Studies on a novel degradation pathway of 4-chloroaniline and chemotaxis to 4-chloroaniline in *Pseudomonas* strains

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CHAPTER 1

General Introduction

Chloroanilines (CAs)

Chlorinated anilines are a group of chlorinated aromatic amines, and widely used as intermediate compounds in the production of polyurethanes, rubber, dyes and pigments, cosmetics (e.g., chlorohexidine, triclocarban, 3,4,4’-trichlorocarbanilid), pharmaceutics, and pesticides (Choy and Chu, 2005; Boon et al., 2001; Gosetti et al., 2010). CAs were used as a precursors in industrial production but also major persistent metabolites of phenyamide herbicides by microbial microbial degradation (phenylurea, acylanilide and phenylcarbamate) (Bartha, 1968; Wallnöfer, 1969; Bartha and Pramer 1970; Kaufman and Blake 1973). Herbicide degradation by microorganism causes CAs releasing and accumulation in soil and degradation of CA-containing products and releasing of industrial wastewater also cause CAs spreading into environment (Bollag et al., 1978; Haggblom, 1992).

![Molecular structure of monochloroaniline (4-chloroaniline)]

Figure 1.1. Molecular structure of monochloroaniline (4-chloroaniline)
4-Chloroaniline (4CA) an aniline chlorinated at the 4 \((\text{para})\) position (Fig1.1). All chloroaniline isomers are haematotoxic and show the same pattern of toxicity but in all cases 4CA shows the most severe effects (Boehncke et al., 2003). About 65% of the global annual 4CA production was processed in pesticides (Srour, 1989), 7.5% was used as dye precursors and 20% as intermediates in the cosmetics industry (Boehncke et al., 2003). There are assessments of consumer exposure to 4CA which report that a number of possible routes leads to total exposure of a maximum 300 ng/kg body weight per day (Boehncke et al., 2003). It was reported that 4CA in agricultural soils was detected with a maximum concentration of 968 \(\mu g/kg\) (Boehncke et al., 2003). The insecticide diflubenzuron and the herbicides monolinuron, buturon, propanil, chlorofenprop-methyl, benzoylpropmethyl, chloroaniformmethane, chlorobromuron, neburon, and oxadiazon can release 4CA as a degradation product.

CAs are toxic (haematotoxic, mutagenic and carcinogenic) (Crabtree et al., 1991; Chung et al., 1997; Bhunia et al., 2003; Boehncke et al., 2003). Due to their toxicity and recalcitrant properties, they have been considered as the important environmental pollutants and are subject to legislative control by the Priority Pollutant List of the United States Environmental Protection Agency (EEC, 1976; Federal Register, 1979). The widespread and large quantity of use of CAs have resulted in release into the environment through industrial wastewaters and their direct application to soil, especially, in agriculture soil (Meyer 1981). To dissimilate the environmentally contaminated CAs, bioremediation has been needed as a primary treatment technique by using degrading bacteria in a detoxification process.
**Dioxygenase enzymes**

Dioxygenase is an enzyme that is able to activate dioxygen, and incorporate oxygen atoms from dioxygen onto the substrate. (Bugg, 2003).

**Toluene dioxygenase**

Toluene dioxygenase is multicomponent enzyme system encoded by *todC1C2BA*. This system three protein components which contains form an electron transfer chain that catalyzes oxidization of toluene in toluene catabolic pathway in *Pseudomonas putida*. Toluene dioxygenase consists of NADH-dependent flavoprotein reductase, [2Fe2S] ferredoxin and iron-sulfur protein (two subunit of terminal dioxygenase) encoded by *todA*, *todB* and *todC1C2* respectively. In the catabolic initial step, toluene is oxidized to (+)-cis-(1S,2R)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol) through the addition of two oxygen atoms into a toluene molecule (Fig. 1.2) (Yeh et al., 1977; Subramanian et al., 1980; Zylstra and Gibson, 1989). This initial reaction is termed the dihydrodiol pathway.

*Cis*-Toluene dihydrodiol is then dehydrogenated mediated by *cis*-toluene dihydrodiol dehydrogenase encoded by *todD* to form 3-methylcatechol which is subsequently subject to *meta* cleavage by 3-methyl-catechol 2,3 dioxygenase encoded by *todE* leads to the formation of 2-hydroxy-6-oxo-2,4-heptadienoate (Zylstra and Gibson, 1989). The toluene degradation (*tod*) pathway (*todC1C2BADE*) is important in bioremediation of environmental pollutants. In this pathway, the aromatic hydrocarbons are oxidized to their corresponding substituted catechols which are further metabolized to TCA cycle intermediates. (Fig.1.3).
Figure 1.2. Initial reactions in the oxidation of toluene by toluene dioxygenase system to from dihydrodial compound (Yeh et al., 1977)

Figure 1.3. Toluene degradation pathway (metabolic pathway for the oxidation of toluene) (Zylstra and Gibson, 1989)
Catechol dioxygenase

Catechol dioxygenase is an enzyme catalyzing incorporation of two oxygen atom from dioxygen into substrates (catechol compounds) leading to a oxidative cleavage of catechol and substituted catechols. Oxidative cleavage of catechol is a key step in the bacterial degradation of aromatic compounds in the environment. There are two families of catechol dioxygenase enzymes which can catalyse the oxidative cleavage of catechol. They are (i) the intradiol dioxygenase (ortho cleavage), catechol 1,2-dioxygenase which cleaves the carbon-carbon bond between the phenolic hydroxyl groups to yield muconic acid as the product, (ii) the extradiol dioxygenase (meta cleavage), catechol 2,3-dioxygenase which cleaves the carbon-carbon bond adjacent to the phenolic hydroxyl groups to yield 2-hydroxymuconaldehyde as the product (Fig. 1.4) (Kojima et al., 1961; Hayaishi, 1966; Bugg, 2003).

![Diagram](image)

**Figure 1.4.** Reaction catalysed by intradiol and extradiol dioxygenaser showing ring-cleavage for the biodegradation of catechol substituted (Bugg, 2003)
Chemotaxis

Motile bacteria have the ability to sense changes in the concentration of chemicals in environments and respond to them by altering their pattern of motility. This behavioral response is called chemotaxis. The fact that bacterial movement is not random but is rather responsive to light (phototaxis), oxygen (aerotaxis), and certain chemicals was already discovered in the 1880s (Eisenbach, 2004). Since prokaryotic bacteria are small size and relative simplicity, their ability to adjust the environment to their needs is very limited. Instead, bacteria apparently adapt a strategy of moving from one environment to another (Macnab and Aizawa, 1984; Eisenbach, 2004). Chemotaxis is assumed to play important roles in environment, for examples, microbe-substrate interaction.

Methyl-accepting chemotaxis protein

Methyl-accepting chemotaxis proteins (MCPs) (Fig. 1.5) are chemotaxis sensory proteins responsible for detection of chemotatic ligands (Kato 2008). MCPs are membrane-spanning homodimers and typical features of MCPs are as follows: a positive charged N terminus followed by a hydrophobic membrane-spanning region, a hydrophilic periplasmic domain, a second hydrophobic membrane-spanning region and a hydrophilic cytoplasmic domain (Falke and Haselbauer, 2001). Chemotactic ligands bind to periplasmic domains of MCPs and their binding initiates chemotaxis signaling. The diverse ligand specificities among MCPs reflect amino acid sequence diversities of periplasmic domains of MCPs. The C-terminal cytoplasmic domains of MCPs are relatively conserved. A 44-amino-acid highly conserved domain (HCD) is located in the cytoplasmic domain. MCPs from phylogenetically diverse bacteria have been shown to
possess the HCD, which is important for the interaction of MCPs from the genomic sequences (Kato, 2008).

Figure 1.5. The structure of typical MCPs. The figure illustrates structural and functional features. MCPs are membrane spanning homodimers.

*Two-component regulatory system in chemotaxis*

Chemotaxis is regulated by two-component regulatory system, called Che system (Fig. 1.6). In *Escherichia coli*, the chemotaxis system consists of MCPs, six cytoplasmic chemotaxis proteins (Che proteins), and flagella (Kato, 2008). CheA and CheY are a histidine protein and a response regulator of the Che system, respectively (Kato, 2008). Chemoreceptors, CheA and coupling protein Che W form a non-covalent but notably
stable signaling complex, multi-component equivalents of single-protein sensor kinases, that couple ligand occupancy to kinase activity (Erbse and Falke, 2009; Hazelbauer and Lai, 2010). Attractant binding to a chemoreceptor inhibits auto phosphorylation activity of CheA, reducing the concentration of short-lived phosphorylated CheY (CheY-P) and switching the flagella rotation (Hazelbauer and Lai, 2010; Kato, 2008). CheZ is involved in dephosphorylation of CheY-P. The system is adapted by covalent modification of chemoreceptors, methylation of specific receptor glutamates by methyltransferase CheR and demethylation by merhylesterase CheB (Hazelbauer and Lai, 2010; Kato, 2008). CheB is another response regulator and receives the phosphoryl group from phosphorylated CheA (Kato, 2008). Phosphorylated CheB increases its methylesterase activity and, thus, the level of methylation of MCPS is controlled in response to environmental stimuli. This reversible methylation of MCPs is required for temporal sensing of chemical gradients. The chemotaxis machinery allows the organism to move toward attractants and away from repellents by a biased random walk (Kato, 2008).

