

## Identification of a *p*-Cresol Degradation Pathway by a GFP-Based Transposon in *Pseudomonas* and Its Dominant Expression in Colonies

Cho, Ah Ra, Eun Jin Lim, Yaligara Veeranagouda, and Kyoung Lee\*

Department of Microbiology, Changwon National University, Changwon-si, Kyongnam 641-773, Korea

Received: April 8, 2011 / Revised: June 29, 2011 / Accepted: July 20, 2011

**In this study, the chromosome-encoded *pcuRCAXB* genes that are required for *p*-cresol degradation have been identified by using a newly constructed green fluorescent protein (GFP)-based promoter probe transposon in the long-chain alkylphenol degrader *Pseudomonas alkylphenolia*. The deduced amino acid sequences of the genes showed the highest identities at the levels of 65–93% compared with those in the databases. The transposon was identified to be inserted in the *pcuA* gene, with the promoterless *gfp* gene being under the control of the *pcu* catabolic gene promoter. The expression of GFP was positively induced by *p*-cresol and was about 10 times higher by cells grown on agar than those in liquid culture. In addition, *p*-hydroxybenzoic acid was detected during *p*-cresol degradation. These results indicate that *P. alkylphenolia* additionally possesses a protocatechuate *ortho*-cleavage route for *p*-cresol degradation that is dominantly expressed in colonies.**

**Keywords:** *Pseudomonas alkylphenolia*, *p*-cresol methylhydroxylase, promoter-probe, *gfp* reporter, *p*-cresol degradation, transposon

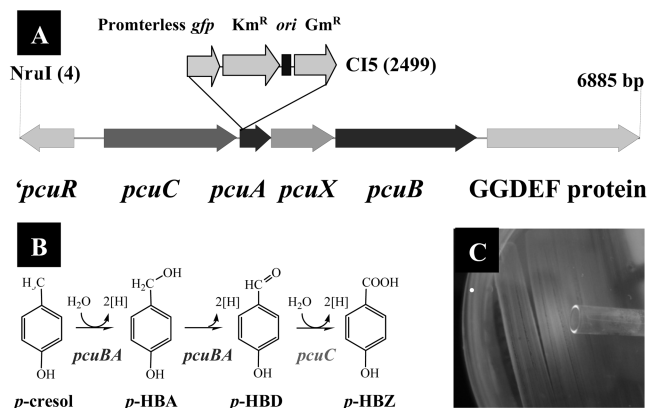
Cresols are isomers of methylphenols. They are manufactured in a large amount for disinfectants and solvents and are raw materials for various chemical products such as perfumes, antioxidants, dyes, and resins [14]. Cresols are found in petroleum, coal tar, auto and diesel exhaust, and tobacco smoke and are also produced in nature. In particular, *p*-cresol is contained in animal urines [21] and is also known as one of the most unpleasant gases in “pig odor” produced from pig farms [3]. Humans exposed to high levels of *p*-cresol can undergo the risk of adverse health effects [14].

Aerobic and anaerobic microorganisms that can use *p*-cresol as the sole source of carbon and energy are easily isolated from nature. Two biodegradation pathways of *p*-cresol by aerobic bacteria have been described [1]. First, *p*-cresol is initially oxidized to 4-methylcatechol by monooxygenases, which either contain flavin or di-iron at the active site. The oxidation product is further degraded via a *meta*-cleavage pathway into TCA cycle intermediates. The representative examples are a single-component enzyme encoded by the *tbuD* gene from *Pseudomonas pickettii* PKO1 [17] and a multicomponent oxygenase from *Pseudomonas* sp. strain CF600 encoded by *dmpKLMNOP* [22], respectively. In the *Pseudomonas alkylphenolia* KL28 strain, the Lap route is known to degrade long-chain alkylphenols including *p*-cresol by a similar degradation pathway present in *Pseudomonas* sp. CF600 [4, 13].

