3- and 4-alkylphenol degradation pathway in *Pseudomonas* sp. strain KL28: genetic organization of the *lap* gene cluster and substrate specificities of phenol hydroxylase and catechol 2,3-dioxygenase

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The enzymes and genes responsible for the catabolism of higher alkylphenols have not been characterized in aerobic bacteria. *Pseudomonas* sp. strain KL28 can utilize a wide range of alkylphenols, which include the 4-4-alkylphenols (C1–C5). The genes, designated as *lap* (for long-chain alkylphenols), encoding enzymes for the catabolic pathway were cloned from chromosomal DNA and sequenced. The *lap* genes are located in a 13.2 kb region with 14 ORFs in the order *lapRBKLMNOPCEHIFG* and with the same transcriptional orientation. The *lapR* gene is transcribed independently and encodes a member of the XylR/DmpR positive transcriptional regulators. *lapB*, the first gene in the *lap* operon, encodes catechol 2,3-dioxygenase (C23O). The *lapKLMNOP* and *lapCEHIFG* genes encode a multicomponent phenol hydroxylase (mPH) and enzymes that degrade derivatives of 2-hydroxymuconic semialdehyde (HMS) to TCA cycle intermediates, respectively. The *PlapB* promoter contains motifs at positions −24(GG) and −12(GC) which are typically found in σ54-dependent promoters. A promoter assay using a *PlapB*: :gfp transcriptional fusion plasmid showed that *lapB* promoter activity is inducible and that it responds to a wide range of (alkyl)phenols. The structural genes encoding enzymes required for this catabolism are similar (42–69 %) to those encoded on a catabolic pVI150 plasmid from an archetypal phenol degrader, *Pseudomonas* sp. CF600. However, the *lap* locus does not include genes encoding HMS hydrolase and ferredoxin. The latter is known to be functionally associated with C23O for use of 4-alkylcatechols as substrates. The arrangement of the *lap* catabolic genes is not commonly found in other *meta*-cleavage operons. Substrate specificity studies show that mPH preferentially oxidizes 3- and 4-alkylphenols to 4-alkylcatechols. C23O preferentially oxidizes 4-alkylcatechols via proximal (2,3) cleavage. This indicates that these two key enzymes have unique substrate preferences and lead to the establishment of the initial steps of the *lap* pathway in strain KL28.

**INTRODUCTION**

Phenol is produced naturally by the breakdown of plant materials during microbial degradation. In contrast, phenols with bulky alkyl group(s) that are found in the environment originate predominantly from anthropogenic sources. For instance, nonyl- and octylphenol are pollutants that are commonly found in aquatic environments. They are recalcitrant intermediates that are formed during the microbial breakdown of alkylphenol ethoxylates (Ferguson *et al*., 2001; Giger *et al*., 1984). Alkylphenol

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Received 7 July 2003
Revised 7 August 2003
Accepted 26 August 2003

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**Abbreviations:** C12O, catechol 1,2-dioxygenase; C23O, catechol 2,3-dioxygenase; GFP, green fluorescent protein; HMS, 2-hydroxymuconic semialdehyde; IHF, integration host factor; lap, long-chain alkylphenol; mPH, multicomponent phenol hydroxylase.

The GenBank accession numbers for the sequences reported in this study are AY324319 (the partial sequence of 16S rDNA) and AY324644 (orf1lapRBKLMNOPCEHIFG).
ethoxylates are highly effective cleaning agents or surfactants used in the textile, petroleum and paper industries. Alkylphenols, which are used for the synthesis of alkylphenol ethoxylates, are also extensively used as lubricating oil additives, antioxidants, stabilizers for rubbers and plastics, dispersants, emulsifiers and plasticizers for resins. Alkylphenols are environmentally hazardous due to their toxicity, and they have been detected in some aquatic biological systems (Ferrara et al., 2001; Keith et al., 2001). Some alkylphenols are also known as endocrine-disrupting chemicals that mimic oestrogenic compounds, causing adverse effects such as developmental and reproductive toxicity in animals (Sharpe et al., 1995; Tanaka & Grizzle, 2002). Studies on the degradation of higher alkylphenols by micro-organisms are therefore receiving significant attention.

Numerous studies on the microbial catabolism of phenol and the methylphenols (e.g. cresols) have been carried out, and have led to a deeper understanding of the microbial degradation of aromatic compounds, in terms of enzymology, genetics, microbial diversity, and the use of microorganisms for the removal of toxic chemicals. Studies on the microbial degradation of higher alkylphenols will provide important information to supplement results obtained from simple phenols. To date, the microbial degradation of higher alkylphenols has been studied in pure cultures, which show that microbial degradation is initiated at the phenolic moiety, rather than at the alkyl chain (Ajithkumar et al., 2003; Fujii et al., 2000; Soares et al., 2003; Tanghe et al., 1999, 2000). However, no information is available on the degradation pathway and the bacterial catabolic genes for higher alkylphenols.

We have isolated a Pseudomonas strain, designated KL28, which can degrade a broad range of 4-n-alkylphenols with an alkyl chain length of C1-C5, and identified the gene cluster, designated as lap (for long-chain alkylphenols), responsible for their complete degradation. The results obtained by comparing the deduced amino acid sequences of the Lap proteins with those of previously reported proteins and of studies on the substrate specificities of multicomponent phenol hydroxylase (mPH; EC 1.14.13.7) and catechol 2,3-dioxygenase (C23O; EC 1.13.11.2) allowed us to determine the degradation pathway of higher alkylphenols (the lap pathway) in strain KL28.

