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Group-Specific Monitoring of Phenol Hydroxylase Genes for a Functional Assessment of Phenol-Stimulated Trichloroethylene Bioremediation

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The sequences of the largest subunit of bacterial multicomponent phenol hydroxylases (LmPHs) were compared. It was found that LmPHs formed three phylogenetic groups, I, II, and III, corresponding to three previously reported kinetic groups, low-K_s (the half-saturation constant in Haldane's equation for trichloroethylene [TCE]), moderate-K_s, and high-K_s groups. Consensus sequences and specific amino acid residues for each group of LmPH were found, which facilitated the design of universal and group-specific PCR primers. PCR-mediated approaches using these primers were applied to analyze phenol/TCE-degrading populations in TCE-contaminated aquifer soil. It was found that the aquifer soil harbored diverse genotypes of LmPH, and the group-specific primers successfully amplified LmPH fragments affiliated with each of the three groups. Analyses of phenol-degrading bacteria isolated from the aquifer soil confirmed the correlation between genotype and phenotype. Competitive PCR assays were used to quantify LmPHs belonging to each group during the enrichment of phenol/TCE-degrading bacteria from the aquifer soil. We found that an enrichment culture established by batch phenol feeding expressed low TCE-degrading activity at a TCE concentration relevant to the contaminated aquifer (e.g., 0.5 mg liter^{-1}); group II and III LmPHs were predominant in this batch enrichment. In contrast, group I LmPHs overgrew an enrichment culture when phenol was fed continuously. This enrichment expressed unexpectedly high TCE-degrading activity that was comparable to the activity expressed by a pure culture of *Methylosinus trichosporium* OB3b. These results demonstrate the utility of the group-specific monitoring of LmPH genes in phenol-stimulated TCE bioremediation. It is also suggested that phenol biostimulation could become a powerful TCE bioremediation strategy when bacteria possessing group I LmPHs are selectively stimulated.

In the last two decades, microbial ecology has developed molecular approaches, especially that known as the rRNA phylogenetic framework, in order to analyze microbial populations in the environment without cultivation (1, 22, 23). Molecular approaches have expanded our knowledge of the diversity and distribution of microbial populations in the environment. Genes coding for catabolic enzymes such as methane monooxygenase (12, 13, 18), ammonia monooxygenase (23, 31), catechol dioxygenase (21), and phenol hydroxylase (38) have also been retrieved from the environment in order to gain insight into the genetic diversity of catabolic populations. It is currently expected that such genetic information could aid in understanding and advancing bioremediation (34, 40, 41).

Contamination of the subsurface environment with chlorinated hydrocarbons, in particular trichloroethylene (TCE) and perchloroethylene, is a potentially serious threat to drinking-water sources. A number of laboratory studies have demonstrated that aliphatic and aromatic hydrocarbon-degrading bacteria, such as methane-, toluene- and phenol-degrading bacteria, cometabolically transform these compounds to readily degradable oxygenated compounds (6, 29). In addition, field trials in which these bacteria were used for TCE bioremediation have been reported (16, 28). We are currently studying phenol-degrading bacteria with the aim of developing efficient TCE bioremediation strategies. It has been found that the kinetics for TCE degradation exhibited by phenol-degrading bacteria are diverse and can be classified into three distinct kinetic groups, low-K_s (the half-saturation constant in Haldane's equation for TCE), moderate-K_s, and high-K_s groups (9). Laboratory axenic culture experiments have suggested that only low-K_s bacteria are capable of efficient TCE degradation at a concentration relevant to contaminated groundwater (9).

It is desirable for phenol-stimulated TCE bioremediation (phenol biostimulation) to develop rapid methods for specifically detecting and quantifying the three groups of phenol-degrading bacteria in the environment. Such a technique would provide useful information for predicting the TCE degradation potential of indigenous bacterial populations, developing effective phenol biostimulation schemes, and evaluating results of enforced phenol biostimulation. For this purpose, this study analyzed genes for the largest subunit of multicomponent phenol hydroxylases (LmPHs) and designed group-specific PCR primers for LmPHs. The utility of PCR approaches with these primers was evaluated by analyzing TCE-contaminated aquifer soil.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. The phenol-degrading bacteria used in this study were *Burkholderia cepacia* E1 (37), *Comamonas* sp. strain E6 (37), *Comamonas testosteroni* R2 (37) and R5 (37), *Pseudomonas* sp. strain WAS2