**Figure 1.6.** Molecular mechanism of che gene cluster in *E. coli*. 
**Pseudomonads**

Pseudomonads is a motile Gram-negative rod-shaped bacterium. Pseudomonas is potential agents of geochemical cycles, bioremediation, and chemotaxis. Some members of Pseudomonas are able to metabolize chemical pollutants in environment and as a result can be used for bioremediation (Rehm, 2008; Shim and Yang, 1999). *P. putida* MT4 is able to degrade 4-n-butylphenol, which is a model pollutant for alkylphenol compounds, via *bup* gene cluster products (Takeo *et al*., 2006). *P. putida* CSV86 is able to degrade and detoxify various aromatics, including naphthalene, salicylate, benzyl alcohol, and methylnaphtatenes, via multiple pathway (Basu and Phale, 2008; Mahajan *et al*., 1994). *P. putida* UCC22 is able to oxidize aniline via *tdn* gene (Fukumori and Saint, 1996). *Pseudomonas acidovorans*, *Pseudomonas* sp. strain G and *Pseudomonas* sp. B13 can utilized aniline and chloroaniline as sole source of carbon and energy however, gene involvement had not been reported. (Loidl *et al*., 1990; Zeyer and Kearney, 1982; Latorre *et al*., 1984). *P. putida* T-57 can utilizes toluene via *tod* gene (Faizal *et al*., 2005), oxidation and reduction reaction whole cell are favored as biocatalysts. Toluene dioxygenase from T-57 exhibited broad substrate specificity. Therefore, solvent-tolerant bacteria as hosts and gene encoding enzymes that catalyze reactions of interest are necessary for the development of bioconversion and biodegradation processes for pullulated substrates.

Pseudomonas is thought to play an important role in microbe-substrate interactions, ecological aspects of chemotaxis have been intensively investigated in *Pseudomonas* species. Bacterial chemotaxis has been intensively studied in *E. coli* and *Salmonella enterica* serovar Typhimurium (Kato, 2008; Stock and Surette, 1996), but understanding the chemotaxis mechanisms in pseudomonads are limited. The 44-amino-acid HCD, which
is located between two methylation regions of MCPs, is highly conserved in prokaryotes. Therefore, it is easy to predict putative MCP genes from the genomic sequences. BLASTP analysis using the HCD sequence of \textit{E.coli} MCP, Tsr (IISV IDGI AFQT NILA LNAA VEAA RAGE QGRG FAVV AGEV RNLA) as a probe predicts that \textit{Pseudomonas aeruginosa} PAO1, possess 26 putative MCP genes, while there are only 5 MCP genes on the \textit{E. coli} genome, suggesting that pseudomonads have the potential to respond a much greater number of chemical stimuli compared to \textit{E. coli}. However, a limited number of MCPs have been characterized in pseudomonads (Kato, 2008).

\textit{Pseudomonas putida} T57

\textit{P. putida} is a motile, Gram-negative, rod-shaped and ubiquitous bacterium frequently isolated from polluted soil and paradigm of a metabolic versatile saprophytic soil bacterium (Kill \textit{et al.}, 2008). \textit{P. putida} T57 was isolated from activated sludge. It is strictly aerobic bacteria and shows high tolerance to organic solvents. \textit{P. putida} T57 strain exhibits the ability to degrade and utilized toluene, \textit{n}-butanol, styrene, \textit{m}-xylene, ethylbenzene, \textit{n}-hexane and propylbenzenes as growth substrates as a carbobe and energy source (Faizal \textit{et al.}, 2005). \textit{P. putida} T57 use the toluene dioxygenase pathway to catabolize toluene. To characterize toluene degrading pathway of \textit{P. putida} T57 strain, southern blot analysis was conducted using toluene dioxygenase genes (\textit{todC1C2BA}) and catechlo 2,3-dioxygenase gene (\textit{todE}) from \textit{P. putida} F1 as probes, \textit{todC1C2BA} and \textit{todE} probe hybridized \textit{EcoRI} fragment of \textit{P. putida} T57 genomic DNA, these result suggest that \textit{P. putida} T57 possesses sequences homologous to \textit{todC1C2BA} and \textit{todE} genes. Moreover, cosmid genomic library of \textit{P. putida} T-57 and screened with the \textit{todC1C2BA} probe and sequence analysis found sequences highly homologous more than 99% similarities to
todC1, todC2, todB, todA, todD, and todE, and gene order was same as that of the P. putida F1 tod operon. These results confirmed the presence of the toluene dioxygenase pathway in P. putida T-57 (Faizal et al., 2005). So toluene-degraded P. putida T57 is concerned to be used as a bacterial host for biodegradation via toluene dioxygenase pathway encoded by todC1C2BADE gene.

Pseudomonas aeruginosa PAO1

P. aeruginosa is a motile Gram-negative rod-shaped bacterium and has the potential pathogenicity for human beings and other mammals. Meanwhile, P. aeruginosa has a diverse metabolic competence (Kill et al., 2008) The first completed genome of P. aeruginosa is the genome of P. aeruginosa PAO1 (Kill et al., 2008; Stover et al., 2000). BLASTP analysis identifies 26 putative MCP genes in P. aeruginosa PAO1, and the characterized MCPs in P. aeruginosa PAO1 are more than other Pseudomonas species (Kato, 2008). Additionally, we have already constructed the MCP gene mutant library in P. aeruginosa PAO1. The MCP gene mutant library and gene-expression Escherichia-Pseudomonas shuttle vector pUCP18 allow heterologous complementally chemotaxis assay to analyze the MCP gene function of pseudomonads. Therefore, P. aeruginosa PAO1 is better model organism to investigate the chemotaxis in pseudomonads.
The scope of this study

CAAs have been extensively used in the industrial production. They are also one of the primary intermediates generated by microbial transformation of insecticides and herbicides. Because of their extensive application, they have been accumulated in the environment including industrial effluent, sludge as well as agricultural soil. Due to their toxicity and recalcitrant properties they have been considered as one of the important environmental pollutants. To dissimilate the environmentally contaminated CAs, bioremediation has been concerned as a one treatment technique for detoxification. Bacterial chemotaxis is one of the most important behavioral adaptation of bacteria to mediate a balance between nutritious and toxic effects of surrounding chemicals. Bacterial chemotactic attraction to pollutant can enhance biodegradation rate by increasing pollutant bioavailability. So, this study includes two main topics. First, biodegradation of CAs via toluene dioxygenas pathway from \( P. \) putida T-57. Second, identification of chemotaxis sensory protein for CAs in \( P. \) aeruginosa PAO1.

In the first topic, \( P. \) putida T57 is able to degrade toluene as a growth substrate (Faizal et al., 2005). Genes involved in toluene degradation was identified and characterized as \( \text{todC1C2BADE} \) encoding toluene dioxygenase, toluene \( \text{cis} \)-dihydodiol dehydrogenase, and 3-methylcatechol 2,3-dioxygenase respectively (Faizal et al., 2005). Until now, there has been no report about CA degradation by toluene dioxygenase pathway. So, It is expected that toluene dioxygenase system involved in CAs degradation and can enhance CAs degradation. In chapter 2, Investigated the ability of \( P. \) putida T57 toluene dioxygenase to degrade CAs and also tried enhancement of the ability of \( P. \) putida T57 to degrade CAs using gene of toluene dioxygenase pathway.
In the second topic, I investigated chemotactic responses of *Pseudomoas aeruginosa* PAO1 to 4CA, because its molecular biology of chemotactxis has been extensively characterized. There have been no reports about bacterial chemotaxis. But, if bacteria have the potential to chemotactically respond to 4CA, it is expected that bioremediation of 4CA could be speeded up by introduction of chemotactic ability toward 4CA into 4CA-degrading bacteria. BLASTP analysis identifies 26 putative MCP genes in *P. aeruginosa* PAO1 and MCP gene mutant library in *P. aeruginosa* PAO1 was already constructed by our laboratory. The MCP gene mutant library and gene-expression *Escherichia-Pseudomonas* shuttle vector pUCP18 allow heterologous complementally chemotaxis assay to analyze the MCP gene function of *Pseudomonads*. It expected that identification of chemotaxis sensory protein for CAs lead to construction of chemotaxis-induced bacterial strain more effective agent for bioremediation. In chapter 3, identification and characterization of chemotaxis sensory protein for CAs in *P. aeruginosa* PAO1 is described.
CHAPTER 2

