible for the low TCE degradation rate of KN1-10A-LX.

Effect of *phyZ* on TCE degradation It was already known from the sequence analysis that *phyZ* was destroyed when KN1-10A was constructed because of the integration of the Tc^r gene and *Ptac* into the middle of *phyZ*. It was considered that the observed low TCE degradation rate of KN1-10A-LX was due to the absence of *phyZ* transcription. In order to investigate the importance of *phyZ*, two vectors were constructed. One was a *phyZ*-lacking vector, pNIN103, and the other a *phyZ*-containing vector, pNIN104. The time courses of TCE degradation by KN1-ΔPH(pNIN103) and KN1-ΔPH(pNIN104) were obtained as shown in Fig. 6. A difference in TCE degradation ability is apparent between these two strains. Although KN1-ΔPH (pNIN103) decomposed only 47% of the TCE in 67 h, KN1-ΔPH (pNIN104) oxidized approximately twice the amount of TCE in the same period of time. In addition, the initial TCE degradation rate by KN1-ΔPH (pNIN103) was extremely slow compared to that by KN1-ΔPH (pNIN104).

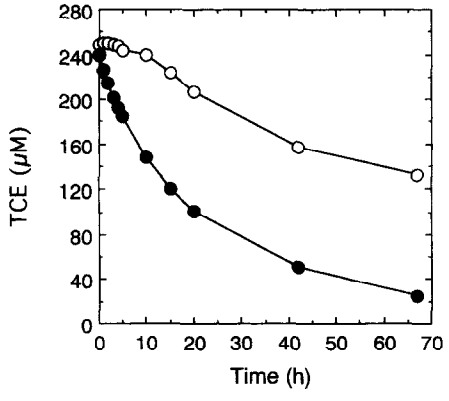

FIG. 6. Degradation of TCE by KN1-ΔPH(pNIN103) (○) and KN1-ΔPH(pNIN104) (●). Cells were cultivated and harvested as described in Materials and Methods, and suspended in MMSB (OD_{600} =1.0). The TCE concentrations were calculated on the basis of the amount of liquid in the vial, without considering the partitioning of TCE into the vapor phase.

Accordingly, KN1-ΔPH(pNIN103) degraded only 4.0% of the TCE in the first 10 h, while KN1-ΔPH(pNIN104) decomposed approximately 10 times the amount of TCE during the same period of time. These results suggest that *phyZ* does not only accelerate TCE degradation but also minimizes the lag period of TCE oxidation.

DISCUSSION

TCE degradation mediated by the phenol hydroxylase from *Ralstonia* sp. KN1 was intensively analyzed using strain KN1 derivatives. When KN1-10A was constructed (17), the tetracycline resistance (Tc^r) gene and *Ptac* were integrated into the *StuI* site (nt 2629) as shown in Fig. 1, rendering *phyZ* nonfunctional. However, since TCE was still degraded by KN1-10A, it was determined that *phyZ* is not essential for TCE oxidation. We already knew from a previous study (11) that the regions encoding *phyA* and *phyE* were required for TCE degradation. Accordingly, the region encoding *phyABCDE* was found to be necessary for TCE degradation.

Ralstonia eutropha JMP134 (10, 29, 30) is a well-analyzed TCE degrader which, among the reported TCE-degrading strains, is the one phylogenetically closest to KN1. Comparison of the sequence data of the KN1 genes to those of the JMP134 *phl* genes (GenBank accession no. AF065891) showed that the gene organization and amino acid sequences in the two were quite different, although both are multicomponent phenol hydroxylases like the *dmp*-encoded phenol hydroxylase from *Pseudomonas* sp. CF600 (27). The deduced amino acid sequence of *phyC*, which is homologous to *dmpN*, having putative ligands for the binuclear iron center (31), revealed only 25% identity and 41% similarity with that of the homologous *phlK*. The genes of *phyZABCDE* were more closely related to *dmpKLMNOP* and particularly to *poxABCDEF* from *R. eutropha* E2 as shown in Table 2, although TCE degradation by these phenol hydroxylases has not been reported. Furthermore, no putative transport facilitator gene similar to *phlX* (29) of JMP134 was found in the sequenced region.

The importance of the small gene, *phyZ*, possibly encoding a 72-amino-acid protein, was indicated by the marked difference in TCE degradation ability between KN1-LX and KN1-10A-LX (Fig. 5). This phenomenon was confirmed by the comparison between TCE oxidation using the *phyZ*-lacking plasmid pNIN103 and the *phyZ*-containing plasmid pNIN104 (Fig. 6). The *phyZ* gene accelerated TCE degradation in two ways. One is the decrease of the lag period in TCE degradation, and the other the general increase in TCE degradation rate. Thus, strain KN1-ΔPH (pNIN104) exhibited a constant degradation rate 9.9 μM/h, and degraded 10 times the amount of TCE as compared to KN1-ΔPH (pNIN103) in the first 10 h (Fig. 6). Although KN1-ΔPH (pNIN103) showed its maximum rate (3.3 μM/h) after 10 h of incubation, this value was still only one-third of that of KN1-ΔPH (pNIN104). The significance of *dmpK* (homologous to *phyZ*) in phenol degradation was previously reported (27, 32). Initially, DmpK was found to be required for growth on a medium with phenol but not for *in vitro* phenol hydroxylase activity, suggesting a role in phenol transport or hydroxylase regulation (25). More recent analysis of DmpK showed that it plays a role in post-translational incorporation of iron into the oxygenase component of the phenol hydroxylase to produce

an active form (32). These analyses suggest that *phyZ* may play the same kind of role in TCE degradation. The observed lag period in the TCE degradation by KN1-ΔPH (pNIN103) may be due to the production of an inactive form of hydroxylase. The subsequent slow TCE degradation may be due to the gradual activation of the produced inactive enzyme by inefficient incorporation of co-existing iron without PhyZ.

The expression analysis carried out using *luxAB* as a reporter showed that *Ptac* integrated into the chromosome was constitutively expressed as shown in Fig. 4. When cultivation was started, light emission (per unit cell, expressed as LU/OD_{600}) decreased rapidly. However, light emission began to increase with detectable cell growth, and continued to increase until the steady state, in 21 h. This result suggests that *Ptac* is a suitable promoter to obtain stable and strong expression of a gene. As shown in Fig. 3, the native phenol-induced promoter terminates production of the enzyme immediately after the exhaustion of phenol. Therefore, when the native phenol-induced strain is applied to bioaugmentation for TCE decontamination, phenol should be added just before augmentation in order to obtain a high level of TCE degradation activity. In addition, phenol should be completely degraded because it is a harmful substrate. On the other hand, when *Ptac* is used for the expression, the timing of cell harvest is more flexible because of the long and high-level expression as shown in Fig. 4. In addition, a safe carbon source such as ethanol or lactate, which can support the growth of KN1 derivatives, can be used for cultivation. Thus, chromosomally integrated *Ptac* is very attractive from the viewpoint of practical application. Our previous results showed that a plasmid carrying *Ptrc*, which is functionally equal to *Ptac*, was not stably maintained in wild-type strain, KN1, without the pressure of antibiotics (17). Therefore, a strong constitutive promoter should be present in the chromosome as described here.

From the above-described results, it is evident that higher TCE degradation ability can be obtained if *Ptac* is integrated upstream of *phyZ*. Therefore, future studies will focus on the development of such a useful strain by homologous recombination.

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