TABLE 1. PCR primers used for amplifying the LmPH fragments

Primer[a]	Nucleotide sequence[b]	Positions[c]	Specificity
phe1f	GA(G/A)GGCATCAA(A/G)AT(C/T)	121–134	Universal
phe2f	CC(C/T/G)TTCATGTC(C/G)GG(T/A/C)GC	628–644	Universal
phe3r	CAG(C/G)CG(A/G)T(A/T)ACC(G/T)CGCCAGAACC	797–819	Universal
phe4r	AT(C/T)TG(G/A)TGCAC(C/A)GGCA(G/A)CC	1355–1373	Universal
pheUf	CCAGG(C/G)(C/G/T)GA(G/A)AA(A/G)GAGA(A/G)GAA(G/A)CT	195–218	Universal
pheUr	CGG(A/T)A(G/A)CCGCGCCAGAACCA	796–815	Universal
pheLr	GG(A/G/C)A(G/T/C)(A/G)TTG(C/T)CCGGGTC	757–774	Group I
pheMHr	GAT(T/C/G)GGCAC(A/G)TTGTCTTC	760–777	Groups II and III
pheHr	GTGGCCATGTCGCCATTGA	660–668	Group III

[a] f, forward primer; r, reverse primer.
[b] Degenerate nucleotides are indicated in parentheses.
[c] Corresponding to the *dmpN* sequence of *Pseudomonas* sp. strain CF600.

(37), *Pseudomonas putida* P-2 (8), *P. putida* P-5 (8), *P. putida* P-6 (8), *P. putida* P-8 (9), and *P. putida* P-10 (9). These bacteria were grown at 25°C in BSM medium (9) supplemented with phenol at 2.0 mM.

Soil sample. A soil sample was obtained from a TCE-contaminated sandy aquifer at a depth between 2.0 and 2.5 m (Kururi, Chiba, Japan), where TCE was detected at between 100 and 500 μg liter^{-1}. One gram of the wet soil was suspended in 10 ml of potassium phosphate buffer (10 mM, pH 7.0), and after vortexing and gently sonicating, it was appropriately diluted with the buffer. The total direct count (TDC) of microbial cells in the suspension was estimated by fluorescence microscopy after staining microorganisms with 4′,6′-diamidino-2-phenylindole (DAPI) (39).

DNA extraction. DNA was extracted from 5 g (wet weight) of the aquifer soil by the method described by Zhou et al. (42) with some modifications. Three cycles of the freeze-thaw treatment (33) were performed after the initial sodium dodecyl sulfate lysis step. Final DNA purification was conducted with a Suprec-2 column (Takara Shuzo). DNA was extracted from each bacterial culture by the method described previously (39). The quality and quantity of the extracted DNA were checked by measuring the UV absorption spectrum (27).

PCR conditions. Repetitive extragenic palindromic sequence PCR (rep-PCR) was conducted by using the primers REP1R-I and REP2-I (3) as described previously (38).

DNA fragments of 16S rRNA genes (16S rDNA) were amplified by using primers 5′-AGAGTTTGATCCTGGCTCAG-3′ (corresponding to *Escherichia coli* 16S rDNA positions 8 to 27 [2]) and 5′-AAGGAGGTGATCCAGCC-3′ (corresponding to *E. coli* 16S rDNA positions 1525 to 1542). Amplification was performed with a Progene thermal cycler (Techne) by using a 50-μl mixture containing 1.25 U of *Taq* DNA polymerase (Amplitaq Gold; Applied Biosystems), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, each deoxynucleoside triphosphate at a concentration of 200 μM, 100 pmol of each primer, and 50 ng of template DNA. The PCR conditions were 10 min for activating the polymerase at 94°C and then 35 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C, and finally 10 min of extension at 72°C.

DNA fragments coding for the largest subunit of multicomponent phenol hydroxylases (LmPH) were amplified by using the Progene thermal cycler and 50-μl mixtures just described. The primers used are listed in Table 1. The PCR conditions for the two primer sets phe1f and phe3r (hereafter described as phe1f/phe3r) and phe2f/phe4r, were as follows: step 1, 10 min of activation at 94°C; step 2, 35 cycles consisting of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C; step 3, 10 min of extension at 72°C. The PCR conditions used for the three primer sets pheUf/pheUr, pheUf/pheMHr, and pheUf/pheHr were as follows: step 1, 10 min of activation at 94°C; step 2, five cycles consisting of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C; step 3, five cycles consisting of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C; step 4, 25 cycles consisting of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C; step 5, 10 min of extension at 72°C. The PCR conditions used for primer set pheUf/pheLr were as follows: step 1, 10 min of activation at 94°C; step 2, five cycles consisting of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; step 3, five cycles consisting of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C; step 4, 25 cycles consisting of 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C; step 5, 10 min of extension at 72°C. The PCR products were checked by electrophoresis through 1.5% (wt/vol) agarose gel (LO3 agarose; Takara Shuzo) in TBE buffer (27) and then staining with ethidium bromide.