Degradation of 4-chloroaniline by toluene dioxygenase from *Pseudomonas putida* T57

Introduction

Chloroanilines (CAs) are industrial chemicals widely used as intermediate compounds in the production of pesticides, polyurethanes, rubber, pharmaceutical products, and dyes (Kearney and Kaufmann, 1975; Gheewala and Annachhatre, 1997). They have been registered in the high production volume chemical program of the Organization for Economic Co-operation and Development (OECD, 2004). In addition, they are generated by microbial transformation of herbicides such as phenylcarbamate, phenylurea, and acylanilides in agricultural soils (Radatingtyas et al., 2003). As a consequence of intensive applications in agriculture and industries, they have been ubiquitously accumulated in the environment including agricultural soil/water, industrial effluents, and sludge. Due to their toxicity and recalcitrant properties, they are subject to legislative control by the Priority Pollutant List of U.S. Environmental Protection Agency (Federal Register, 1979). To dissipilate the environmental contaminated chloroanilines, bioremediation has been noted as a primary treatment technique in which a detoxification process is depending on the microbial biodegradation. To develop bioremediation technologies for chloroaniline-polluted sites, several chloroaniline-degrading bacteria have been isolated. They include *Pseudomonas* sp. JL2, *Delftia acidovorans* CA28, *Comamonas testosterone* 12, *Acinetobacter baumannii* CA2, *Pseudomonas putida* CA16, *Delftia tsuruhatensis* H1, and *Acinetobacter baylyi* GFJ2 (Latorre et al., 1984; Loidl et al., 1990; Boon et al., 2001; Vangnai and Petchkroh, 2007; Zhang et al., 2010; Hongsawat and
Metabolite analysis, together with analogy with the reaction catalyzed by aniline dioxygenase (Aoki et al., 1983), indicated that the initial step of chloroaniline degradation is an oxidative deamination by chloroaniline dioxybenzene to convert chloroanilines to corresponding chlorocatechols (Latorre et al., 1984; Hongsawat and Vangnai, 2011; Breugelmans et al., 2010). Chlorocatechols are then metabolized by ortho ring cleavage enzymes or meta ring cleavage enzyme. Although there are several reports concerning characterization of ring cleavage enzymes of chlorocatechols and their genes (Loidl et al., 1990; Vangnai and Petchkroh, 2007; Zhang et al., 2010; Hongsawat and Vangnai, 2011; Kim et al., 2007; Kaschabek et al., 1998; Hinteregger et al., 1992; Stockiger et al., 1992), information on the initial step and chloroaniline dioxygenase is very limited and only dcaA1, dcaA2, and dcaB gene in C. testosterone have been functionally demonstrated to encode chloroaniline dioxygenase (Król et al., 2012).

P. putida T57 was isolated from activated sludge of chemical factory as toluene-utilizing bacterium (Faizal et al., 2005). It metabolizes toluene via toluene dioxygenase pathway. In toluene dioxygenase (products of the todC1C2BA gene), which is oxidized to 3-methylcatechol by toluene cis-dihydrodiol dehydrogenase (the gene product of todD), and 3-methylcatechol is further metabolized by meta-cleavage enzyme, 3-methylcatechol 2,3-dioxygenase (the gene product of todE). Amino acid sequences of the P. putida T57 todC1C2BADE products are completely identical to those of P. putida F1 counterparts. P. putida toluene dioxygenase has a broad substrate specificity (Semak et al., 2012). In addition, we revealed that tod operon in both P. putida F1 and P. putida T57 was induced by 4-chloroaniline (4CA) as well as toluene (Vangnai et al., 2012). Therefore, we speculate that toluene dioxygenase could degrade/oxidize chloroanilines although there have been no reports about transformation of chloroanilines by toluene dioxygenase. In
this study, we investigated the ability of *P. putida* toluene dioxygenase to degrade chloroanilines. We also tried enhancement of the ability of *P.putida* T57 to degrade chloroanilines using genes of toluene dioxygenase pathway.
Experiment procedures

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 2.1. Escherichia coli DH5α was used for plasmid construction and DNA manipulation. E.coli was grown at 37˚C with shaking in Luria-Bertani (LB) medium (Samrook et al., 1989) supplemented with appropriate antibiotic. For solid medium, 2% agar was added to LB medium. P. putida strains were grown at 28˚C with shaking in 2xYT (Samrook et al., 1989) medium and MSBY medium supplemented with 0.5% ethanol (MSBYE medium). MSBY medium consisted of 4.3 g of K₂HPO₄, 3.4 g of KH₂PO₄, 2.0 g of (NH₄)₂SO₄, 0.34 g of MgCl₂.6H₂O, 0.001 g of MnCl₂.4H₂O, 0.006g of FeSO₄.7H₂O, 0.026 g of CaCl₂.2H₂O, 0.02 mg of Na₂MoO₄.2H₂O, 0.01 mg of ZnCl₂.7H₂O, 0.01 mg of CoCl₂.6H₂O, 0.01 mg of CuSO₄, 0.001 mg of NiSO₄.6H₂O and 0.001 mg of Na₂SeO₄ and 0.1 g of yeast extract per liter of deionized water. When necessary, kanamycin and ampicillin were added at 50 μg/ml and 50 μg/ml, respectively.
<table>
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<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tr>
<td><strong>Pseudomonas putida</strong></td>
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<tr>
<td>T57</td>
<td>wild type strain, <em>todC1C2BADE</em></td>
<td>Faizal et al., (2005)</td>
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<td><strong>Escherichia coli</strong></td>
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<tr>
<td>DH5α</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, φ80dlacZΔM15, Δ(lacZYA-argF)&lt;br&gt;U169, deoR, recA1, endA1, hsdR17&lt;br&gt;(r&lt;sub&gt;b&lt;/sub&gt;&lt;sup&gt;−&lt;/sup&gt;, m&lt;sub&gt;b&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;), phoA, supE44, λ&lt;sup&gt;−&lt;/sup&gt;, thi-1,&lt;br&gt;gyrA96, relA1</td>
<td>Takara Bio</td>
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<td><strong>Plasmids</strong></td>
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<td>pHA10</td>
<td><em>Escherichia-Pseudomonas</em> shuttle vector; Cb&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Arai et al., (1991)</td>
</tr>
<tr>
<td>pHK1</td>
<td><em>Escherichia-Pseudomonas</em> shuttle vector; Km&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pUC4K</td>
<td>pUC4 containing a 1.3-kb kanamycin resistance gene (<em>kan</em>) cassette; Ap&lt;sup&gt;−&lt;/sup&gt;, Km&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Vieira and Messing (1982)</td>
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<tr>
<td>pUC4K-C1C2BA</td>
<td>pUC4K with a 3.7-kb PCR fragment containing <em>todC1C2BA</em> from <em>P.putida</em> T57; Ap&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pUC4K-C1C2BADE</td>
<td>pUC4K with a 5.5-kb PCR fragment containing <em>todC1C2BADE</em> from <em>P.putida</em> T57; Ap&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pHK-C1C2BA</td>
<td>pHK1 with a 3.8-kb <em>BamHI-NsiI</em> fragment containing kan promoter-<em>todC1C2BA</em>; Km&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>pHK-C1C2BADE</td>
<td>pHK1 with a 5.6-kb <em>BamHI-NsiI</em> fragment containing <em>kan</em> promoter-<em>todC1C2BADE</em>; Km&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This study</td>
</tr>
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Ap<sup>−</sup>, ampicillin resistance; Cb<sup>−</sup>, carbenicillin resistance; Km<sup>−</sup>, kanamycin resistance.
**DNA manipulation**

Standard procedures were used for plasmid DNA preparations, restriction enzyme digestion, ligation, transformations, and agarose gel electrophoresis (Sambrook et al., 1989). PCR reactions were carried out using KOD Plus DNA polymerase (Toyobo, Tokyo, Japan) according to the manufacturer’s instruction. *P. putida* was transformed by electroporation as described previously (Faizal et al., 2005). DNA sequencing analysis was performed for PCR products to validate accuracy of PCR amplification.