**METHODS**

**Bacterial strains and culture conditions.** Alkylphenol-degrading strains were initially isolated from soils in an industrial complex in Changwon, Korea. Enrichment was carried out using 4-ethylphenol as a carbon and energy source in minimal salts basal (MSB) medium (Stanier et al., 1966). One strain, designated KL28, which showed the highest growth, was isolated for further study. Strain KL28 was routinely grown in Luria–Bertani (LB) medium (Sambrook & Russell, 2001). Alkylphenols were supplied in the vapour phase in plates or added directly to liquid culture at 0.04% (v/v) as sole sources of carbon and energy in MSB medium. Other solid chemicals were supplemented at 0.04% (w/v) and volatile chemicals such as benzene, toluene and styrene were supplied in the vapour phase. Escherichia coli DH5α (Ausubel et al., 1990) was used as a host strain for the maintenance of plasmids. To culture transformed E. coli or Pseudomonas, ampicillin (Ap), gentamicin (Gm) or tetracycline (Tc) was incorporated into the culture media at the amounts indicated previously (Choi et al., 2003). E. coli and Pseudomonas cells were grown at 37°C and 28°C, respectively. In liquid culture, cells were grown in a 50 ml culture volume in 250 ml Erlemeyer flasks in a shaking incubator at 180 r.p.m. Pseudomonas putida G7.C-1 is an NAH-7 plasmid cured derivative of strain G7 (Dunn & Gunsalus, 1973).

**Construction of the genomic library and the cloning of alkylphenol catabolic genes.** The total DNA of strain KL28 was purified as previously described (Ausubel et al., 1990). The DNA was partially digested with Sau3A1. Fragments in the range 25–35 kb were isolated by ultracentrifugation over a sucrose density gradient and ligated into cosmid pLAFR3 (Staskawicz et al., 1987) that had been treated with BamHI and calf intestine dephosphatase. After *in vitro* packaging into λ bacteriophage, using a Gigapack III Packaging extract (Stratagene), the E. coli VCS257 (Stratagene) host strain was transfected with the phage. Cosmid clones in pLAFR3 were mobilized from the E. coli strain to *P. putida* G7.C-1, by triparental mating in the presence of E. coli HB101 (pRK2013) (Figurski & Helinski, 1979). Recombinant *P. putida* G7.C-1 cells containing the alkylphenol catabolic genes were positively selected in Tc-containing MSB medium with 4-ethylphenol supplied in the vapour phase. One recombinant plasmid, designated pJ2, conferred upon *P. putida* G7.C-1 the ability to grow on higher alkylphenols and was selected for further study. Other molecular genetic techniques were performed using standard procedures (Sambrook & Russell, 2001) and as recommended by the reagent suppliers.

**DNA sequence analysis.** The insert in pJ2 was digested with various restriction enzymes and subcloned in pBluescript SK(−) (Stratagene). Subclones were used as templates for DNA sequencing. Nucleotide sequences were determined by Genotech Co. (Taejeon, Korea) using an automated sequencing unit (ABI PRISM 377, PE Biosystems) with M13 and sequence-based primers. Searches for specific nucleotide or amino acid sequences were carried out using the BLAST program (Altschul et al., 1997) provided by DDBJ/GenBank/EMBL and the ExPASY Interface to EMBnet/CH/SIB/SCS provided by the Swiss Institute of Bioinformatics (SIB), as available on the internet. The nucleotide sequence of the partial 16S rDNA gene of strain KL28 was determined by direct sequencing of the PCR product amplified using 27F and 1522R primers (Johnson, 1994) with Ex-Taq DNA polymerase (TaKaRa, Japan).

**Construction of plasmids.** pJJPMO2 (Gm') was constructed by cloning the 5-8 kb *XhoI–EcoRI* fragment carrying the *mPH* genes from pJJ2 into the corresponding restriction sites of pBBR1CMCS-5 (Kovach et al., 1995) (see Fig. 1). pJJRI (Gm') is a *PapGII–gfp* vector and was constructed by cloning a 1-3 kb *SalI–BamHI* fragment of pJJ2 into the same restriction sites of pPROBE-GT (Miller et al., 2000) (see Fig. 1). pJJR2 (Gm') is a *PapGII–gfp* vector and was constructed by cloning the 1-0 kb *PstI–DraI* fragment of pJJ2 digested with *SalI* and *PstI* (see Fig. 1). pJJXYLE (Ap') was constructed by cloning the 1-0 kb PCR fragment of the *xylE* gene amplified with the primers 5′-CTATGAAAGGTGACGTCATGAAC-3′ and 5′-CAGTCCATCAATCCTCCTGAAAT-3′ from the total DNA of *P. putida* mt-2 (Murray et al., 1972) into a PCR product cloning vector, pEZ-T (RNA Co.). pJC23O (Ap') was made by cloning a 1-0 kb PCR fragment carrying the *lapB* gene amplified with the primers 5′-CCGCACTACAGCCATTATTGAG-3′ and 5′-GGCTGATGCCTCAGTCACTC-3′ from pJJ2 into pEZ-T. PCR was carried out as previously described (Choi et al., 2003) but with a polymerization time of 1 min.

**RT-PCR.** c-DNA from the total RNA of strain KL28, grown on
4-ethylphenol as sole source of carbon and energy, was produced and used for PCR as previously described (Cho et al., 2000), but with a polymerization time of 1-5 min. Positive controls contained the chromosomal DNA of strain KL28 as a template in the PCR reaction. Negative controls were tested by PCR without the reverse transcription step for possible DNA contamination in the sample preparations. The products formed by PCR were confirmed by DNA sequence analysis. The primers used were 790, 5'-CCGAGCTAGACAGATAGGTC-3'; 2120, 5'-CAGCAGCAGCACTGTTATC-3'; 2550, 5'-CTGCGTCATCGTAAGTCGGAT-3'; 3937, 5'-GGTCTAGCTGATTGGGAG-3'; 4195, 5'-AACGTGGACATCGGTCCAA-3'; 5197, 5'-GTGCCGTAGCCATAGCTACAG-3'.

Induction studies with green fluorescent protein (GFP) as the reporter. Pseudomonas sp. strain KL28(pJJR1) was grown in MSB liquid medium containing pyruvate (10 mM) and Gm. After 24 h, alkylphenols (final concentration 1 mM from 1 M stock in methanol) were added to the culture media. Cells were further grown and 2 ml aliquots were harvested every 24 h by centrifugation, washed twice with saline, and resuspended in saline at an OD600 of around 0.2. The expression level of GFP was measured using a spectrofluorophotometer (model RF-5391PC, Shimadzu Co.) as previously described (Choi et al., 2003). Pseudomonas sp. strain KL28(pJJR2) and its control strain Pseudomonas sp. strain KL28(pPROBE-GT) were grown in LB liquid medium with Gm. The expression level of GFP was measured 24 h after inoculation.