Sequence analysis. The PCR products were ligated into pUC18 (27) and cloned into *E. coli* JM109 by using a Sure clone ligation kit (Amersham Pharmacia Biotech.). Plasmids were purified from the JM109 colonies by the standard miniprep procedure (27) and used as templates for nucleotide sequencing. The nucleotide sequences were determined by using a DNA sequencing kit (Dye Terminator Cycle Sequencer; Applied Biosystems), an appropriate PCR primer (1 pmol), and a model 377 DNA sequencer (Applied Biosystems). The primers used for sequencing 16S rDNA fragments have been described by Edwards et al. (4). The GenBank database search was conducted with the Blast program. The sequences were aligned by using ClustalW version 1.7 (36), and the alignment was refined by visual inspection. A neighbor-joining tree (26) was constructed by using the njplot software in ClustalW version 1.7.

Competitive PCR. Competitor fragments were produced by using a competitive DNA construction kit (Takara Shuzo). The sizes of the competitor fragments were 444 bp for pheUf/pheUr, 441 bp for pheUf/pheLr, 442 bp for pheUf/pheMHr, and 343 bp for pheUf/pheHr. The PCR conditions were as described above except for the addition of an appropriate amount of the competitor fragment. The PCR products were separated by electrophoresis through 2.0% (wt/vol) agarose gel, and stained with ethidium bromide. The band intensity was quantified by using image-processing software (NIH Image, version 1.60; National Institutes of Health), and the copy number of a target sequence in the PCR mixture was determined by comparing band intensities.

Isolation of bacteria. (i) Direct plating. The diluted soil suspensions described above were spread on agar plates containing 1/10th-strength TSB medium (Difco) supplemented with phenol at 2.0 mM (1/10TSB200 plate). After the plates had been incubated at 25°C for 14 days, all the colonies that appeared on one plate were picked and purified by restreaking.

(ii) Chemostat enrichment. Eight hundred milliliters of an inorganic medium (MP medium [37]) in a TBR-2 fermentor (2-liter capacity; Sakura Fine Technical) was supplemented with phenol at 0.5 mM and inoculated with 10 g (wet) of the aquifer soil. The vessel was agitated at 150 rpm for 24 h at 25°C, and MP medium containing 1,500 mg of phenol liter^{-1} (16 mM) was then continuously supplied at a rate of 670 ml day^{-1}. Air was supplied at a rate of 2 liters min^{-1}. The culture volume was maintained at 1.5 liters, and the temperature was kept at 25°C. Eight days after commencing the cultivation, when the culture parameters (phenol concentration, optical density at 660 nm [OD_{660}], and dissolved oxygen concentration [DO]) had become stable, a small portion of the culture was sampled. This was then appropriately diluted and streaked onto agar plates containing MP medium supplemented with phenol at 0.5 mM. The plates were incubated at 25°C for 14 days, and all the colonies on one plate were picked and purified.

TCE-degrading activity. This study employed the pseudo-first-order degradation rate constant k_1 (32) to describe the TCE-degrading activity according to previous studies (9, 10, 30, 32). The k_1 value was determined by the method described previously (9) at a TCE concentration of 0.5 mg liter^{-1}, since this is the typical TCE concentration in a contaminated aquifer (10, 17).

Enrichment of phenol-degrading bacteria from aquifer soil. (i) Batch phenol feeding. One liter of BSM medium in a TBR-2 fermentor was inoculated with the aquifer soil (20 g wet), and phenol was then added at 0.2 mM. The vessel was agitated at 150 rpm and 25°C. Air was supplied at 1.5 liters min^{-1}. When the OD_{660} had stopped increasing, a small portion of the culture was sampled. TDC of the culture was determined by the epifluorescence microscopy method after the cells had been stained with DAPI.