**Plasmid construction**

Plasmid pHA10 is a broad-host-range vector with carbenicillin resistance gene as a selectable marker gene (Arai et al., 1991). Since *P. putida* T57 is inherently resistant to carbenicillin, carbenicillin resistance gene was replaced with *kan* gene from pUC4K (Vieira and Messing, 1982) to construct broad-host-range vector plasmid pHK1 for *P. putida* T57. A 3.7-kb DNA fragment containing the *todC1 todC2 todB todA* gene and a 5.5-kb DNA fragment containing the *todC1 todC2 todB todA todD todE* genes were amplified from *P. putida* T57 genome by PCR with C1-f (5’-ACAGTAATACAAGGGCGGCAATTGCTGCCAAGTC-3’)/A-r (5’-GTGTCCCGTTGAATAATGCATCGCAGCTCAAGTCTCAGTTA-3’) primer set and C1-f/E-r (5’-GTGTCCCGTTGAATAATGCATCGCAGCTCAAGTCTCAGTTATGGAAT-3’) primer set, respectively. A 3.9-kb DNA fragment was amplified from pUC4K by PCR with 4K-f (5’-CCCTTGGTATTACTGTTTTATGGAATGTTA-3’)/4K-r (5’TATTCAACGGGAAACGTTCTTCCCTCG-3’) primer set. Resulting 3.7-kb and 5.5-kb DNA fragment from *P. putida* T57 were fused with the 3.9-kb PCR product from pUC4K.
by an In-Fusion® HD cloning kit (Takara Bio, Shigma, Japan) to obtain pUC4K-C1C2BA and pUC4K-C1C2BADE, respectively. A 3.8-kb BamHI-NsiI fragment containing *kan* promoter- *todC1* *todC2* *todB* *todA* *todD* *todE* from pUC4K-C1C2BADE were cloned between BamHI and PstI site in pHK1 to obtain pHK-C1C2BA and pHK-C1C2BADE. In pHK-C1C2BA and pHK-C1C2BADE, the *tod* genes are under the control of strong and constitutive *kan* promoter.

**Biotransformation of chloroaniline and 4-chlorocatechol**

Biotransformation of chloroanilines and 4-chlorocatechol was performed with resting-cell suspension. *P. putida* cells, grown overnight in 2xYT medium, were inoculated (a 1% inoculums) into 200 ml MSBYE medium into 200 ml MSBYE medium in a 500-ml screw-capped Erlenmeyer flask. When necessary, toluene was provided in the vapor phase. After 14 h of cultivation, cells were harvested by centrifugation (6,000 g, 10 min, 4°C), and washed with MSBY twice and resuspended in the same medium at an optical density at 600 nm (OD600) of 5. Ten ml of the cell suspension and substrates were added to a 50-ml screwed capped vial, which was then incubated with shaking (150 strokes/min) at 28°C. Samples were periodically taken and the supernatant was subjected to metabolite analyses. When *E. coli* DH5α (pHK-C1C2BA) was examined for transformation of 4CA, bacterial cell, grown overnight in LB medium with kanamycin, were inoculated to 200 ml LB medium and cultivated at 37°C with shaking. After additional 3 h cultivation, cells were harvested by centrifugation (6,000 g, 10 min, 4°C), and washed with MSBY twice and resuspended in the same medium at OD500 of 5. This cell suspension was used for biotransformation of 4CA.
**Thin layer chromatography (TLC) analysis**

Supernatant of transformation mixture was extracted equal volume of ethyl acetate. The ethyl acetate extract was concentrated under vacuum. The residue was suspended in small amount of ethyl acetate and used for analysis. The metabolites were analyzed by TLC on silica gel 60 F254 plates (Merck KGaA, Darmstadt, Germany) using the solvent system chloroform: ethyl acetate: acetic acid (50:50:1 v/v/v). Metabolites were detected by ferric chloride (detection of phenolic compounds) (Malbaša et al., 2004). Metabolites were further analyzed by mass-spectrometry (MS). Each TLC spot was scrapped off and extracted with methanol. The extract was analyzed by MS using an LTQ Orbitrap XL (Thermo Fischer Scientific Inc., MA, USA) in the flow-injection mode.

**High performance liquid chromatography (HPLC) analysis**

Substrates and their metabolites were also analyzed by HPLC system (JASCO Co., Tokyo, Japan) with a reverse phase column (TSK gel ODS-80TM, Tosoh, Yamaguchi, Japan) at a flow rate of 0.8 ml/min. UV absorption was measured at 240 nm. The compounds were eluted by using linear gradient of 10-70% acetonitrile-water over 30 min.
Results

Toluene-induced *P. putida* T57 degraded 4CA

*P. putida* T57 possesses toluene-inducible toluene dioxygenase (Tod) pathway (Vangnai, 2012) and utilizes several aromatic compounds through Tod pathway (Faizal, 2005). Since toluene dioxygenase has broad substrate specificity (Semak et al., 2012), we supposed that toluene-induced *P. putida* T57 could degrade 4CA. We performed resting-cell reaction to examine *P. putida* T57 for its ability to degrade 4CA. Toluene-non-induced cells showed very weak, but significant activity of 4CA degradation (Fig. 2.1). Toluene-induced *P. putida* T57 cells degraded 4CA more rapidly than toluene-non-induced cells. This result indicates that toluene induces the additional ability to degrade 4CA in *P. putida* T57, which supports the idea that Tod pathway is involved in 4CA degradation.
**Figure 2.1.** 4CA degradation by *P. putida* T57. *P. putida* T57 cells grown in the presence of toluene (toluene-induced) (closed squares) and in the absence of toluene (toluene-non-induced) (closed triangles) were subjected to resting cell reaction using 0.1 mM 4CA as a substrate. Autoclaved cells were used as a negative control (closed circles). Data are means of the results at least three individual experiments. Error bars indicate standard deviations (SDs).
**P. putida T57 toluene dioxygenase oxidizes 4CA**

Initial step of Tod pathway, oxidation of aromatic compound, is catalyzed by toluene dioxygenase (Faizal et al., 2005). To confirm that Tod pathway is involved in 4CA degradation, we introduced *todC1C2BA* gene encoding toluene dioxygenase complex (toluene dioxygenase, ferredoxin and ferredoxin reductase) into *E. coli* DH5α and examined the resulting recombinant *E. coli* strain to oxidize 4CA. Resting-cell reaction indicated that *E. coli* DH5α (pHK-C1C2BA) degraded 4CA (Fig. 2.2A). TLC followed by ferric chloride staining found three phenolic metabolites (Fig. 2.2B) and subsequent MS analysis identified them as 4-chlorocatechol, 2-amino-5-chlorophenol, and 5-chloropyrogallol (Fig. 2.2C). In the previous studies, it was reported that the initial step of 4CA degradation in chloroaniline-degradation bacteria is oxidative deamination and its product is 4-chlorocatechol (Latorre at al., 1984; Król et al., 2012), 4-Chlorocatechol formation in *E. coli* DH5α (pHK-C1C2BA) resting cell reaction indicates that toluene dioxygenase catalyzes 1,2 dioxygenation of 4CA, which generates 4-chloroaniline-1,2-*cis*-dihydrodiol and it converts to 4-chlorocatechol by spontaneous deamination (Fig. 2.3A). Generation of 2-amino-5-chlorophenol suggests that toluene dioxygenase also catalyzes 2,3-dioxygenation of 4CA, which generates 4-chloroaniline-2,3-*cis*-dihydrodiol and it then converts to 2-amino-5-chlorophenol by spontaneous dehydration. It is supposed that 5-chloropyrogallol was generate by further dioxygenation of 4-chlorocatechol. In *E.coli* DH5α (pHK-C1C2BA) resting cell reaction using 4-chlorocatechol as a substrate, we also detected 5-chloropyrogallol (data not shown), supporting this idea.
Figure 2.2. 4CA degradation by *E. coli* DH5α strains (A), TLC analysis of metabolites (B), and MS analysis of metabolites (C). (A) Time course of 4CA concentrations in resting cell reaction using *E. coli* DH5α (pHA10) (closed circles) and *E. coli* DH5α (pHK-C1C2BA) (closed squares). Data are means of the results at least three individual experiments. Error bars indicate SDs. (B) Samples were taken at 0, 1.5, 2, 3, and 4 h after initiation of resting cell reaction and subjected to TLC analysis. TLC plate was visualized for detection of phenolic compounds by spraying FeCl₃ solution. Metabolites were recovered from TLC spots and analyzed by MS. (C) Mass spectra of metabolites from spots I to III. Spot II was contaminated by 4CC and 5-chloropyrogallol and their molecular ion peaks (m/z 142.99 and 158.99) as well as that of 2-amino-5-chlorophenol (m/z 142.01) were detected (panel “Spot II”).
**Figure 2.3.** Proposed metabolic pathways of 4CA in *E. coli* DH5α (pHK-C1C2BA) (A) and *P. putida* T57 (pHK-C1C2BADE) (B). I, 4-chloroaniline; II, 4-chloroaniline-1,2-*cis*-dihydrodiol; III, 4-chloroaniline-2,3-*cis*-dihydrodiol; IV, 4-chlorocatechol; V, 2-amino-5-chlorophenol; VI, 5-chloropyrogallol; VII, 3-amino-6-chlorocatechol. Bold arrows indicate routes which were experimentally confirmed. Absolute stereochemistry is not intended.
Król et al demonstrated that multi component chloroaniline dioxygenase encoded by dcaA1, dcaA2 and dcaB are responsible for conversion of 3CA to 4-chlorocatechol in Comamonas testosteroni WDL7 (Król et al., 2012). The dcaA1, dcaA2 and dcaB genes code for large and small subunits of chloroaniline dioxygenase, and chloroaniline dioxygenase reductase, respectively. Chloroaniline dioxygenase large subunit (DcaA1) shares high amino acid sequence identity (79-98% identity) with aniline dioxygenase large subunits of aniline-utilizing bacteria, Delftia acidovorans 7N (Urata et al., 2004), P. putida UCC22 (Fukumori and Saint, 1997), Delftia tsuhatensis AD9 (Liang et al., 2005), and Frateuria sp. ANA-18 (Murakami et al., 2003) while chloroaniline dioxygenase small subunit (DcaA2) shows 71-97% identity to aniline dioxygenase small subunits of these aniline-utilizing bacteria. Large (TodC1) and small subunits (TodC2) of P.putida T57 toluene dioxygenase show much lower identity to DcaA1 and DcaA2 (28% and 26%, respectively). The phylogenetic analysis also revealed that subunits of P. putida T57 toluene dioxygenase are phylogenetically distant from those of aniline/chloroaniline dioxygenases (Fig. 2.4). Nonetheless P. putida T57 toluene dioxygenase still catalyzes oxidative deamination from 4CA to 4-chlorocatechol. It is the first finding that toluene dioxygenase catalyses oxidative deamination of 4CA.
**Figure 2.4.** Phylogenetic trees of chloroaniline, aniline, and toluene dioxygenase large and small subunits. The phylogenetic trees were generated using Kimura method (Kimura, 1983) with GenBank COBALT multiple alignment software. The bars indicate a branch length equivalent to 0.5 change per amino acid. Dioxygenases analyzed (accession numbers of large and small subunits) are *C. testosteroni* WDL7 chloroaniline dioxygenase (AEV91088.1 and AEV91089.1), *D. acidovorans* 7N aniline dioxygenase (BAD61049.1 and BAD6150.1), *P. putida* UCC22 aniline dioxygenase (BAA12807.1 and BAA12808.1), *D. tsuruhatensis* AD9 aniline dioxygenase (AAX47241.1 and AAX47242.1), *Frateuria* sp. ANA-18 aniline dioxygenase (BAC82526.1 and BAC82527.1), and *P. putida* T57 toluene dioxygenase (BAN59728.1 and BAN59729.1).
Even after toluene induction, *P. putida* T57 showed only weak 4CA degradation ability. To enhance 4CA degradation ability of *P. putida* T57, we introduced plasmid pHK-C1C2BADE harboring the entire *tod* operon into *P. putida* T57 and resulting *P. putida* T57 (pHK-C1C2BADE) was subjected to resting cell reaction to evaluate 4CA degradation. *P. putida* T57 (pHK-C1C2BADE) showed approximately 250-fold higher 4CA degradation rate (1.5 mM/h) than toluene-induced parent strain did (5.7 μM/h) (Fig. 2.1 and Fig. 2.5A). Resting cell reaction was conducted at different initial concentrations of 4CA (1 to 4 mM) and it was revealed that *P. putida* T57 (pHK-C1C2BADE) completely degraded up to 2 mM 4CA (Fig. 2.5A). Thus, 4CA degradation ability can be enhanced by increasing gene dosage of *tod* operon in *P. putida* T57.