The other pathway involves the oxidation of the methyl group of *p*-cresol to yield *p*-hydroxybenzaldehyde via an intermediate *p*-hydroxybenzyl alcohol by *p*-cresol methylhydroxylase [12] (Fig. 1). Then, the formed product is oxidized to *p*-hydroxybenzoate by a dehydrogenase and to protocatechuate by a flavin-containing monooxygenase. Protocatechuate undergoes *ortho*-cleavage to be channeled into the TCA cycle intermediates [11]. The genetic organization (Fig. 1) encoding *p*-cresol methylhydroxylase and *p*-hydroxybenzaldehyde dehydrogenase is known to be conserved and forms an operon in *Pseudomonas* [27]. The gene clusters are named *pchACXF* in *P. putida* NCIMB 9866 and NCIMB 9869 [15] and *pcuCAXB* in *Pseudomonas mendocina* KR1 [27] and *Pseudomonas fluorescens* PC18 and PC24 [14]. The genes *pchA/pcuC* are known to code for *p*-hydroxybenzaldehyde dehydrogenase and *pchCF/pcuAB* for *p*-cresol methylhydroxylase. The latter enzyme is present in the periplasmic space as an  $\alpha_2\beta_2$  subunit composition. The large and the small subunits of the *p*-cresol methylhydroxylase contain FAD and a c-type cytochrome, respectively [7]. The function of the gene product encoded on *pchX/pcuX* is still unknown. The

\*Corresponding author

Phone: +82 55 213 3486; Fax: +82 55 213 3480;  
E-mail: kyounglee@changwon.ac.kr



**Fig. 1.** Characterization of the CI5 mutant.

(A) Genetic organization of the transposon-inserted chromosome segment in the CI5 mutant. The transposon portion from promoterless *gfp* to Gm<sup>R</sup> is also included. (B) Biodegradation pathway of *p*-cresol to *p*-hydroxybenzoic acid (*p*-HBZ) through *p*-hydroxybenzyl alcohol (*p*-HBA) and *p*-hydroxybenzaldehyde (*p*-HBD) by the *pcu* gene products. (C) Exhibition of green fluorescence by spread colonies exposed to *p*-cresol vapor for 15 days. The supply of *p*-cresol in a tube was previously described [19].

promoter for the *pch/pcu* catabolic operons is shown to be under the control of a  $\sigma^{54}$ -dependent XylR-type transcriptional activator, which is the gene product of *pchR/pcuR*. These genes are located upstream of and are transcribed divergently from the catabolic genes [14, 27].

In this study, a newly constructed GFP-based promoter probe transposon has been applied to tag the genes expressed by *p*-cresol on agar plates in strain *P. alkylphenolia*. As a result, the genes encoding *p*-cresol methylhydroxylase have been identified and the expression level in different culture conditions has been characterized.

## MATERIALS AND METHODS

### Strains and Culture Conditions

*P. alkylphenolia* KL28 (KCTC22206) as an alkylphenol degrader has been previously described. Plasmids have not been identified in this strain [13]. Strain KL28 was cultured on Luria–Bertani (LB) medium [2] or minimal salts basal medium (MSB) [23] with appropriate carbon source(s) at 30°C. *Escherichia coli* cells were grown on LB medium at 37°C. Whenever required, cells were cultured on agar plates prepared from media with 1.5% agar. For maintenance of the plasmids in cells, an appropriate antibiotic was added in the media with amounts as previously described [28].

### Construction of a Mutant Library with New Transposon

To facilitate recovery of the transposon-mutated gene, an *E. coli* replication origin *oriR6K* was introduced inside the transposon in the previous GFP-based transposon vector, mini-Tn5*gfp*-Km [25]. To this end, the DNA fragment containing the replication origin (*oriR6K*) was retrieved following *Sac*I digestion of pRL27 and cloned in the *Sac*I site in p34S-Km [9], and then the *oriR6K* cassette was cloned into the *Kpn*I site in mini-Tn5*gfp*-Km. The constructed

transposon vector was named as pSH2. The genetic organization of pSH2 inside the inverted repeats is depicted in Fig. 1.

For transposon mutagenesis of strain *P. alkylphenolia* KL28, tri-parental mating with *P. alkylphenolia* KL28, *E. coli* DH5 $\alpha$ / $\lambda$ *pir*(pSH2), and *E. coli* HB101(pRK2013) was carried out as previously described [10]. To isolate transconjugants, the mated-cell pools were spread on MSB agar with 5 mM sodium pyruvate, ampicillin, and gentamicin and incubated at 30°C. After 3 days, the colonies producing GFP were marked and then dried on 3MM papers that had been immersed in 0.01% *p*-cresol in acetone and placed in the lid of the plates. The plates were incubated upside down at 30°C for 3 days, and colonies producing new GFP were isolated. One of the strong GFP producers was isolated and designated as CI5 mutant.