Biotransformation of (alkyl)phenols by recombinant E. coli DH5α(pJJPMO2) cells expressing mPH. E. coli DH5α(pJJPMO2) was grown in LB broth with Gm at 28°C. Expression of the cloned genes was induced by adding IPTG (final concentration 0.25 mM), as described previously (Lee et al., 1997). Cells harvested by centrifugation were suspended in 50 mM sodium phosphate buffer (pH 7.0) with 20 mM glucose to an OD600 of 1. Phenol or alkylphenol was then added to each flask to a concentration of 0-0.5% (v/v) in a culture volume of 50 ml. Biotransformation was carried out at 28°C with shaking at 180 r.p.m. for 48 h. The culture supernatants were extracted with ethyl acetate and the extracts were concentrated by rotary evaporation under vacuum at 45°C. The concentrated extracts were analysed by TLC using silica gel 60 F254 (2 mm thickness) with chloroform/acetone (95:5, v/v). GC-MS analysis and oxygen consumption assays with cell extracts were carried out as previously described (Cho et al., 2000).

Measurement of substrate preference of C23O. E. coli DH5α harbouring pDTG617 (Zylstra & Gibson, 1991), pJXYLE and pJJC23O are recombinant cells expressing catechol dioxygenase encoded by todE, xylE and lapB, respectively. Cell extracts were obtained as previously described (Cho et al., 2000) and used to determine if the three C23Os could form the same product from a given catechol substrate. Assay mixtures (total 1 ml) contained potassium phosphate buffer (0.1 M, pH 7.5), catechol substrate (final concentration 0.1 mM) and cell extract (75 μg) and incubated...
at 28 °C with gentle agitation for 30 min. Product formation was monitored by scanning with a spectrophotometer (model 2130, Scinco, Korea). The specific activity of LapB was determined using a cell extract of E. coli DH5α(pJJ230) in the same phosphate buffer by measuring the rate of formation of meta-cleavage products from (substituted) catechols. The wavelengths (absorption coefficients in mM$^{-1}$ cm$^{-1}$) used to monitor the formation of the meta-cleavage products of catechol, 3-methylcatechol, 4-methylcatechol, 4-ethylcatechol and 2,3-dihydroxybiphenyl were 376 (40), 389 (11–9), 382 (24–5), 381 (36–0) and 434 (19–8) nm, respectively (Bayly et al., 1966; Duggleby & Williams, 1986; Ramos et al., 1987; Seah et al., 1998). Protein concentrations were determined using the BCA protein assay (Fierce) with BSA as the standard. Specific enzyme activities are reported as μmol product formed min$^{-1}$ (mg protein)$^{-1}$. Activity assays were conducted in triplicate, and the initial rates of the assays were determined and used to calculate mean and standard deviations.

**Chemicals.** The aromatic compounds used in this study were obtained from Aldrich, except for the following: 4-n-butylphenol and 4-n-pentylphenol from Lancaster Synthesis, Morecambe, UK; 4-n-hexylphenol from Kanto Chemical, Japan; 3-ethylphenol, 2-hydroxy- and 4-hydroxybiphenyl from Fluka Chemica; and 2,3-dihydroxybiphenyl from Wako Pure Chemicals. Enzymes and reagents used for DNA manipulation were purchased from Takara, KOSCO, Promega and Pharmacia. The commercial phenotype identification API 20NE kit was obtained from API Analytab Products.

**RESULTS**

**Identification of strain KL28 and its growth on various alkylphenols**

The almost complete nucleotide sequence of 16S rDNA (1453 bp) of strain KL28 showed 99% sequence identity to P. putida strains ATCC 12633 and ATCC 11172 and P. asplenii ATCC 23835$^T$. In addition, biochemical testing with API 20NE showed that strain KL28 is closest to the genus *Pseudomonas*. In MSB liquid medium, strain KL28 was tested for its ability to degrade a range of alkylphenols as the sole sources of carbon and energy. It grew from an OD$_{600}$ of 0–2 to an OD$_{600}$ of 0.4–0.5 with a doubling time of 5–7 h within 2 days in the presence of m-cresol, p-cresol, 3-ethylphenol, 4-ethylphenol or 4-propylphenol. The same level of growth was obtained within 4–5 days in the presence of 4-butylphenol or 4-pentylphenol with doubling times of 15 and 21 h, respectively. However, the strain did not grow in the presence of phenol, o-cresol, 2-ethylphenol, 4-n-alkylphenols (C$_4$–C$_6$), 4-hydroxyacetophenone, 2- or 4-hydroxybiphenyl, 2,3-, 2,6- or 3,5-dimethylphenol, 2-, 3- or 4-chlorophenol, benzene, toluene, styrene, biphenyl or naphthalene. The strain also grew in the presence of 4-methylcatechol or 4-ethylcatechol, but not in the presence of catechol. During growth with p-cresol and 4-ethylphenol, strain KL28 produced a yellow-coloured diffusible product, presumably a meta-ring cleavage product.

