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 Methyllosinus 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.

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...
The PCR products were ligated into pUC18 (27) and checked by electrophoresis through 1.5% (wt/vol) agarose gel (LO3 agarose; 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, 35 cycles consisting of 1 min at 94 °C, 1 min at 54 °C, and 1 min at 72 °C; step 3, 10 min of extension at 72 °C; step 4, 25 cycles consisting 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. In this study, the pseudo-first-order degradation rate constant k1 (32) was used to describe the TCE-degrading activity according to previous studies (9, 10, 30, 32). The k1 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 BM medium in a TBR-2 fermentor was inoculated with the aquifer soil (20 g wet), and phenol was then added at 0.2 mM. After culture parameters (OD600 and DO) had become stable, a small portion of the culture was sampled. The culture volume was maintained at 1.5 liters, and the temperature was kept at 25 °C. Eight days after commencing the cultivation, the culture parameters (phenol concentration, optical density at 660 nm [OD600], and dissolved oxygen concentration [DO]) had become stable, a small portion of the culture was sampled. This study employed the pseudo-first-order degradation rate constant k1 (32) to describe the TCE-degrading activity according to previous studies (9, 10, 30, 32).

(ii) Continuous phenol feeding. Air was supplied at a rate of 2 liters min−1. TDC was conducted by using a Competitor PCR. The PCR products were purified by using a competitive DNA construction kit (Takara Shuzo Co., Tokyo, Japan). The PCR conditions were as described previously (9, 10, 30, 32). The k1 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).
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), PhID 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...
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/pheUr 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
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/pheUr. 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 grew 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 2. Characteristics of isolated strains

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Closely related organism based on 16S rRNA gene sequence (% identity)</th>
<th>Rep-PCR pattern no.</th>
<th>Group-specific PCR(^b) with primers:</th>
<th>Mean (k_1) ± SD (liter g(^{-1}) h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Universal</td>
<td>Group I</td>
</tr>
<tr>
<td>HAB-01</td>
<td>Ralstonia sp. strain BKME-6 (99)</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HAB-02</td>
<td>Ralstonia sp. strain BKME-6 (99)</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HAB-08</td>
<td>Ralstonia eutropha KT-1 (99)</td>
<td>3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HAB-11</td>
<td>Ralstonia eutropha KT-1 (99)</td>
<td>4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HAB-18</td>
<td>Ralstonia eutropha KT-1 (99)</td>
<td>5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HAB-21</td>
<td>Variovorax sp. strain WFF52 (99)</td>
<td>6</td>
<td>+</td>
<td>+</td>
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<tr>
<td>HAB-22</td>
<td>Variovorax sp. strain WFF52 (99)</td>
<td>7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HAB-23</td>
<td>Variovorax sp. strain WFF52 (99)</td>
<td>9</td>
<td>+</td>
<td>+</td>
</tr>
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<td>HAB-24</td>
<td>Variovorax sp. strain WFF52 (99)</td>
<td>11</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HAB-27</td>
<td>Variovorax sp. strain WFF52 (99)</td>
<td>12</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HAB-29</td>
<td>Variovorax sp. strain WFF52 (99)</td>
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<td>+</td>
</tr>
<tr>
<td>HAB-30</td>
<td>Variovorax sp. strain WFF52 (99)</td>
<td>10</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LAB-05</td>
<td>Pseudomonas sp. strain IpA-2 (99)</td>
<td>11</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>LAB-06</td>
<td>Pseudomonas sp. strain BKME-9 (98)</td>
<td>12</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>LAB-08</td>
<td>Pseudomonas sp. strain Dha-51 (99)</td>
<td>13</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>LAB-16</td>
<td>Pseudomonas sp. strain PsF (99)</td>
<td>14</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>LAB-18</td>
<td>Pseudomonas sp. strain Dha-51 (99)</td>
<td>15</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>LAB-20</td>
<td>Pseudomonas sp. strain IpA-2 (99)</td>
<td>16</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>LAB-21</td>
<td>Pseudomonas putida ATCC 17484 (99)</td>
<td>17</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>LAB-23</td>
<td>Pseudomonas sp. strain PsF (99)</td>
<td>18</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>LAB-26</td>
<td>Pseudomonas jessenii (99)</td>
<td>19</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>LAB-27</td>
<td>Pseudomonas sp. strain Dha-51 (99)</td>
<td>20</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>LAB-36</td>
<td>Pseudomonas agarici (97)</td>
<td>21</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>LAB-44</td>
<td>Pseudomonas rhodesiae (100)</td>
<td>22</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

\(^a\) 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.
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 $k_i$ 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 $k_i$ 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-$K_i$-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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