Resting cell reaction using *E. coli* DH5α (pHK-C1C2BA) suggests that toluene dioxygenase converts 4CA to 4-chloroaniline-1,2-*cis*-dihydrodiol and 4-chloroaniline-2,3-*cis*-dihydrodiol (Fig. 2.3A). Toluene-2,3-*cis*-dihydrodiol oxidoreductase (the gene product of *todD*) oxidizes toluene-2,3-*cis*-dihydrodiol to 3-methylcatechol (Faizal et al., 2005). Therefore, this enzyme is supposed to convert 4-chloroaniline-2,3-*cis*-dihydrodiol to 3-amino-6-chlorocatechol (compound VII in Fig. 2.3B). *P. putida* F1, which possess identical Tod pathway, can degrade 4-chlorocatechol, but not 3-chlorocatechol (Spain and Gibson, 1988; Klecka and Gibson, 1981). In resting cell reaction using 4-chlorocatechol as a substrate, *P. putida* T57 (pHK-C1C2BADE) completely degraded 0.5 mM 4-chlorocatechol and produced a yellow color compound (data not shown), suggesting that catechol 2,3-dioxygenase (the gene product of *todE*) catalyzes meta-cleavage of 4-chlorocatechol. Therefore, *P. putida* T57 (pHK-C1C2BADE) is supposed to degrade 4-chlorocatechol but not 3-amino-6-chlorocatechol nor 5-chloropylogallol. From these
result, the degradation pathway of 4CA in *P. putida* T57 (pHK-C1C2BADE) is proposed in Fig. 2.3B. It is noteworthy that *P. putida* T57 (pHK-C1C2BADE) could not utilize 4CA and 4-chlorocatechol as sole carbon and energy sources (data not shown).

*Substrate specificity*

Various chloroaniline, including 2-chloroaniline (2CA), 3-chloroaniline (3CA), 3,4-dichloroaniline (34DCA), and 3,5-dichloroaniline (35DCA) were tested for biodegradation by *P. putida* T57 (pHK-C1C2BADE). In resting cell reaction, degradation rates of 2CA, 3CA, 4CA, and 34DCA by *P. putida* T57 (pHK-C1C2BADE) were found to be 0.15mM/h, 0.5mM/h, 1.5mM/h, and 0.1mM/h, respectively (Fig. 2.5B). This strain hardly degraded 35DCA.
**Figure 2.5.** Effect of the initial 4CA concentration on its degradation (A) and resting cell reaction using different chloroanilines (B) by *P. putida* T57 (pHK-C1C2BADE). (A) The initial 4CA concentrations were 1 mM (closed circles), 2 mM (closed squares), 3 mM (closed triangles), and 4 mM (closed diamonds). (B) Closed triangles, 2CA; closed squares, 3CA; closed circles, 4CA; open squares, 34DCA; open circles, 35DCA.
Discussion

We, for the first time, demonstrated that *P. putida* toluene dioxygenase can oxidized chloroanilines. It catalyzes 1,2- and 2,3-dioxygenation of 4CA. 1,2-Dioxygenation of 4CA results in oxidative deamination of 4CA and 4-chlorocatechol is generated. Introduction of tod operon considerably enhanced the ability of *P. putida* T57 to degrade chloroanilines. *P. putida* T57 (pHK-C1C2BADE) degraded 2CA, 3CA, and 34DCA as well as 4CA. Among chloroanilines tested, 4CA was the best substrate followed by 3CA, 2CA, and 34DCA, but it hardly degraded 35DCA. These results suggest that toluene dioxygenase pathway would be useful for bioremediation of CA-contaminated sites. But *P. putida* T57 (pHK-C1C2BADE) could not utilize CAs as sole carbon and energy source in spite of its strong activity degrade CAs, indicating that it could not mineralize CAs and that it would accumulate dead end products. To completely degrade CAs, it is necessary to provide *P. putida* T57 recombinant strain with the ability to efficiently catabolize chlorocatechols.
CHAPTER 3

Identification of CtpL as a chromosomally-encoded chemoreceptor for 4-chloroaniline and catechol in *Pseudomonas aeruginosa* PAO1

**Introduction**

Chemotaxis is one of the most important behavioral adaptations of bacteria to mediate a balance between the nutritious and toxic effects of surrounding chemicals. Bacterial chemotactic attraction to environmental pollutants can enhance biodegradation rates by increasing pollutant bioavailability, and this potentially leads to an improvement in the bioremediation efficiency (Pandey et al., 2009). Several studies have reported the isolation and characterization of bacteria with a chemotactic response toward a variety of hazardous aromatic pollutants, including pesticides (Hawkins and Harwood, 2002; Liu and Parales, 2009), petroleum-associated monoaromatic (Harwood et al., 1984; Lacal et al., 2011; Parales et al., 2000), polyaromatic (Gordillo et al., 2007; Grimm and Harwood, 1997), and aromatic derivatives (Pandey et al., 2012; Parales, 2004). However, such information is relatively scarce for some of the recently recognized recalcitrant xenobiotics, such as chloro-nitroaromatics, furan, chlorinated anilines, and so on (Pandey et al., 2012; Nichlos et al., 2012). Despite the numerous reports of the isolation and phenotypic characterization of chemotactic bacteria, the specificity of bacterial chemotactic response to chemicals is actually determined by their chemoreceptors (Lacal et al., 2013). However, so far only three chemoreceptors for aromatic pollutants have been described; the plasmid-encoded NahY in the naphthalene-degrading *Pseudomonas putida* G7 (Grimm and Harwood, 1999), the plasmid-encoded NbaY in the 2-nitrobenzoate-
degrading *P. fluorescens* KU-7 (Iwaki et al., 2007), and the plasmid-encoded McpT in the toluene, benzene, and ethylbenzene-degrading *P. putida* DOT-T1E (DOT-T1E) (Lacal et al., 2011; Lacal et al., 2013).