**Cloning of alkylphenol catabolic genes from strain KL28**

Various efforts to detect a plasmid in strain KL28 failed, indicating that the genes for alkylphenol degradation are encoded on its chromosome. In order to identify the genes responsible for the degradation of alkylphenols in strain KL28, a genomic library was made using pLAFR3, as described in Methods. pLAFR3 is a cosmid vector (Staskawicz et al., 1987). It can be mobilized by conjugation and can replicate in various Gram-negative bacteria. The genomic library in E. coli cells was transferred via conjugation to *P. putida* G7.C-1, which cannot grow on alkylphenols. The recombinant strains obtained were screened for their ability to grow on 4-ethylphenol as a carbon and energy source. Eight different transconjugants were isolated and found to contain recombinant plasmids, all of which were apparently identical in size and orientation in the insert. One of the recombinant strains, containing a recombinant plasmid named pJJ2, was selected for further studies. *P. putida* G7.C-1(pJJ2) could grow on 4-n-alkylphenols (C$_4$–C$_6$) as the sole sources of carbon and energy at growth rates similar to those by KL28 (data not shown). The transconjugant strain also grew on 4-n-hexylphenol as the sole source of carbon and energy. When plasmid pJJ2 was introduced into E. coli DH5α, the recombinant strain obtained did not degrade alkylphenols and did not produce indigo from indole. The latter biochemical reaction is caused by *E. coli* cells that express mPHKL28 (see below). These results showed that the alkylphenol catabolic genes cloned from strain KL28 are not expressed in *E. coli*, indicating that cis or/and trans elements necessary for the transcription of the cloned genes in *E. coli* and *Pseudomonas* may be different.

**Analyses of the nucleotide and deduced amino acid sequences of the lap genes**

Subcloning and restriction analysis of pJJ2 showed that the insert was approximately 25–5 kb in size. When a subcloned plasmid (pJJPMO2) (Fig. 1) was introduced into E. coli DH5α, the recombinant *E. coli* strain was able to produce indigo on LB agar even in the absence of IPTG. This result indicated that the DNA fragment contains genes encoding an oxygenase with expression under control of the *lac* promoter from the vector. The production of indigo is due to indole formation by tryptophanase in *E. coli* and indoxyl formation by the oxygenase from the plasmid, as was previously shown by *E. coli* cells expressing naphthalene dioxygenase (Ensley et al., 1983). Sequence analysis of the fragment showed that it contains six complete genes (designated *lapKLMNOP*) for mPH, which was first identified in *Pseudomonas* sp. CF600 by Nordlund et al. (1990). Comparison of the amino acid sequence of the oxygenase component with those of other non-haem iron oxygenases revealed that the active site of the oxygenase component contains a dinuclear iron centre that is liganded by a pair of a conserved motif (Asp/Glu)-Glu-Xaa-Arg-His (Fox et al., 1993). The role of the amino acid sequence motif was further confirmed in methane monoxygenase crystal structures (Elango et al., 1997; Rosenzweig et al., 1997). Recently, Cadieux et al. (2002) reported experimental evidence for the presence of the binuclear iron centre in a
purified mPH. The conserved amino acid sequence fingerprints were found at positions 138–142 and 233–237 in LapN, which constitutes a catalytic oxygenase component with LapL and LapO in a putative \( \alpha_2\beta_2\gamma_2 \) hexamer (Cadieux et al., 2002). In most cases, the phenol catabolic genes are clustered. Thus, we further subcloned and sequenced in both directions from the oxygenase genes including the AvrII site to the downstream end (Fig. 1). The sequenced region consisted of 14 471 bp with a G+C content of 59.8 mol%. It contained 13 complete and 2 incomplete ORFs (Table 1). The gene products showed some degree of sequence identity (33–74%) with their counterparts in the (methyl)phenol catabolic \( dmp \) operon in \textit{Pseudomonas} sp. CF600 (Bartilson & Shingler, 1989; Nordlund et al., 1990; Shingler et al., 1992, 1993) and the phenol catabolic \( aph \) operon from \textit{Comamonas testosteroni} TA441 (Arai et al., 1998, 1999, 2000) (Table 1). The complete nucleotide sequences containing mPH genes are only known for the \( dmp \) and \( aph \) operons. Thus, the functions of the regulatory gene product (LapR) and of the catabolic gene products (LapBKLMNOPCEHIFG) can be readily inferred as indicated in Table 1. The identified genes constitute a catabolic pathway for the degradation of alkylphenols to TCA cycle intermediates. The lapG gene

### Table 1. Properties of the lap genes identified from pJJ2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Coding region</th>
<th>G + C (mol%)</th>
<th>Protein*</th>
<th>No. of amino acid residues and size (kDa)</th>
<th>Equivalent to Dmp/Aph†</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>orf1</td>
<td>1–1249</td>
<td>61</td>
<td>Putative methyl-accepting protein (truncated)</td>
<td>417</td>
<td>43-6</td>
<td>–</td>
</tr>
<tr>
<td>lapR</td>
<td>1362–3041</td>
<td>58</td>
<td>XylR/DmpR-type regulator</td>
<td>540</td>
<td>42</td>
<td>DmpR</td>
</tr>
<tr>
<td>lapB</td>
<td>3478–4044</td>
<td>59</td>
<td>C23O</td>
<td>309</td>
<td>51</td>
<td>DmpB</td>
</tr>
<tr>
<td>lapK</td>
<td>4417–4707</td>
<td>61</td>
<td>mPH (assembly)</td>
<td>97</td>
<td>43</td>
<td>DmpK</td>
</tr>
<tr>
<td>lapL</td>
<td>4758–5747</td>
<td>61</td>
<td>mPH (( \beta ) subunit)</td>
<td>11-2</td>
<td>41</td>
<td>DmpL</td>
</tr>
<tr>
<td>lapM</td>
<td>5760–6026</td>
<td>56</td>
<td>mPH (activator)</td>
<td>89</td>
<td>54</td>
<td>DmpM</td>
</tr>
<tr>
<td>lapN</td>
<td>6045–7562</td>
<td>58</td>
<td>mPH (( \alpha ) subunit)</td>
<td>506</td>
<td>61</td>
<td>DmpN</td>
</tr>
<tr>
<td>lapO</td>
<td>7620–7973</td>
<td>60</td>
<td>mPH (( \gamma ) subunit)</td>
<td>118</td>
<td>44</td>
<td>DmpO</td>
</tr>
<tr>
<td>lapP</td>
<td>7987–9042</td>
<td>62</td>
<td>mPH (reductase)</td>
<td>379</td>
<td>60</td>
<td>DmpP</td>
</tr>
<tr>
<td>lapC</td>
<td>9258–10715</td>
<td>63</td>
<td>HMS dehydrogenase</td>
<td>486</td>
<td>69</td>
<td>DmpC</td>
</tr>
<tr>
<td>lapE</td>
<td>10726–11520</td>
<td>61</td>
<td>2-Hydroxypent-2,4-dienoate hydratase</td>
<td>265</td>
<td>60</td>
<td>DmpE</td>
</tr>
<tr>
<td>lapH</td>
<td>11520–12320</td>
<td>59</td>
<td>4-Oxalocrotonate decarboxylase</td>
<td>267</td>
<td>55</td>
<td>DmpH</td>
</tr>
<tr>
<td>lapI</td>
<td>12347–12556</td>
<td>58</td>
<td>4-Oxalocrotonate isomerase</td>
<td>70</td>
<td>43</td>
<td>DmpI</td>
</tr>
<tr>
<td>lapF</td>
<td>12556–13467</td>
<td>62</td>
<td>Acetaldehyde dehydrogenase (acylating)</td>
<td>304</td>
<td>55</td>
<td>DmpF</td>
</tr>
<tr>
<td>lapG</td>
<td>13470–14471</td>
<td>63</td>
<td>4-Hydroxy-2-oxovalerate aldolase (truncated)</td>
<td>334</td>
<td>55</td>
<td>DmpG</td>
</tr>
</tbody>
</table>