(ii) Continuous phenol feeding. After the sampling, BSM medium containing 1,500 mg of phenol liter^{-1} (16 mM) was continuously supplied to the batch-fed culture at a rate of 500 ml day^{-1}, and the culture volume was maintained at 1.0 liter. The phenol concentration was measured by high-performance liquid chromatography as described previously (9). The detection limit was 2.5 μg liter^{-1} (approximately 26 nM). After culture parameters (OD_{660} and DO) had become

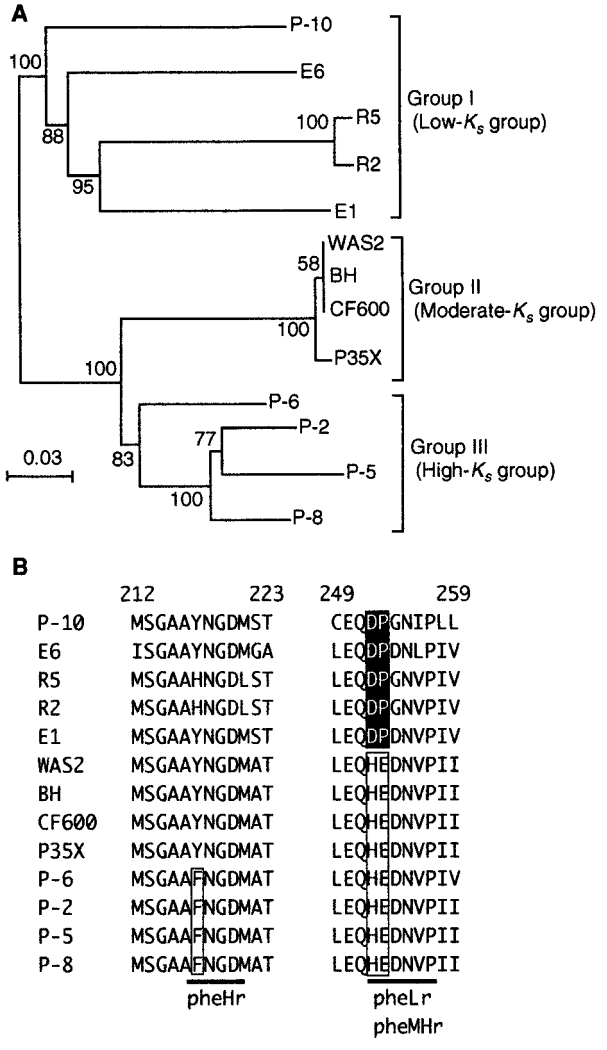

FIG. 1. Sequence analyses of LmPHs used for designing the group-specific PCR primers. (A) An unrooted neighbor-joining tree based on the nucleotide sequences of LmPHs, showing the phylogenetic relationship among 13 phenol-degrading bacteria. Sequences corresponding to nucleotide positions 121 to 1373 of the *dmp* sequence (20) were used for calculation. Nucleotide positions at which any sequence had a gap were not included in the calculations. Numbers at the branch nodes are bootstrap values (per 100 trials); only values greater than 50 are indicated. The bar represents 0.03 substitution per site. (B) Signature amino acids for the three groups of LmPH. Regions used for designing the group-specific degenerate primers are underlined. Numbers above the sequence correspond to the numbering in the DmpN sequence (20).

stable and the phenol concentration dropped below the detection limit, a small portion of the culture was sampled.

Statistics. Data were statistically analyzed by the Student *t* test. A value of $P = 0.05$ was considered significant.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been deposited in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases under accession numbers AB051680 to AB051754.

RESULTS AND DISCUSSION

Design of PCR primers. In phenol-degrading bacteria, phenol hydroxylase (2-monooxygenase) catalyzes the cometabolic transformation of TCE (6). Two types of phenol hydroxylase are known, single-component and multicomponent enzymes (11); among them, multicomponent enzymes are considered the major ones in the environment (24, 38). The catalytic domain of multicomponent phenol hydroxylase has been found to exist within LmPH, as exemplified by DmpN of *Pseudomonas* sp. strain CF600 (7, 15). We thus compared the amino acid sequences of LmPHs of six previously cloned phenol hydroxylases, DmpN (20), PhhN from *P. putida* P35X (19), PhlD from *P. putida* H (14), PheA4 from *P. putida* BH (35), PoxD from *Ralstonia eutropha* E2 (15), and MopN of *Acinetobacter calcoaceticus* NCIB8250 (5). We identified consensus amino acid sequences which were used to design the degenerate PCR primers phe1f, phe2f, phe3r, and phe4r (Table 1). These primers enabled the LmPH fragments of strains E1, E6, R2, R5, WAS2, P-2, P-5, P-6, P-8, and P-10 to be amplified and sequenced, although fragments with improper sizes were also amplified. Figure 1A shows the phylogenetic relationship among LmPHs of the 13 phenol-degrading bacteria that were used in our previous study (9). It was found that LmPHs formed three groups (I, II, and III), corresponding to the three kinetic groups identified in our previous study (9). Group I comprised only LmPHs of the low-K_s-type bacteria, group II comprised only LmPHs of moderate-K_s-type bacteria, while group III comprised only LmPHs of high-K_s-type bacteria.