### Identification of the Mutated Gene in CI5 Mutant

Genomic DNA isolated from mutant CI5 was partially digested with *Bcl*II or *Nru*I and purified using a GFX purification kit (Amersham Biosciences). The digested DNA fragments were self-ligated with T4 DNA ligase and then introduced into *E. coli* DH5 $\alpha$ / $\lambda$ *pir* competent cells [20]. Transformants were selected on LB agar with gentamicin and ampicillin. The transposon junction plasmids obtained by this method were sequenced at Genotech Co. Ltd. (Taejeon, Korea) using an automated sequencing apparatus (ABI PRISM 377, PE Biosystems Inc.) with primers that were designed to read DNA sequences outward from the inverted repeat sequences, GFP-1 reverse primer (5'-TCACCCTCTCCACTGACAGAA-3') and Gm-end forward primer (5'-GCAGTGGCTCTCTATACAAAG-3'). ORFs were identified using the GETORF program (<http://bioweb.pasteur.fr>). The deduced amino acid sequences were compared with those in the protein sequence database (GenBank) using the BLASTX algorithm (<http://www.ncbi.nlm.nih.gov>). The nucleotide sequence obtained in this study was deposited in the NCBI nucleotide sequence database under the accession number JF773352.

### Biotransformation of *p*-Cresol and Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

To detect metabolites during utilization of *p*-cresol by strain KL28, a resting cell system with cells grown on *p*-cresol was used for biotransformation. To this end, strain KL28 was initially grown on 4 mM *p*-cresol in MSB liquid culture at 28°C in a rotary shaker at 160 rpm for 48 h, and then 4 mM *p*-cresol was additionally added to increase the cell density, and the culture was further incubated for 24 h. The cells harvested by centrifugation were resuspended to an OD<sub>600</sub> of 1 in 50 mM Na-phosphate buffer (pH 7.0) containing 10 mM glucose and 2 mM *p*-cresol. Biotransformation was conducted in 250 ml Erlenmeyer flasks at 28°C with shaking at 160 rpm for 2 h. Following incubation and centrifugation of the culture, the supernatants were extracted with ethyl acetate and concentrated as previously described [5]. To identify the metabolites in the extract, GC–MS was performed with a capillary column (Perkin Elmer Elite-5ms, 0.25 mm  $\times$  30 m, 0.25  $\mu$ m film thickness) as previously described [5] with the following modifications. The column temperature was initially kept at 60°C for 2 min and then programmed from 60 to 270°C at 10°C/min with a helium flow of 1 ml/min.

### GFP Expression Measurements

As a starting inoculum, CI5 mutant was cultured in LB medium in a reciprocal shaking incubator for 12 h at 30°C (160 rpm). Then cells

were recovered by centrifugation and the OD<sub>660nm</sub> of the cell suspension was initially adjusted to 0.2 with sterile saline and diluted to 10<sup>-3</sup>. Fifty µl of the final dilution was spread on MSB agar containing designated carbon source(s) (described in the figure legend) and incubated at 30°C for 3 days. For measurements of GFP expression, cells were scraped in 2 ml of sterile saline and removed from the plates, pelleted by centrifugation, and resuspended in saline. For liquid culture, the initial cell suspension (50 µl) obtained from LB medium was inoculated in 250 ml Erlenmeyer flasks containing 50 ml of MSB with designated carbon source(s) and incubated at 30°C on a reciprocal shaker at a speed of 160 rpm for 3 days. For fluorescence measurements, culture samples were washed with saline by centrifugation and the fluorescence intensity of cells in saline was analyzed using a spectrofluorophotometer (Model RF-5391PC; Shimadzu Co.) at excitation and emission wavelengths of 450 and 509 nm, respectively, with 3.0 nm wavelength splits. For background fluorescence measurements, the *P. alkylphenolia* KL28 strain was cultured under the same culture conditions and the fluorescences produced were subtracted. The specific fluorescence intensity of each sample was defined as previously described [6].