4-Chloroaniline (4CA) is a chlorinated aromatic amine (i.e. a chlorinated aniline), that is now ubiquitous in the environment (ATSDR, 2011) because it has not only been intensively used in and released from chemical industries, but it is also a key intermediate in the natural transformation of pesticides (Dom et al., 2010). Due to its toxicity, it has been recognized as one of the priority hazardous substances and is subject to cleanup using biological treatment (Hongsawat and Vangnai, 2011). Biodegradation of the pollutant can be enhanced by using motile bacteria with suitable chemotactic capabilities to target the pollutant chemical(s), but 4CA chemotaxis has yet to be reported.

In this study, the potential chemotactic response to 4CA was initially examined in three well characterized chemotactic *Pseudomonas* species (*P. putida* F1, *P. fluorescens* Pf01 and *Pseudomonas aeruginosa* PAO (PAO1)). The positive chemotactic response of PAO1 to 4CA led to further investigations on quantitative analysis of bacterial chemotaxis and the biotransformation of 4CA by this bacterium. The molecular analysis revealed that CtpL is the chemoreceptor responsible for chemoattraction of PAO1 to the non-metabolizable 4CA as well as to the metabolizable catechol.

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Experiment procedures

Bacterial strains, plasmids, growth conditions and culture media

The bacterial strains, mutants, and plasmids used in this study are listed in Table 3.1 (Compeau et al., 1988; Hong et al., 2004; Kato et al., 2008; Kato et al., 1999, Royle et al., 1981; Sambrook and Russell, 2001; Schweizer, 1991; Spain and Gibson, 1988; Nikata et al., 1992; Kuroda et al., 1995). Each strain of the three *Pseudomonas* species, and the series of PAO1 mutants, was initially cultured in 2xYT medium (1.6% (w/v) tryptone, 1.0% (w/v) yeast extract, 0.5% (w/v) NaCl) at 28 °C for 18 h. For the chemotaxis assay, a 2% (v/v) culture inoculum (2%, v/v) culture inoculum was then transferred to the Pi-free T0 medium (g/liter: Tris-HCl (pH 7.6) 10, glucose 2, NaCl 2, NH4Cl 1, KCl 0.1, Na2SO4 0.1, CaCl2$$\cdot$$2H2O 0.01, MgCl2$$\cdot$$6H2O 0.01, FeCl3 0.001) (Wu et al., 2000), or the 5 mM-Pi-containing T5 medium (as indicated), with or without the addition of the target chemical (either 4CA or catechol). After 6 h of growth, cells were harvested, washed twice at room temperature and re-suspended in chemotaxis buffer (10 mM N-2-hydoxyethylpiperazine-$$N'$$-2-ethanesulfonic acid (HEPES) buffer, pH 7.0) (Wu et al., 2000).

Quantitative chemotaxis assay

Computer-assisted capillary assays were conducted as described previously (Nikata et al., 1992). Cell movement was observed under an inverted microscope within the area of approximately 0.8 x 0.8 mm$$^2$$ around the capillary orifice and was recorded as MP4 file. Still digital images captured in each frame were used to count the numbers of bacterial cells at the initial time (N0) and at each time interval (Nt) around the mouth of a capillary tube containing a known concentration of the test compound solidified with 1% (w/v)
agarose. Casamino acid at 2% (w/v) was used as a positive control. The strength of the chemoeffector was determined in terms of the normalized cell number per frame \( \frac{N_t}{N_0} \) as a measure of the magnitude of cell chemotactic response toward the test chemical.

**DNA manipulation, plasmid construction and electroporation**

Standard techniques for DNA preparation, plasmid construction, and all cloning were used (Sambrook and Russell, 2001). The enzymes were all from Toyobo, Tokyo, Japan. PAO1 was transformed by electroporation as described previously (Wu et al., 2000).

**RNA extraction and quantitative real time reverse transcriptase PCR (qrtRT-PCR)**

Overnight-grown cells in 2xYT medium were used as an inoculum for cell growth in the following mediums: 11-mM glucose-containing T0 or T3 medium, or T medium, each with or without 4CA (1 mM). After 6 h incubation, cells were harvested and the total RNA from them using NucleoSpin® RNA II kit (Macherey-Nagel, PA, USA) according to the manufacturer’s instructions. Two-stage quantitative real time (qrt)RT-PCR was performed by first using RT-PCR to generate the cDNA from the total RNA using the One-Step SYBR PrimeScript RT-PCR kit (Takara Bio Inc., Japan), and then using this cDNA for the qrt-PCR stage on a LightCycler 1.5 thermocycler (Roche Diagnostics, USA). Thermal cycling for the qrt-PCR was performed at 42 °C (5 s) followed by 40 cycles of 95 °C (5 s), 57 °C (10 s), and 72 °C (6 s), using the ctpL or gyrB primers. The gene expression data were normalized to the expression of the endogenous reference gyrB gene, and reported as the relative value. The primers for ctpL, which were designed between transmembrane regions 1 and 2 to give an expected product size of 119 bp, were 5’-GGAGTTGCGCGAATTCAG-3’ and 5’-CGGACCATTGTCCAGGTT-3’. The
primers for \textit{gyrB} were 5’-TGCTGCTGACCTTCTTCTTCC-3’ and 5’-CTTGCTTGCTTTTCTTGACCTT-3’ giving an expected product size of 98 bp. Subsequently, gene expression data was subjected to one-way ANOVA analysis with Student–Newman–Keuls post test using GraphPad InStat version 3.00 (GraphPad, SD, USA). Data were means of at least three independent experiments with standard deviation. \( P \)-values < 0.05 were considered significant.

4CA Biotransformation

4CA transformation was conducted using a resting-cell suspension of PAO1. Cells were grown at 28 \(^\circ\)C, 150 rpm, for 14 h in mineral salt basal (MSB) medium (4.3 g of \( \text{K}_2\text{HPO}_4 \), 3.4 g of \( \text{KH}_2\text{PO}_4 \), 2.0 g of \( \text{(NH}_4\text{)}_2\text{SO}_4 \), 0.34 g of \( \text{MgCl}_2\cdot6\text{H}_2\text{O} \), 1 mg of \( \text{MnCl}_2\cdot4\text{H}_2\text{O} \), 6 mg of \( \text{FeSO}_4\cdot7\text{H}_2\text{O} \), 26 mg of \( \text{CaCl}_2\cdot2\text{H}_2\text{O} \), 0.02 mg of \( \text{Na}_2\text{MoO}_4\cdot2\text{H}_2\text{O} \), 0.01 mg of \( \text{ZnCl}_2\cdot7\text{H}_2\text{O} \), 0.01 mg of \( \text{CoCl}_2\cdot6\text{H}_2\text{O} \), 0.01 mg of \( \text{CuSO}_4 \), 0.001 mg of \( \text{NiSO}_4\cdot6\text{H}_2\text{O} \) and 1 \( \mu \text{g} \) of \( \text{Na}_2\text{SeO}_4 \), per liter of deionized water) supplemented with 0.1g/L of yeast extract (MSBY). The overnight-grown cells were then harvested. The cell pellet was washed twice with MSB medium and re-suspended in MSBY to the optical density of 5. Then, 4CA was added at 2 mM into the suspension culture and incubated at 28 \(^\circ\)C and 150 rpm for 30 h. At the specified time interval, the samples were taken for chromatography analysis.