–, No corresponding gene was found in strains CF600 and TA441.

* Deduced from the corresponding genes of \textit{Pseudomonas} sp. CF600 and \textit{C. testosteroni} TA441 (see text for references).

† The arrangement and composition of the catabolic genes are not equal in the three (alkyl)phenol degradation operons (see Discussion).

‡ Results of NCIB BLAST 2 sequences.
enodes 4-hydroxy-2-oxovalerate aldolase and is located at the end of the cloned DNA fragment. However, it lacks 16-18 amino acids at the carboxyl terminal compared to known LapG homologues, but may be functionally active because its insert in pJJ2 supported the growth of strain G7.C-1 on alkylphenols. Interestingly, the lap gene cluster does not contain genes encoding ferredoxin, which are generally located adjacent to a gene encoding C23O, and 2-hydroxymuconic semialdehyde (HMS) hydrolase for the hydrolytic branch of the meta pathway. Furthermore, the order of the lap genes is not found in other meta-cleavage operons.

**RT-PCR analysis of the transcription of lap genes**

DmpR in the dmp operon belongs to the XylR/DmpR subfamily of the NtrC family of positive transcriptional activators (Inouye et al., 1988; Shingler et al., 1993). The putative transcriptional regulatory protein LapR is homologous to DmpR (Table 1) and also shares 43% sequence identity with XylR, which controls the upper pathway operon of the TOL plasmid pWW0 at the Pu promoter (Abril et al., 1991), suggesting that LapR also belongs to the same family of transcriptional activators. The lapR gene is transcribed in the same direction as the flanking genes (Fig. 1). In order to determine if the lapR gene is co-transcribed with the adjacent genes, RT-PCR was carried out as described in Methods. Although positive and negative controls gave the expected PCR results, only PCR primers of set 3 in Fig. 1 generated the expected RT-PCR product (data not shown). This result indicates that the lapR gene is independently transcribed from adjacent genes, and that the gene encoding C23O is co-transcribed with genes encoding mPH. The possibility of the presence of a promoter upstream of the lapR gene was investigated with a GFP reporter, as constructed in pJJ2 (Fig. 1). The level of the specific GFP expression of *Pseudomonas* sp. KL28(pJJ2) was 6.7 ± 1, whereas the control level of the specific GFP expression by *Pseudomonas* sp. KL28 (pPROBE-GT) was 1.8 ± 0.3. This result further indicates the presence of a promoter in front of the lapR gene.

**Nucleotide sequence features in PlapB and the induction of the lap catabolic operon by (alkyl)phenols**

The nucleotide sequence upstream of lapB has the following sequence features: a putative $\sigma^{34}$-dependent −24/−12-type promoter $5'$-TGGCACCATCCTTGCA-3' of consensus sequence $5'$-TGGC-N$_k$-TGCA-3', where $N$ represents any nucleotide (Thony & Hennecke, 1989), the putative IHF-binding region $5'$-GATCATGCTTTA-3' of consensus sequence $5'$-WATCAAN$_n$TTR-3' where $W=A$ or $T$; $R=A$ or G (Friedman, 1988), and two palindromic elements (Fig. 2). These sequence features, except for the second inverted repeat sequence, are well characterized in the Po and Pu promoter regions, which are controlled by the aromatic effector-responsive XylR and DmpR transcriptional activators, respectively (Abril et al., 1991; de Lorenzo et al., 1991; Sze et al., 2001). The promoter sequence and the amino acid sequence similarity of LapR to DmpR and XylR suggest that the expression of $P_{lapB}$ is controlled by the regulatory protein LapR. In order to determine the range of effectors of the lap operon, the transcriptional activity of the lapB promoter ($P_{lapB}$) in response to various chemicals was assessed by monitoring the expression of GFP from the gfp-fusion plasmid, pJJR1 (Fig. 1). The expression of GFP was determined from *Pseudomonas* sp. KL28(pJJR1) as described in Methods. Expression of GFP was induced by phenol and by a broad range of (alkyl)phenols, which served as carbon and energy sources for strain KL28 (Table 2). GFP expression was high in the presence of 3-ethylphenol, 4-ethylphenol, m-cresol and phenol, with a preference for 3- and 4-alkylphenols over 2-alkylphenols. However, GFP was not expressed in the presence of aromatic hydrocarbons such as benzene, p-xylene, toluene, biphenyl and naphthalene (data not shown). This induction pattern differs from those of DmpR, PhhR and MopR (Ng et al., 1995; Schirmer et al., 1997; Shingler & Moore, 1994), all of which are found in phenol degraders. These regulatory proteins show no preference for 3- and 4-alkylphenols and are more responsive to simple phenols.