## RESULTS AND DISCUSSION

### Selection of Mutants with New Transposon

Tn5-derived minitransposons with a promoterless reporter gene have been substantially used to probe promoters that are specifically induced under special conditions [8]. For their many advantages, including easy detection, GFP has been frequently used as a reporter. For instance, the minitransposon vectors such as pAG408 [24] and mini-Tn5*gfp*-Km [25] adopted the modified *gfp* gene, showing stronger fluorescence signal in *E. coli* (18 ~ 45-fold) than that exhibited by the natural GFP, making this more suitable for monocopy gene analysis in single cells. The promoterless *gfp* gene is located directly inside one end of the inverted repeat sequence in an orientation that affords the generation of gene-operon fusions. By using these minitransposons, the *bph* gene cluster from *Comamonas testosteroni* NCIMB 10643 [16] and the genes induced at lower pH from *Agrobacterium tumefaciens* [25] have been identified. In these cases, to identify the gene mutated by the transposons, an experimental step involving a time-consuming shotgun cloning of the mutated gene to a vector should be followed. However, this step can be avoided by introducing a conditional origin of replication such as a  $\pi$ -protein-dependent replication origin, *oriR6K*, inside the transposon, such as constructed in plasmids [9] and pRL27 [18]. Nevertheless, these reported vectors do not contain the *gfp* gene for promoter probing. Thus, the transposon delivery vector pSH2 was newly constructed by introducing the *oriR6K* origin in the transposon of mini-Tn5*gfp*-Km to facilitate subsequent cloning of transposon insertion mutants, as described in Materials and Methods. The transposon delivery vector pSH2 still maintains the original features of mini-Tn5*gfp*-Km for

transposon delivery and mutant selection with gentamycin, kanamycin, and ampicillin resistance genes [25]. The procedure for identification of the mutated gene is as follows. The DNA isolated from mutants is first digested with a restriction enzyme that does not cut inside the transposon. The resulting DNA will include the transposon insertion along with the adjacent genomic DNA and is then ligated by T4 DNA ligase to be a transposon junction plasmid that replicates in *E. coli* expressing the  $\pi$ -protein, encoded by the *pir* gene. Here, simply the *E. coli* transformants that grow on LB with gentamicin or kanamycin can be selected, and the plasmids obtained from the transformants can be used to identify the mutated genes by nucleotide sequencing using the primers described in Materials and Methods.

### Identification of the Mutated Gene in CI5 Strain

To identify the mutated gene induced by *p*-cresol in the isolate CI5, the transposon junction plasmids from the mutant were obtained by one-step cloning as described in Materials and Methods. The transposon junction plasmids obtained from CI5 yielded the same restriction patterns (data not shown), which indicated that CI5 has a single mutation at one location. The nucleotide sequence containing 6,885 bp around the Tn5 transposon in one of the retrieved plasmids was then determined (Fig. 1). Sequence analysis revealed the transposon was inserted into the 5'-terminus of the gene encoding PcuA (at the 17<sup>th</sup> cysteine), along with a direct duplication of 9 bp (5'-GCCTTGCCC-3') that directly flanked the borders of Tn5. Further nucleotide sequence analysis of the retrieved chromosome identified the *pcuRCAXB* gene cluster that encodes a regulator and catabolic enzymes for the conversion of *p*-cresol to *p*-hydroxybenzaldehyde (Fig. 1). This result indicated that *P. alkylphenolia* also possesses a protocatechuate *ortho*-cleavage route [11] in addition to the Lap catabolic pathway for *p*-cresol catabolism. This gene cluster has been identified in *P. putida* NCIMB 9866, NCIMB 9869, *P. mendocina* KR1, and *P. fluorescens* PC18 and PC24 [14, 15, 27]. In addition, the same gene cluster has been identified in the genomes of *Aromatoleum aromaticum* EbN1 (strain: EbN1; old-name: *Azoarcus* sp. EbN1) and *Nitrobacter* sp. Nb-311A. The deduced amino acid sequences of the *pcuRCAXB* from *P. alkylphenolia* showed the highest identities at the levels of 65–93% compared with those in the database. The gene located downstream of the *pcuC* gene was analyzed to encode an enzyme containing a GGDEF motif (Fig. 1). However, the deduced amino acid sequence of the protein has limited homology to the proteins found in *Pseudomonas* in databases. The promoter located upstream of the *pcuC* was found to contain a  $\sigma^{54}$ -binding -24 and -12 nucleotide sequence elements TGGCAC-N<sub>5</sub>-TAGCT (consensus sequence 5'-TGGCAC-N<sub>5</sub>-TTGC(A/T)) [26] at the positions from 875 to 890. In addition, an inverted repeat nucleotide