By comparing the deduced amino acid sequences of these 13 LmPHs, we found specific amino acid residues for each of the three LmPH groups (Fig. 1B), which were then used to design group-specific PCR primers (Table 1). The universal PCR primers pheUf and pheUr for all LmPH genes were also designed (Table 1). LmPH fragments could be amplified by using pheUf/pheUr from all 13 of the phenol-degrading bacteria, while the combination of the group-specific primers with pheUf allowed the specific amplification of each group of LmPH (Fig. 2, for example).

Diversity of LmPH in TCE-contaminated aquifer. The four sets of primers were used to analyze LmPHs in TCE-contaminated aquifer soil that had no history of exposure to aromatic compounds, including phenol. The PCR primers successfully amplified LmPH fragments of the expected sizes from DNA

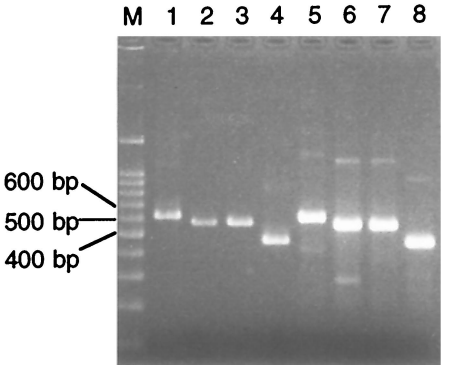

FIG. 2. PCR amplification of LmPH fragments from bacterial strains and the aquifer soil. Lane M, DNA size markers (100-bp DNA ladder from 100 to 1,500 bp; Takara Shuzo); lanes 1 to 4, soil DNA; lanes 5 and 7, *Pseudomonas* sp. strain CF600; lane 6, *C. testosteroni* R5; lane 8, *P. putida* P-2. The PCR primers used were pheUf/pheUr (lanes 1 and 5), pheUf/pheLr (lanes 2 and 6), pheUf/pheMHr (lanes 3 and 7), and pheUf/pheHr (lanes 4 and 8).