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**Fig. 2.** Regulatory region of the lap operon. The ATG initiation codon of lapB is indicated in bold. Putative ribosome-binding (RBS) and IHF-binding (IHFBS) regions are indicated by the thin and double underlines, respectively. The −12 and −24 sequences of the lapB promoter, $P_{lapB}$, are boxed and labelled accordingly. Arrows in both directions indicate an inverted repeat.
Table 2. Expression of the lap operon based on $P_{lapB}$ in response to different alkylphenols

*Units are arbitrary and represent the mean of duplicate determinations in two independent experiments.

Substrate preference of mPHKL28

While the products of the lap genes are compatible with those in the dmp and aph operons, the first two steps of the alkylphenol degradation, which involve the hydroxylation of alkylphenols and the ring cleavage of catechols, can show variations in regioselectivity and in the mode of ring cleavage, respectively. First, to determine the substrate preference and regioselectivity of mPHKL28, biotransformations were carried out as described in Methods with E. coli DH5$\alpha$(pPJM02). The products formed were identified by TLC and GC-MS. mPH catalysed the formation of catechols from a broad-range of (alkyl)phenols with a preference for 3- and 4-alkylphenols over 2-alkylphenols (Table 3). In the case of 3-alkylphenols, hydroxylation occurred at the carbon at the distal position from the alkyl substitution, yielding 4-alkylcatechols. Products from 2-ethylphenol and 4-alkylphenols with an alkyl group greater than C4 were not detected under the given experimental conditions. This result may be due either to the inability of substrate and/or product to be transported across the E. coli membrane or to the diminished activity of mPH with higher alkylphenols. In contrast to whole-cell transformations, which were carried out in 48 h, the mPH activity of cell extract was too low to be distinguished from the basal activity in an oxygen consumption assay (data not shown).

Substrate preference and the mode of ring cleavage by C23O$_{KL28}$, LapB

Extradiol dioxygenases catalyse the incorporation of molecular oxygen into a carbon–carbon bond adjacent to the hydroxyl group of catechols. In terms of the meta-cleavage of 4-alkylcatechols by extradiol dioxygenases, molecular oxygen can be incorporated at a position near to the alkyl substitution or at a position distant from the substitution, which are called (2,3) proximal and (1,6) distal cleavages, respectively (Nozaki et al., 1970). The site of ring cleavage of the C23O of strain KL28 (LapB) was determined

Table 3. Identification of products formed from (alkyl)phenols by E. coli DH5$\alpha$(pPJM02) expressing mPH

Conditions for biotransformation and GC/MS analysis are described in Methods. –, No products were detected.

*Yield was obtained from the mean of duplicate determinations in two independent experiments.

†Tentatively identified.
by comparing the visible spectra of the ring fission products with those formed by TodE and XylE. The latter two enzymes are found in the degradation pathways of toluene and xylenes and are known to catalyze the dioxygenation of 3- and 4-methylcatechol by (2,3) proximal cleavage (Cerdan et al., 1994; Cho et al., 2000; Klecka & Gibson, 1981; Ramos et al., 1987). In addition, XylE and TodE are known to catalyse the dioxygenation of 4-ethyloxybenol and 2,3-dihydroxybiphenyl, respectively, via the same mode of ring cleavage (Cerdan et al., 1994; Cho et al., 2000; Ramos et al., 1987). TodE and/or XylE produced maximum absorbance values ($\lambda_{\text{max}}$) from 3- and 4-methylcatechol, 2,3-dihydroxybiphenyl and 4-ethylcatechol, which were the same as the $\lambda_{\text{max}}$ values previously determined (Table 4). In addition, LapB yielded the same absorbance spectra with different levels of absorbance yielding the same $\lambda_{\text{max}}$ values as those formed by TodE and XylE from the same substrates, indicating a (2,3) proximal cleavage mode for LapB. LapB preferentially oxidized 4-methylcatechol and 4-ethylcatechol, with relatively weak activity upon 3-methylcatechol, catechol and 2,3-dihydroxybiphenyl. The meta-ring cleavage product of 4-ethylcatechol was not degraded in the absence of NAD$^+$ or NADP$^+$ by the cell extract of KL28 cells grown on 4-ethylphenol, indicating a lack of HMS hydrolase in the lap pathway.

**DISCUSSION**

_Pseudomonas_ sp. strain KL28 is an alkylphenol degrader, which can assimilate 4-n-alkylphenols with a side chain length of C$_3$–C$_5$. The range of compounds that are degraded by strain KL28 is similar to that found for an activated sludge isolate of _Pseudomonas veronii_ INA06 (Ajithkumar et al., 2003), which can assimilate phenol, p-cresol and other 4-n-alkylphenols (C$_3$–C$_5$). However, because strains KL28 and INA06 cannot use octyl- or nonylphenol as a carbon source, they are distinguished from other strains which include the _P. putida_ phh operon from _R. eutropha_ P35X (Ng et al., 1994), the _phi_ operon from _P. putida_ H (Herrmann et al., 1995), the _pxo_ operon _R. eutropha_ E2 (Hino et al., 1998), the _phc_ operon from _C. testosteroni_ R5 (Teramoto et al., 1999), the _phe_ operon from _Ralstonia_ sp. KN1 (Nakamura et al., 2000), and the _phk_ operon from _Burkholderia kururiensis_. While the deduced amino acid sequences of the _lap_ genes are 33–74% identical to the corresponding proteins in the _dmp_ and _aph_ operons or show identities similar to isofunctional proteins from other phenol catabolic operons, the arrangement of the _lap_ catabolic genes differs from those of known phenol catabolic genes that include mPH genes. In most cases, mPH genes are followed by genes encoding a XylT-type ferredoxin (Hugo et al., 1998) and C23O, which are equivalent to _dmpQB_ and _aphQB_ in _P. putida_ and _TA441_ (Fig. 3). Other examples include the _phh_ operon from _P. putida_ P35X (Ng et al., 1994), the _phi_ operon from _P. putida_ H (Herrmann et al., 1995), the _pxo_ operon _R. eutropha_ E2 (Hino et al., 1998), the _phc_ operon from _C. testosteroni_ R5 (Teramoto et al., 1999), the _phe_ operon from _Ralstonia_ sp. KN1 (Nakamura et al., 2000), and the _phk_ operon from _Burkholderia kururiensis_.