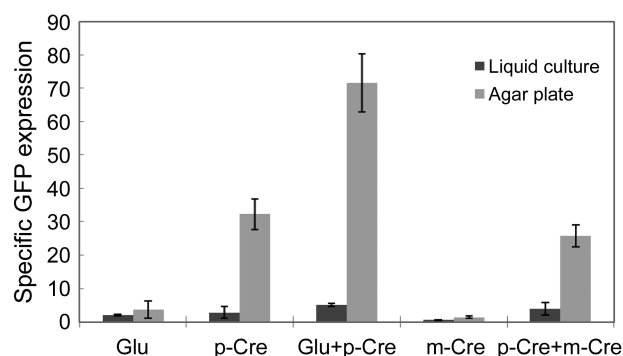
sequence 5'-CCCGCCAGGACGCACCTGGCGGG-3' (underlined) located downstream of the *pcuB* has been identified at the positions from 5,084 to 5,106, indicating that the transcription of the gene cluster could be terminated at the location.

### Identification of Metabolites Accumulated by Biotransformation of *p*-Cresol

In order to provide chemical evidence of a metabolite for the transposon-based experiment, the presence of an *ortho*-cleavage route recruiting the enzymes encoded on the *pcu* genes in *P. alkylphenolia* KL28 was investigated by surveying the intermediates accumulated during growing on *p*-cresol. Because our initial attempt to detect intermediates accumulated from ordinary culture supernatants was not successful, a resting cell system was adopted as described in Materials and Methods. During the incubation, a light-yellow color appeared, further supporting the presence of a *Lap meta*-cleavage route for *p*-cresol degradation [13]. In addition, GC-MS analysis with the ethyl acetate extract of the culture supernatant showed a peak identified to be *p*-hydroxybenzoic acid ( $R_t = 16.8$  min, a molecular ion at  $m/z$  138), as was the authentic compound. This result clearly showed the presence of a *p*-cresol degradation pathway involving *p*-cresol methylhydroxylase in strain KL28, as shown in Fig. 1B.

### Characterization of the Expression of *gfp* Gene Tagged on the *pcu* Catabolic Gene Cluster

Although the expression of *pcu/pch* catabolic genes has been known to be positively controlled by *p*-cresol [14, 15, 27], its expression on solid media has not been characterized. Thus, in this study, the *pcu* gene expression was determined with the CI5 mutant by monitoring the tagged GFP expression with different carbon sources when cells were growing in liquid and solid media. On MSB agar plates, the expression of the *gfp* gene was not induced by glucose or *m*-cresol and only induced by *p*-cresol, even in the presence of other carbon source such as glucose or *m*-cresol (Fig. 2). Interestingly, the expression was enhanced by *p*-cresol with glucose more than that expressed by *p*-cresol alone. GFP expression on MSB agar plate could be detected with the naked eye under a UV light emitting 365 nm (Fig. 1). In addition, the GFP expression was detected by cells grown on LB agar when *p*-cresol was supplied. In liquid cultures, although the expression of the *gfp* gene was stimulated in the presence of *p*-cresol, the expression levels were about 10 times less than those on agar plates (Fig. 2). The differences in cell density and the availability of substrates such as *p*-cresol, oxygen, and water between the two culture conditions may result in the difference in the expression of the catabolic genes. In particular, the concentration of oxygen in air will be much



**Fig. 2.** Specific GFP expression by the CI5 mutant.

The experimental details are described in the Materials and Methods. The carbon sources supplied in the media were Glu (for 2 mM glucose); p-Cre (for 1 mM *p*-cresol); and m-Cre (for 0.5 mM *m*-cresol). Mean values for three independent cultures are shown with standard deviation.