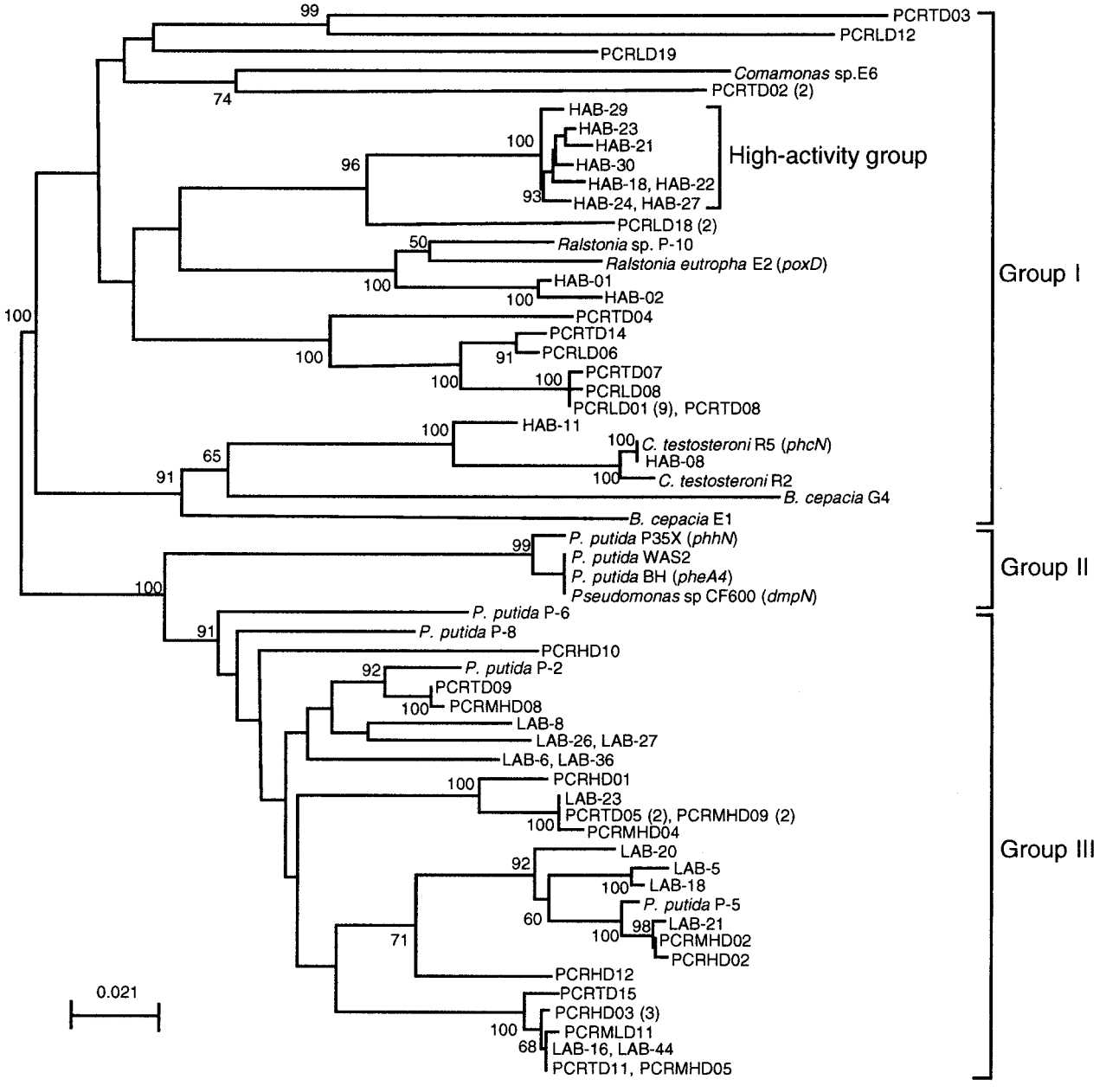

FIG. 3. Unrooted neighbor-joining tree based on the nucleotide sequences of LmPHs, showing the phylogenetic relationship among phenol-degrading bacteria isolated from the aquifer soil (HAB and LAB strains), LmPH fragments amplified directly from the aquifer soil DNA (PCRTD amplified by using pheUf/pheUr, PCRLD amplified by using pheUf/pheLr, PCRMHD amplified by using pheUf/pheMHr, and PCRHD amplified by using pheUf/pheHr) and known phenol-degrading bacteria. Sequences corresponding to nucleotide positions 195 to 668 of the *dmp* sequence (20) were used for calculation. Nucleotide positions at which any sequence had a gap were not included in the calculations. Numbers at the branch nodes are bootstrap values (per 100 trials); only values greater than 50 are indicated. Numbers in parentheses indicate the number of identical sequence types. The bar represents 0.021 substitution per site.

extracted from the soil (Fig. 2). The nucleotide sequences of 41 LmPH fragments were then determined, and 24 different sequence types were obtained (Fig. 3). This figure shows that LmPH fragments amplified by using pheUf/pheUr were distributed in groups I and III; among them, LmPHs in group I were very diverse. All LmPHs amplified by using pheUf/pheLr were affiliated with group I, while all LmPHs amplified by using pheUf/pheMHr or pheUf/pheHr were affiliated with group III.

Bacteria were isolated in parallel from the aquifer soil by direct plating or plating after enrichment in a chemostat culture. Among the 84 colonies isolated by direct plating, LmPH fragments were amplified by using pheUf/pheUr from 12 strains (the LAB strains in Table 2). Most of the remaining 72 strains are considered not to be phenol-degrading bacteria, since none of 20 strains randomly selected from these 72 strains could grow on phenol (data not shown). Among the 28 strains isolated after chemostat enrichment, 12 strains were