The chromosomally encoded _lap_ catabolic gene cluster of strain KL28 can be compared to the _dmp_ and _aph_ phenol catabolic gene clusters, where all the operonic genes including those for mPH have been cloned and sequenced. The _dmp_ gene cluster located in the pV1150 catabolic plasmid of _Pseudomonas_ sp. strain CF600 allows strain CF600 to grow on phenol, o-, m- or p-cresol, 3,4-dimethylphenol or 2-ethylphenol as sole sources of carbon and energy (Shigler et al., 1989). Regulatory mutants of strain CF600 also have the ability to grow on 4-ethylphenol as a carbon and energy source (Sandar et al., 2001). However, the wild-type and mutant strains have not been reported to grow on higher alkylphenols. _C. testosteroni_ TA441, an isolate from the gut of the wood-feeding termite _Reticulitermes speratus_, does not grow on phenol as a sole carbon source initially, but it is able to utilize phenol by derepression of the _aph_ gene cluster after adaptation in a medium containing phenol as a sole source of carbon and energy (Arari et al., 1998).

While the deduced amino acid sequences of the _lap_ genes are 33–74% identical to the corresponding proteins in the _dmp_ and _aph_ operons or show identities similar to isofunctional proteins from other phenol catabolic operons, the arrangement of the _lap_ catabolic genes differs from those of known phenol catabolic genes that include mPH genes.

**Table 4. Activity of C23Os in cell extracts of cloned E. coli**

Experimental conditions are described in Methods. –, No detectable activity; NA, not applicable.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$\lambda_{\text{max}}$ (nm) of product/absorbance by</th>
<th>Cleavage mode of LapB</th>
<th>Activity of LapB (mU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TodE</td>
<td>XylE</td>
<td>LapB</td>
</tr>
<tr>
<td>Catechol</td>
<td>376/0·75</td>
<td>376/0·86</td>
<td>376/0·97</td>
</tr>
<tr>
<td>3-Methylcatechol</td>
<td>389/0·60</td>
<td>389/0·58</td>
<td>389/0·41</td>
</tr>
<tr>
<td>4-Methylcatechol</td>
<td>382/0·38</td>
<td>382/0·82</td>
<td>382/0·81</td>
</tr>
<tr>
<td>4-Ethylcatechol</td>
<td>–</td>
<td>381/0·21</td>
<td>381/0·82</td>
</tr>
<tr>
<td>2,3-Dihydroxybiphenyl</td>
<td>434/0·49</td>
<td>434/0·57</td>
<td>434/0·58</td>
</tr>
</tbody>
</table>

*(2,3) Proximal cleavage.
Fig. 3. Comparison of the lap gene cluster of strain KL28 with the (methyl)phenol degradative genes of Pseudomonas sp. CF600 (dmp) and of C. testosteroni TA441 (aph). Gene designations are described in Table 1 with the following additions: dmpO and aphO, ferredoxin genes; dmpD, HMS hydrolase gene; aphS and aphT, transcriptional regulator genes; aphX, aphY and aphJ, unknown genes.

(unfinished, GenBank accession no. AB063252). In the case of the mop operon from Acinetobacter calcoaceticus NCIB 8250, the genes encoding mPH are followed by a catechol 1,2-dioxygenase (C12O) gene (Ehrt et al., 1995). Furthermore, in the mph operon from A. calcoaceticus PHEA-2, the genes encoding mPH and C12O are separated by six ORFs that are related to benzoate degradation (Xu et al., 2003). In contrast, in the lap genetic organization, the gene encoding C23O precedes the genes encoding mPH, which are followed by genes encoding enzymes that degrade HMS derivatives to TCA cycle intermediates. In addition, the order of the rest of the genes for the meta pathway operon of strain KL28 differs from those of Pseudomonas sp. CF600 and C. testosteroni TA441 (Fig. 3). The deduced gene products from a DNA fragment encoding bupBA1A2A3A4A5A6 from the butylphenol degrader P. putida MT4 (GenBank accession no. AB107791) showed highest sequence identity (65–98%) to lapBKLNMNOP with the same gene order, indicating that its gene organization may be similar to that of the lap operon. The lap gene products also showed high sequence identities (61–87%) with unassigned gene products found in the Azoto-
bacter vinelandii genome (GenBank accession number NZ_AAAU02000027). In the latter case, the order of the gene cluster is the same as the lap catabolic operon, but a group of eight unrelated ORFs is inserted between the genes corresponding to lapP and lapC. These results indicate that the genetic organization of the lap operon is an emerging module for meta-cleavage operons.