higher than in water. Further studies are warranted to determine the mechanism resulting in the difference in the expression between the two culture conditions. Since the *Lap* catabolic pathway is induced at very low levels by *p*-cresol on MSB agar [13], on solid media *p*-cresol is assumed to be predominantly degraded by a route encoded by the *pcu* genes in *P. alkylphenolia* KL28. This strain with two different pathways for *p*-cresol degradation may have advantages to adapt to changing nutrient availability, because each pathway may have different regulatory and induction characteristics, as shown in this study, and different kinetic parameters.

In this study, the newly constructed transposon delivery vector pSH2 has been proved to be useful for promoter probing and one-step cloning of transposon insertion mutants. Specifically, by *gfp* tagging of the gene encoding *p*-cresol methylhydroxylase using pSH2, the expression of the *pcu* genes in *P. alkylphenolia* could be characterized in addition to obtaining the nucleotide sequence information of the mutated gene and its flanking genes.

*P. alkylphenolia* KL28 has been shown to form mushroom-like aerial structures when cells were growing on agar plates with *p*-cresol being provided in a vapor phase [19]. The aerial structures could maintain their viability for several months to a year in air by forming ultramicrocells in the aerial structures. Combining this capability to the predominant expression of *pcu* genes on solid media would make this strain a good candidate to be used as an agent that can be incorporated in a biofilter to clean up *p*-cresol odor generated from pig farms and manufacturers.

### Acknowledgment

This research was financially supported by Changwon National University in 2009–2010.