TABLE 2. Characteristics of isolated strains

Isolate	Closely related organism based on 16S rRNA gene sequence (% identity)	Rep-PCR pattern no.[a]	Group-specific PCR[b] with primers:				Mean k_1 ± SD (liter g^{-1} h^{-1})[c]
			Universal	Group I	Group II	Group III	
HAB-01	*Ralstonia* sp. strain BKME-6 (99)	1	+	+	−	−	18 ± 0.5
HAB-02	*Ralstonia* sp. strain BKME-6 (99)	2	+	+	−	−	18 ± 0.5
HAB-08	*Ralstonia eutropha* KT-1 (99)	3	+	+	−	−	NA
HAB-11	*Ralstonia eutropha* KT-1 (99)	4	+	+	−	−	22 ± 3.5
HAB-18	*Ralstonia eutropha* KT-1 (99)	5	+	+	−	−	32 ± 2.7
HAB-21	*Variovorax* sp. strain WFF52 (99)	6	+	+	−	−	24 ± 0.8
HAB-22	*Variovorax* sp. strain WFF52 (99)	7	+	+	−	−	ND
HAB-23	*Variovorax* sp. strain WFF52 (99)	7	+	+	−	−	NA
HAB-24	*Variovorax* sp. strain WFF52 (99)	8	+	+	−	−	98 ± 0.3
HAB-27	*Variovorax* sp. strain WFF52 (99)	9	+	+	−	−	99 ± 5.8
HAB-29	*Variovorax* sp. strain WFF52 (99)	9	+	+	−	−	91 ± 4.6
HAB-30	*Variovorax* sp. strain WFF52 (99)	10	+	+	−	−	200 ± 6.7
LAB-05	*Pseudomonas* sp. strain IpA-2 (99)	11	+	−	+	+	NA
LAB-06	*Pseudomonas* sp. strain BKME-9 (98)	12	+	−	+	+	1.7 ± 0.2[d]
LAB-08	*Pseudomonas* sp. strain DhA-51 (99)	13	+	−	+	+	NA
LAB-16	*Pseudomonas* sp. strain PsF (99)	14	+	−	+	+	NA
LAB-18	*Pseudomonas* sp. strain DhA-51 (99)	15	+	−	−	+	1.4 ± 0.04[d]
LAB-20	*Pseudomonas* sp. strain IpA-2 (99)	16	+	−	+	+	0.3 ± 0.2
LAB-21	*Pseudomonas putida* ATCC 17484 (99)	11	+	−	+	+	1.3 ± 0.04[d]
LAB-23	*Pseudomonas* sp. strain PsF (99)	17	+	−	+	+	1.3 ± 0.18
LAB-26	*Pseudomonas jessenii* (99)	18	+	−	+	+	1.0 ± 0.05
LAB-27	*Pseudomonas* sp. strain DhA-51 (99)	19	+	−	+	+	ND
LAB-36	*Pseudomonas agarici* (97)	20	+	−	+	+	0.3 ± 0.1
LAB-44	*Pseudomonas rhodesiae* (100)	21	+	−	+	+	1.4 ± 0.06[d]

[a] Identical numbers indicate identical rep-PCR patterns.
[b] +, fragment of the expected size amplified; −, fragment of the expected size not amplified.
[c] The values were determined using cells grown in chemostat cultures as described previously (8); data are means for groups of three. NA, not assayed; ND, not detected.
[d] Value determined using cells grown in batch culture and harvested at the late exponential growth phase.

positive in PCR by using pheUf/pheUr (the HAB strains in Table 2). The pheUf/pheUr PCR-positive strains could grow on phenol as the sole carbon source, except for HAB-22 and LAB-27.

The characteristics of the LAB and HAB strains are summarized in Table 2. Judging from the 16S rRNA and LmPH gene sequences and rep-PCR patterns, it was concluded that none of these 24 strains had identical features. Sequence analyses of the LmPH fragments amplified by using pheUf/pheUr show that the HAB strains possessed group I LmPHs, while the LAB strains possessed group III LmPHs (Fig. 3). This clear discrimination between the HAB and LAB strains was confirmed by PCR analyses with the group-specific primers (Table 2), demonstrating the accuracy of these primers. In addition, phenotypic data, i.e., the TCE-degrading activity (at 0.5 mg $liter^{-1}$) expressed by the k_1 value, further supported this discrimination between the HAB and LAB strains (Table 2). Our previous study found that the three kinetic groups of phenol-degrading bacteria could be rapidly discriminated by their k_1 values (9); i.e., $k_1 < 2$ liters g^{-1} h^{-1} for the high-K_s group, $2 < k_1 < 10$ for the moderate-K_s group, and $k_1 > 10$ for the low-K_s group. Based on this criterion, the HAB strains could be affiliated with the low-K_s group, while the LAB strains were affiliated with the high-K_s group. These results indicate that the LmPH genotype is correlated with TCE degradation activity.

We found that a group of phenol-degrading bacteria (the high-activity group in Fig. 3), including strains HAB-24, HAB-27, HAB-29, and HAB-30, expressed unexpectedly high TCE-degrading activities (Table 2). The k_1 values of known TCE-degrading bacteria have been reported, e.g., 69 liters g^{-1} h^{-1} for *Methylosinus trichosporium* OB3b (10), 22 for *B. cepacia* G4 (10), and 35 for *C. testosteroni* R5 (9). The present results thus expand our knowledge of the physiological diversity of TCE-degrading bacteria in the environment. In addition, we suggest that our group-specific PCR is useful for screening phenol-degrading bacteria that exhibit high TCE-degrading activities. The potential of the high-activity strains, particularly strain HAB-30, for bioaugmentation is also suggested.