Chromatography analysis

The transformation of 4CA was analyzed by high performance liquid chromatography (HPLC) (JASCO Co., Tokyo, Japan) with a reverse phase column (TSKgel ODS-80TM, Tosoh, Yamaguchi, Japan) at a flow rate of 0.8 mL/min. UV absorption was measured at 240 nm. The compounds were eluted using a linear gradient of
10 - 70% (v/v) acetonitrile-water over 30 min. Qualitative and quantitative analyses were performed using a range of standard concentrations of 4CA (Nacalai Tesque, Japan) for comparative peak area evaluation, which had a HPLC retention time of 19.18 min under these test conditions. The transformation intermediate formed during the reaction was further analyzed using a $p$-chloroacetanilide (4CD) standard (Sigma-Aldrich, USA), which had two HPLC retention times of 14.50 and 14.75 min under these test conditions. The sample fraction collected from the transformation reaction was then extracted and concentrated with ethyl acetate, and dried over anhydrous sodium sulfate. The dried sample was re-dissolved with ethyl acetate prior to identification using liquid chromatography-mass spectroscopy (LC-MS) on a LTQ Orbitrap XL (J108) instrument (Thermo Fischer Scientific Inc., MA, USA) in flow-injection mode. Methanol and ethyl acetate (Riedel-de Haen) were used as controls. The mass spectrum was recorded by monoisotopic negative mode (MS2 of [M−H]$^-$) and then the expected mass of each intermediate were determined with the Xcalibur program (Thermo Scientific, USA).
<table>
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<th>Strain/Plasmid</th>
<th>Relevant characteristics</th>
<th>Reference</th>
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<tr>
<td><strong>Pseudomonas putida</strong> strain F1</td>
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<td>Spain and Gibson, (1988)</td>
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<tr>
<td><strong>Pseudomonas fluorescens</strong> strain PfO1</td>
<td>Wild type strain</td>
<td>Compeau et al., (1988)</td>
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<tr>
<td><strong>Pseudomonas aeruginosa</strong> PAO1</td>
<td>Wild type strain</td>
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<td>ΔCHE4</td>
<td>PAO1 derivative, ΔChe2 cluster (cheY2 cheA2 cheW2 aer-2 cheR2 PA0174\textsuperscript{b} cheB2)</td>
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<td>recA1, endA1, gyrA96, thi-1, hsdR17(\textsuperscript{r} m+)\textsuperscript{k}, e14 (mcrA), supE44, relA1, Δ(lac-proAB)/F\textsuperscript{−}[traD36, proAB\textsuperscript{−}, lacIq, lacZΔM15]</td>
<td>Sambrook and Russell, (2001)</td>
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<td><strong>mcp</strong>-disrupted PAO1 mutants</td>
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<td>Wu et al., (2000)</td>
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<td><strong>Escherichia coli-Pseudomonas</strong> shuttle vector; Cb\textsuperscript{′}</td>
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<td>pUCP18 containing a 1,974-kb PCR fragment of ctpL (PA4844); Cb\textsuperscript{′}</td>
<td>This study</td>
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Results

*Chemotactic response of Pseudomonas strains to 4CA*

Members of the genus *Pseudomonas* are ubiquitous in a large variety of natural environments, which reflects not only that they are considered one of the most metabolically diverse microorganisms, but also that they have a range of physiological and genetic adaptabilities, such as chemotactic responses to surrounding chemicals, for their growth and survival (Spiers et al., 2000). Several *Pseudomonas* spp. have been reported to chemotactically respond to aromatic hydrocarbons and their substituted derivatives, but their sensing capabilities to each of these chemoeffectors are varied, presumably due to their chemoreceptor diversity (Liu and Parales, 2009; Lacal et al., 2011; Parales et al., 2000; Gordillo et al., 2007; Grimm and Harwood, 1997; Grimm and Harwood, 1999; Iwaki et al., 2007). In this study, the well-studied motile *P. putida* F1 (F1), *P. fluorescens* Pf01 (Pf01) and *P. aeruginosa* PAO1 (PAO1) (Table 3.1), were grown without chemical induction and analyzed for their chemotactic response to 4CA using a computer-assisted capillary assay. All strains showed normal motility and a positive chemotactic response to 2% (w/v) casamino acid, the positive control, but they responded differently to 4CA. Under the test conditions, F1 and Pf01 exhibited very weak chemotactic responses to 4CA with the N_{60}/N_{0} value ranging from 1 – 1.2 (data not shown), while PAO1 showed a stronger chemotactic attraction (Fig. 3.1A). This result is in agreement with the fact that the different *Pseudomonas* species/strains can respond differently to the same chemoattractant (Nichols et al., 2012). Since PAO1, one of the bacteria with well characterized chemosensory system (Kato et al., 2008), positively responded to the environmental pollutant 4CA, PAO1 was selected for further studies.
Figure 3.1. Quantitative analysis and induction of the chemotactic response of PAO1 to 4CA and catechol. (A, B) Non-induced PAO1 cells were prepared in T₀ medium (as described in Materials and methods) prior to exposure to (A) 4CA at (▲) 0.5, (■) 1, (●) 2, (◆) 4 mM, or (B) catechol at (▲) 1, (■) 2, (●) 10 mM, solidified with 1% (w/v) agarose in a capillary tube. The results were expressed as the normalized cell number (Nₜ/Nₜ₀). The chemotaxis buffer (10 mM HEPES) in agarose ( *, dashed line) was the negative control. Casamino acid was the positive control giving the Nₜ₆₀/Nₜ₀ value of approximately 4. (C) Concentration response curve for the chemotaxis of non-induced PAO1 cells to 4CA (■) or catechol (●) measure at 60 s of exposure and expressed as Nₜ₆₀/Nₜ₀. (D) Induction of PAO1 chemotaxis to 4CA. Cells were cultured overnight in
2xYT medium, and then in 11 mM glucose-containing T₀ medium, without (Non-ind) or with induction by either 1 mM 4CA (4CA-ind) or 1 mM catechol (Cat-ind). Chemotaxis, using a computer-assisted capillary assay, was then performed with 1 mM 4CA or catechol. The strength of chemotactic attraction is shown as the normalized 𝑁𝑡_{60}/𝑁𝑡₀ cell ratio. In A – D, the data are shown as mean ± SD and are derived from at least three independent experiments conducted in triplicates.

Chemotactic response and metabolic activity of PAO1 to 4CA and aromatic analogues

As chemotactic responses of bacteria allow them to move toward a microenvironment that is optimal for their growth and survival (or away from detrimental ones), several bacterial growth-supporting chemicals generally act as their chemoattractants (Alexandre and Zhulin, 2001). To determine the metabolic activity and chemotactic behavioral responses of PAO1 to 4CA and other aromatic analogues, cell growth was determined in MSB medium supplemented with each chemical tested as a sole carbon source, while the chemotactic response was examined from cells grown under a non-induced condition. The results revealed that PAO1 was attracted by almost all of the metabolizable, growth-supporting test chemicals, the exception being 4-hydroxybenzoic acid (Table 3.2). Of the four growth-supporting aromatics tested, catechol was the strongest attractant for PAO1. In addition, PAO1 showed positive chemotactic responses to some non-metabolizable chemicals, such as 3-chloroaniline (3CA), 4CA, 34DCA, and 4-aminobenzoic acid. Further investigation showed that although PAO1 was not able to utilize 4CA as a carbon source, it could stoichiometrically transform 4CA to 4CD (Fig. 3.2A and 3.2B), which appeared in PAO1 to be a dead end product in this degradation pathway (Fig. 3.2C). Chemotaxis assays also revealed that 4CD was not a chemoeffect
for PAO1 (Table 3.2). Thus, the motility behavior of PAO1 towards 4CA was solely a positive chemotactic response of cells to 4CA.

The quantitative analysis of PAO1 chemotaxis with various concentrations of the non-metabolizable 4CA and the metabolizable catechol were expressed as bell-shaped concentration response curves, which indicated that cell chemotactic response was dependent on the chemoeffector concentration (Fig. 3.1A - C). The strength of the chemotactic response of PAO1 to both 4CA and catechol increased as the concentration increased up to the optimal concentration at 1 mM for 4CA and 2 mM for catechol and then decreased at higher concentrations (Fig. 3.1C). The difference in the cell chemotactic behavior towards the non-metabolizable 4CA and the metabolizable catechol was that the cell chemoattraction to catechol rapidly declined after 45 s of exposure (Fig. 3.1B), whereas it tended towards reaching a plateau for 4CA (Fig. 3.1A).

The above results indicated that the chemotactic response of PAO1 to 4CA was constitutive under the conditions tested. Nevertheless, generally this natural ability can be enhanced if it is inducible (Pandey et al., 2012). Therefore, an induction test was conducted by growing cells in the presence and absence of its chemoeffector as an inducer (1 mM of either 4CA or catechol) for 6 h prior to chemotaxis assay with each corresponding chemoeffector. The results indicated that there was no noticeable difference in the apparent chemotaxis strength towards 4CA among non-induced cells, 4CA-induced cells, and catechol-induced cells (Fig. 3.1D).
### TABLE 3.2 Chemotaxis and growth of PAO1 and the ΔCTPL on chloroanilines and aromatic analogues

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Chemotactic response&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cell growth&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>PAO1</td>
<td>dCTPL</td>
</tr>
<tr>
<td>Aniline</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2-Chloroaniline (2CA)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3-Chloroaniline (3CA)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4-Chloroaniline (4CA)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2,4-Dichloroaniline (24DCA)</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>3,4-Dichloroaniline (34DCA)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4-Chloroacetanilide</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Catechol</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>4-Chlorocatechol</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sodium salicylate</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Anthranilic acid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4-Aminobenzoic acid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sodium benzoate</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Nitrobenzene</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>4-Chloronitrobenzene</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Benzene</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Toluene</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Casamino acid (positive control)</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>HEPES (10 mM) (negative control)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O (negative control)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Concentration of the test compound was at 1 mM for chemotaxis test and 10 mM for growth determination.