One of the intriguing features in the lap gene cluster is that it lacks a gene encoding ferredoxin, which is generally located adjacent to the gene for C23O, as shown in the above examples of the dmp, aph, phi, phi, pox, phi, phe and phk operons for phenol catabolism. The role of ferredoxin (XylT) from the TOL plasmid has been studied in detail. XylT contains a plant-type [2Fe–2S] cluster, and is known to reactivate C23O (XylE), when XylE is inactivated by the oxidation of the ferrous iron at the active site during catalysis (Hugo et al., 1998). The inactivation of XylE by 4-methylcatechol was dramatic and hence it was shown that such a ferredoxin gene is necessary for degradation pathways using 4-methylcatechol as an intermediate. This property enables XylE to degrade 4-methylcatechol in addition to 3-methylcatechol and thus to extend the catabolic capacity of the degradation pathway (Polissi & Harayama, 1993). It was unexpected that a ferredoxin gene was not found adjacent to the gene for C23O in the lap gene cluster, because the lap pathway contains enzymes that metabolize alkylphenols that form 4-methylcatechol and other 4-alkylcatechol intermediates. However, LapB maintained high activity with 4-methyl-
catechol and 4-ethylcatechol with the cell extract obtained from recombinant E. coli (Table 4), suggesting the enzyme is active even in the absence of XylT-type ferredoxin. The requirement for ferredoxin might be absolute for the catalysis of 4-methylcatechol by C23Os that are found in degradation pathways utilizing both 3- and 4-methylcatechols. However, enzymes encoded by the lap operon constitute a degradation pathway utilizing exclusively 4-alkylcatechols; thus it is assumed that LapB might have been optimally evolved to catalyse 4-alkylcatecols without the aid of ferre-
doxin. In the available databases, more than 250 extradiol-
type dioxygenases were found. The amino acid sequence of LapB is closest to Avin0687 from an A. vinelandii genome and the partial sequence of BupB from strain MT4, with identities of 84 and 65 %, respectively. These three enzymes might have arisen from the same origin because the gene organizations in their operons are similar, as mentioned above. LapB is next closest to the C23Os from P. putida UCC22(pTDN1) (GenBank accession no. BAB62050), P. stutzeri OX1 (GenBank accession no. CAD43168) and P. putida plasmid pDK1 (GenBank accession no. A42733), with identities of 62, 52 and 52 %, respectively. These genes participate in the degradation of aniline, o-xylene and tolene/xylene, respectively (Benjamin et al., 1991; Bertoni et al., 1996; Saint & Venables, 1990) and have XylT-type ferredoxin associated with the genes for C23O. In terms of the divergence in amino acid sequence and catalytic properties, LapB, probably with Avin0687 and BupB, appears to belong to its own subfamily of extradiol dioxygenases, which were categorized by Eltis & Bolin (1996).
The first step in *lap* degradation is the conversion of 3- and 4-*n*-alkylphenols to 4-alkylcatechols, which is catalysed by mPH (LapKLMNOP) (Table 1). Phenol is also oxidized to catechol by flavin-containing single-component phenol hydroxylases (Ballou, 1982). It is important to note that the mPH in strain KL28 forms only 4-alkylcatechols from hydroxylation of 3-alkylphenols (Table 3). Many single and multicomponent phenol hydroxylases characterized to date catalyse *m*-cresol to 3-methylcatechol or a mixture of 3- and 4-methylcatechol (Arenghi et al., 2001; Johnson & Olsen, 1997; Kukor & Olsen, 1992; Shingler, 1996). An exception is C23O from the thermophilic *Bacillus stearothermophilus*, which catalyses the conversion of *m*-cresol to 4-methylcatechol (Buswell, 1975). In addition, the aforementioned phenol hydroxylases oxidize 2-alkylphenols to 3-alkylcatechols with high activity, whereas the mPH of KL28 shows weak or no activity toward 2-alkylphenols (Table 3), indicating that mPH in the *lap* pathway possesses a unique specificity. It is further noted that the ability of mPHKL28 to oxidize indole to indigo is dissimilar from that of mPHCF600, which has been reported to produce a red pigment, not indigo (Powlowski & Shingler, 1994). Understanding of the reaction mechanism and the regio-specificity of mPH will be greatly aided by elucidation of its structure.

The ring fission products (variably substituted HMS) formed by C23O can be further degraded by dehydrogenation (oxalocrotonate branch) or hydrolysis (hydrolytic branch), where the former needs an electron acceptor such as NAD$^+$ (Fig. 4). It was first observed in *P. putida* U that the ring fission products formed from 3-methylcatechol and (4-methyl)catechol follow the hydrolytic and oxaloacronate branches, respectively (Sala-Trepat et al., 1972; Wigmore et al., 1974). This was further confirmed in *Pseudomonas* sp. CF600 and *P. putida* containing the TOL plasmid pWWO, with mutants defective in the specific genes (Harayama et al., 1987; Powlowski & Shingler, 1994).

The lack of formation of 3-alkylcatechol intermediates in the *lap* pathway may have led evolutionarily to a deficiency of the hydrolytic branch enzyme (equivalent to *dmpD* in strain CF600). The latter enzyme is also not found in the *aph* pathway in *C. testosteroni* TA441, which degrades phenol but not alkylphenols (Arai et al., 2000) (Fig. 3).

On the basis of the substrate specificities of mPH and C23O in the *lap* operon, and of the amino acid sequence similarities between the *lap* gene products and known enzymes, the 3- and 4-alkylphenol degradation pathway present in strain KL28 can be depicted as shown in Fig. 4. This pathway contrasts with the degradation pathways for *p*-cresol by *P. putida* NCIMB 9869 and NCIMB 9866 (Kim et al., 1994) and 4-ethylphenol by *Pseudomonas* sp. JD1 (Reeve et al., 1989), which start degradation from the side chain, but is compatible with the *p*-cresol degradation pathways present in *P. putida* U (Bayly et al., 1966), *Alcaligenes eutrophus* 345 (Hughes et al., 1984) and *Pseudomonas* sp. CF600 (Shingler, 1996), and part of the degradation pathway for alkylbenzoates in *P. putida* containing the TOL plasmid (Ramos et al., 1987). The results obtained from assays of GFP-based promoter activity and the substrate specificities of the first two enzymes (mPH and C23O) revealed that the *lap* pathway present in strain KL28 is best suited for the catabolism of *m*- and *p*-cresol and 3- and 4-ethylphenol, and that it may be expanded further to accommodate larger side chains due to the relaxed specificities of catabolic enzymes, as well as a regulatory protein.

**ACKNOWLEDGEMENTS**

This work was supported by a Korea Research Foundation Grant (KRF 200-005-D20020). We would like to thank Professor David T. Gibson (University of Iowa) for providing plasmid pDTG617 and strains *P. putida* G7.C-1 and *P. putida* mt-2 for this study, and Dr Daniel J. Lessner (University of Iowa) for critical reading and suggestions.

**Fig. 4.** The 3- and 4-*n*-alkylphenol degradation (*lap*) pathway in *Pseudomonas* sp. KL28. HMS derivatives undergo the 4-oxalocrotonate branch that is initiated by LapC but do not follow the hydrolytic branch because of the lack of HMS hydrolase.
REFERENCES