Group-specific monitoring of LmPHs. The competitive PCR assay was developed to estimate the total copy number of LmPH genes belonging to each of the three groups. The total number of group II LmPHs was estimated by subtracting the copy number obtained by using pheUf/pheHr from that obtained by using pheUf/pheMHr. We found that the group III LmPHs were most abundant in the original aquifer soil (Table 3) and that the copy number was not significantly different from the copy number of total LmPH (determined by using pheUf/pheUr). When soil bacteria were grown aerobically after being supplemented with 0.2 mM phenol (batch feeding), the group II and III LmPHs increased vigorously to over 10^8 copies ml^{-1} (Table 3). In contrast, when phenol was supplied continuously (continuous feeding), cluster I LmPH overgrew the soil culture, and its copy number was 67% of the TDC value. The data presented in Table 3 illustrate that the majority of phenol-degrading bacteria in the aquifer soil could be detected by the PCR assay developed in this study when phenol was supplied.

The k_1 value for the enrichment culture established by con-

TABLE 3. Group-specific monitoring of LmPHs in the TCE-contaminated aquifer soil and in enrichment cultures growing on phenol[a]

Soil consortium	TDC (cells ml^{-1})	Group-specific monitoring (mean copies $ml^{-1} \pm$ SD, $n = 3$)				Mean k_1 (liter g^{-1} h^{-1}) $\pm$ SD ($n = 3$)
		Universal	Group I	Group II	Group III	
Original aquifer soil	$1.0 \times 10^9 \pm 0.8 \times 10^9$	$5.3 \times 10^6 \pm 2.3 \times 10^6$	$2.9 \times 10^5 \pm 1.8 \times 10^5$	$3.0 \times 10^5 \pm 1.7 \times 10^5$	$3.7 \times 10^6 \pm 2.5 \times 10^6$	ND[a]
Batch fed	$5.1 \times 10^8 \pm 0.9 \times 10^8$	$1.1 \times 10^8 \pm 0.8 \times 10^8$	$5.6 \times 10^4 \pm 4.2 \times 10^4$	$3.0 \times 10^8 \pm 1.4 \times 10^8$	$1.1 \times 10^8 \pm 0.5 \times 10^8$	2.8 ± 0.5
Continuously fed	$4.3 \times 10^9 \pm 0.5 \times 10^9$	$2.2 \times 10^9 \pm 1.9 \times 10^9$	$2.9 \times 10^9 \pm 0.6 \times 10^9$	$1.9 \times 10^6 \pm 1.4 \times 10^6$	$1.2 \times 10^7 \pm 0.6 \times 10^7$	58 ± 1.9

[a] ND, not detected.

tinuous phenol feeding was much higher than that established by batch feeding (Table 3). This result was considered to be consistent with the results of the LmPH population analysis (Table 3). The k_1 value for the batch-fed consortium was considered insufficient for the degradation of TCE at a concentration relevant to that in a contaminated aquifer (9). In contrast, the k_1 value for the continuously fed consortium was unexpectedly high and is comparable to the value expressed by a pure culture of *M. trichosporium* OB3b (10). Molecular population analyses have suggested that the high-activity group bacteria were major members of this consortium (data not shown). During the continuous-feeding experiment, phenol was almost completely degraded (below the detection limit). This is likely to have been achieved by bacteria possessing group I LmPHs, since they correspond to low-K_s-type bacteria that also exhibit high affinities for phenol (9). The data thus suggest that phenol biostimulation could be a powerful TCE bioremediation strategy if bacteria possessing group I LmPHs can be selectively stimulated.

Conclusions. The phylogenetic analyses (Fig. 1 and 3) in combination with analyses of the TCE degradation activities of the isolated bacteria (8) (Table 2) revealed a clear correlation between the LmPH genotypes and TCE degradation activities, which facilitated group-specific monitoring of the different types of phenol-degrading bacteria. It must be impossible to trace all the different species of diverse microbial populations in the natural environment; we thus suggest that group-specific analyses as conducted in this study would be a practical way for understanding and managing natural microbial consortia.

When phenol-degrading bacteria possessing group I LmPHs were dominant, the soil enrichment culture expressed very high TCE degradation activity (Table 3); this was achieved by the continuous feeding of phenol to the aquifer soil. Shih et al. have also shown that the phenol feeding pattern altered the microbial community structure and cometabolic TCE-degrading activity (30). In contrast to the results from the present study, after long-term operation, a consortium established by the pulse addition of phenol showed a much higher TCE transformation rate than a consortium established by continuous phenol feeding. They observed that the continuous culture became overgrown by filamentous microorganisms, especially fungi, which were incapable of TCE degradation or only slowly degraded TCE, suggesting that complex microbial successions may occur during long-term operation. Further studies are thus needed to develop effective phenol-feeding schemes for the enrichment and maintenance of microbial consortia which express high TCE-degrading activity.

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