## REFERENCES

- Bayly, R. C., S. Dagley, and D. T. Gibson. 1966. The metabolism of cresols by species of *Pseudomonas*. *Biochem. J.* **101**: 293–301.
- Bertani, L. E. and G. Bertani. 1970. Preparation and characterization of temperate, non-inducible bacteriophage P2 (host: *Escherichia coli*). *J. Gen. Virol.* **6**: 201–212.
- Borrell, B. 2009. Why study pig odor? *Sci. Am.* Accessed at <http://www.scientificamerican.com/article.cfm?id=why-study-pig-odor>.
- Cho, J. H., D. K. Jung, K. Lee, and S. Rhee. 2009. Crystal structure and functional analysis of the extradiol dioxygenase LapB from a long-chain alkylphenol degradation pathway in *Pseudomonas*. *J. Biol. Chem.* **284**: 34321–34330.
- Cho, M. C., D.-O. Kang, B. D. Yoon, and K. Lee. 2000. Toluene degradation pathway from *Pseudomonas putida* F1: Substrate specificity and gene induction by 1-substituted benzenes. *J. Ind. Microbiol. Biotechnol.* **25**: 163–170.
- Choi, E. N., M. C. Cho, Y. Kim, C. K. Kim, and K. Lee. 2003. Expansion of growth substrate range in *Pseudomonas putida* F1 by mutations in both *cymR* and *todS*, which recruit a ring-fission hydrolase CmtE and induce the *tod* catabolic operon, respectively. *Microbiology* **149**: 795–805.
- Cunane, L. M., Z. W. Chen, N. Shamala, F. S. Mathews, C. N. Cronin, and W. S. McIntire. 2000. Structures of the flavocytochrome *p*-cresol methylhydroxylase and its enzyme–substrate complex: Gated substrate entry and proton relays support the proposed catalytic mechanism. *J. Mol. Biol.* **295**: 357–374.
- de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis. 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in Gram-negative eubacteria. *J. Bacteriol.* **172**: 6568–6572.
- Dennis, J. J. and G. J. Zylstra. 1998. Plasposons: Modular self-cloning minitransposon derivatives for rapid genetic analysis of Gram-negative bacterial genomes. *Appl. Environ. Microbiol.* **64**: 2710–2715.
- Figurski, D. H. and D. R. Helinski. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. *Proc. Natl. Acad. Sci. USA* **76**: 1648–1652.
- Harwood, C. S. and R. E. Parales. 1996. The beta-ketoadipate pathway and the biology of self-identity. *Annu. Rev. Microbiol.* **50**: 553–590.
- Hopper, D. J. 1976. The hydroxylation of *p*-cresol and its conversion to *p*-hydroxybenzaldehyde in *Pseudomonas putida*. *Biochem. Biophys. Res. Commun.* **69**: 462–468.
- Jeong, J. J., J. H. Kim, C. K. Kim, I. Hwang, and K. Lee. 2003. 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. *Microbiology* **149**: 3265–3277.
- Joesaar, M., E. Heinaru, S. Viggor, E. Vedler, and A. Heinaru. 2010. Diversity of the transcriptional regulation of the *pch* gene cluster in two indigenous *p*-cresol-degradative strains of *Pseudomonas fluorescens*. *FEMS Microbiol. Ecol.* **72**: 464–475.
- Kim, J., J. H. Fuller, G. Cecchini, and W. S. McIntire. 1994. Cloning, sequencing, and expression of the structural genes for the cytochrome and flavoprotein subunits of *p*-cresol methylhydroxylase from two strains of *Pseudomonas putida*. *J. Bacteriol.* **176**: 6349–6361.
- Kim, J. S., J. H. Kim, E. K. Ryu, J.-K. Kim, C.-K. Kim, I. Hwang, and K. Lee. 2004. Versatile catabolic properties of the *Tn4371*-encoded *bph* pathway in *Comamonas testosteroni* (formerly *Pseudomonas* sp.) NCIMB 10643. *J. Microbiol. Biotechnol.* **14**: 302–311.
- Kukor, J. J. and R. H. Olsen. 1992. Complete nucleotide sequence of *tbuD*, the gene encoding phenol/cresol hydroxylase from *Pseudomonas pickettii* PKO1, and functional analysis of the encoded enzyme. *J. Bacteriol.* **174**: 6518–6526.
- Larsen, R. A., M. M. Wilson, A. M. Guss, and W. W. Metcalf. 2002. Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. *Arch. Microbiol.* **178**: 193–201.
- Lee, K. and Y. Veeranagouda. 2009. Ultramicrocells form by reductive division in macroscopic *Pseudomonas* aerial structures. *Environ. Microbiol.* **11**: 1117–1125.
- Miller, V. L. and J. J. Mekalanos. 1988. A novel suicide vector and its use in construction of insertion mutations: Osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* **170**: 2575–2583.
- Schmidt, E. G. 1949. Urinary phenols; the simultaneous determination of phenol and *p*-cresol in urine. *J. Biol. Chem.* **179**: 211–215.
- Shingler, V., J. Powlowski, and U. Marklund. 1992. Nucleotide sequence and functional analysis of the complete phenol/3,4-dimethylphenol catabolic pathway of *Pseudomonas* sp. strain CF600. *J. Bacteriol.* **174**: 711–724.
- Stanier, R. Y., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic pseudomonads: A taxonomic study. *J. Gen. Microbiol.* **43**: 159–271.
- Suarez, A., A. Guttler, M. Stratz, L. H. Staendner, K. N. Timmis, and C. A. Guzman. 1997. Green fluorescent protein-based reporter systems for genetic analysis of bacteria including monocopy applications. *Gene* **196**: 69–74.
- Tang, X., B. F. Lu, and S. Q. Pan. 1999. A bifunctional transposon mini-Tn5*gfp-km* which can be used to select for promoter fusions and report gene expression levels in *Agrobacterium tumefaciens*. *FEMS Microbiol. Lett.* **179**: 37–42.
- Thony, B. and H. Hennecke. 1989. The -24/-12 promoter comes of age. *FEMS Microbiol. Rev.* **5**: 341–357.
- Wright, A. and R. H. Olsen. 1994. Self-mobilization and organization of the genes encoding the toluene metabolic pathway of *Pseudomonas mendocina* KR1. *Appl. Environ. Microbiol.* **60**: 235–242.
- Yun, J. I., K. M. Cho, J. K. Kim, S. O. Lee, K. Cho, and K. Lee. 2007. Mutation of *rpoS* enhances *Pseudomonas* sp. KL28 growth at higher concentrations of *m*-cresol and changes its surface-related phenotypes. *FEMS Microbiol. Lett.* **269**: 97–103.