<sup>b</sup> Chemotactic response was measured by a computer-assisted capillary assay as described in Materials and methods. Cell numbers (N) in the analysis frame area were captured at a starting time (t<sub>0</sub>) and a 60-second chemical exposure time (t<sub>60</sub>). The strength of chemotactic attraction is shown by the increase of cell number in the analysis frame area at t<sub>60</sub> after normalized with those at t<sub>0</sub> (N<sub>t60</sub>/N<sub>t0</sub>). The value of the normalized cell number is represented by the + symbol: 4 ≥ +++ > 3; 3 ≥ ++ > 2; 2 ≥ + > 1.2; 1.2 ≥ - > 0; ND, not determined.

<sup>c</sup> Cell growth in MSB medium was determined as optical density of cells at 600 nm (OD<sub>600</sub>) after 24h of incubation. The cell optical density value is represented by the + symbol: ++ > 0.6; 0.6 ≥ + > 0.1; 0.1 ≥ - > 0 (no growth); ND, not determined.
Figure 3.2. Biotransformation of 4CA and the formation of transformation product in PAO1. (A) Biotransformation of 4CA (■) (initial concentration of 2 mM) by PAO1 using a resting-cell technique, and the formation of 4-chloroacetanilide (4CD, ○) as a transformation product. (B) LC-MS spectra of the transformation product obtained from the PAO1 culture supernatant of media containing 4CA after 15-h of incubation. The m/z value was 168.02, which indicates 4CD (C₈H₇ONCl) as the transformation product. (C) Biotransformation of 4CD (initial concentration of 2 mM) (○) by PAO1 using a resting-cell technique.
Identification of the che gene involved in chemotactic response of PAO1 to 4CA

PAO1 has a complex chemosensory system with several known chemotaxis (che) genes (Kato et al., 2008; Croft et al., 2000; Stover et al., 2000). To identify the che gene(s) potentially involved in the chemoattraction of PAO1 to 4CA, five Che-cluster defective mutants (Table 3.1) were examined for their responses to 4CA. All of these mutants were confirmed to be fully motile. Upon testing with 4CA (1 mM) using the computer-assisted capillary assay, the ΔCHE1 mutant, which has a deletion of the che cluster 1 (cheY cheZ cheA cheB motA2 motB2 cheW), and PC4, a cheR mutant, completely lost chemoattraction to 4CA, while the chemotactic behavior of ΔCHE3 (ΔPil-Chp cluster), ΔCHE4 (ΔChe2 cluster), and ΔCHE5 (ΔWsp cluster) remained normal and similar to the response of the wildtype (PAO1-WT) (Fig. 3.3A). The results indicated that chemoattraction of PAO1 to 4CA appears to essentially involve the action of the che cluster 1 and cheR. This indicated that PAO1 chemotaxis to 4CA is in fact methyl-accepting chemotaxis protein (MCP)-dependent.

*CtpL was identified as a chemotactic transducer for 4CA and catechol in PAO1*

Bacterial cells detect chemoeffectors mainly using cell surface MCP chemoreceptors. PAO1 contains 26 known-mcp-like genes in its genome (Kato et al., 2008; Croft et al., 2000; Stover et al., 2000). To identify which mcp gene(s) was/were potentially involved in the 4CA chemoattraction, a series of 26 mcp mutants, which had previously been generated by either deletion or a kanamycin-cassette insertion of an individual mcp-like gene in the PAO1 genome (Wu et al., 2000; Hong et al., 2004; Taguchi et al., 1997), were each examined for their chemotactic behavior towards 4CA. Twenty-five of these mcp mutants exhibited a normal chemoattractive behavior towards 4CA that was similar to that of the PAO1-WT. The responses of the tlpQ (PA2573)- and
pctA(PA4309)-deficient mutants are shown as examples (Fig. 3.3B). In contrast, the ctpL-deficient mutant (ΔCTPL) showed no detectable chemotactic ability to 4CA (Fig. 3.3B).

To elucidate the potential role of CtpL in the chemoattraction in PAO1 to 4CA, gene complementation was conducted prior to assaying the transformant for chemotactic ability. A plasmid harboring a functional copy of ctpL (pCTPL) was constructed (Table 3.1) and introduced into the corresponding ΔctpL single mutant. Positive chemotaxis to 4CA was fully restored in the transformed ΔCTPL(pCTPL) (Fig. 3.3B). Furthermore, the chemotaxis response of the ctpL-deficient mutant with and without ctpL complementation to catechol (2 mM) revealed similar results; ΔCTPL showed no positive response to catechol, while the ΔCTPL(pCTPL) transformant fully regained normal chemoattraction (data not shown). This result suggests that CtpL is responsible for the positive chemotaxis towards 4CA and catechol in PAO1.

In addition, to further examine the gene-dosage dependency in the chemotactic response, the chemotaxis assay with 4CA was conducted with the ctpL-over-expressing PAO1 transformant (PAO1(pCTPL)). Chemoattraction to 4CA in the ctpL overexpressing cells was not significantly increased (data not shown), suggesting that additional of ctpL gene copies did not markedly enhance the positive chemotactic response in PAO1.
Figure 3.3. Chemotactic response of PAO1 mutants to 4CA. Non-induced PAO1 mutants and the wildtype (PAO1-WT) were prepared prior to the exposure to 4CA (1 mM) in a computer-assisted capillary assay. Chemotactic response of PAO1-WT to 4CA was used as the reference positive control. The results are expressed as the normalized cell number (Nt/Nt₀). (A) Chemotactic behavior of the che mutants: ΔCHE1, ΔCHE3, ΔCHE4, ΔCHE5, and PC4. Response of the PAO1-WT to chemotaxis buffer (HEPES)-containing agar was the negative control. (B) Chemotactic behavior of the single Δmcp mutants. Responses of tlpQ-deficient mutant (ΔTLPQ) and pctA-deficient mutant (ΔPCTA) are shown as representative example of the mcp mutants exhibiting a similar chemotaxis to that of PAO1-WT, while no chemotactic response to 4CA was found in the ctpL-deficient
mutant (ΔCTPL), but was restored by ctpL gene complementation, ΔCTPL(pCTPL). In A and B, the data are shown as mean ± SD and are derived from at least three independent experiments conducted in triplicates.

Expression of ctpL and cell response toward 4CA and catechol in the presence of inorganic phosphate (Pi)

CtpL has previously been identified as a chemoreceptor in PAO1 for the chemotaxis to Pi at low Pi concentrations (Wu et al., 2000). The synthesis of CtpL is transcriptionally regulated by the phosphate starvation regulon (Pho regulon) under Pi limitation, but not in conditions of excess Pi (Wu et al., 2000; Vershinina and Znamenskaia, 2002). In this study, the effect of the chemoeffectors (4CA and catechol) was investigated on ctpL expression using qrt-RT-PCR analysis, and on the cell chemotactic response under conditions of Pi starvation (T₀ medium) or excess Pi (T₅ medium). Cultures were initially grown in 2xYT medium with excess Pi to suppress expression of ctpL and then cells were re-inoculated and grown for 6 h in 11 mM glucose-containing T₀ medium to induce ctpL expression, or in T₅ medium to keep the ctpL levels suppressed. In both media, 1 mM 4CA or 2 mM catechol was added at the previously determined optimal concentration for cell chemoattraction. In the Pi-deficient T₀ medium, the ctpL transcript level was relatively high as expected and also positively detectable in the presence of catechol or 4CA (although the expression level was 3.5- and 2.5 fold lower than that in the absence of the test chemoeffector) (Fig. 3.4). In the Pi-rich T₅ medium, the ctpL transcript level was very low as expected (six-fold less than that in T₀ medium) but was upregulated 1.3- and 2.4 fold in the presence of catechol or 4CA, respectively (Fig. 3.4).
Analysis of the chemotactic responses of PAO1 towards 4CA and catechol in the presence of Pi at 0, 1, 10 and 100 μM in chemotaxis buffer revealed a positive chemoattraction of cells towards 4CA and catechol at low (0 and 1μM) Pi concentrations (Fig. 3.5). However, essentially no chemoattraction to the chemoeffector was noted at the higher Pi concentrations of 10-μM and 100-μM (Fig. 3.5).

Figure 3.4. Quantitative real time RT-PCR analysis of ctpL mRNA expression in PAO1. Overnight-grown cells in 2xYT medium were used as an inoculum for cell growth in 11 mM glucose-containing T₀ (open bar) or T₅ (solid bar) medium, each in the presence of the solvent only control (None), catechol (2 mM) or 4CA (1 mM). After 6 h incubation, total RNA was isolated and qrtRT-PCR analysis of the ctpL and gyrB transcript levels was performed. The results are expressed as the relative ctpL gene expression value after normalizing to the gyrB transcript level. Data are shown as mean ± 1SD and are derived from at least three independent experiments. Italic letters (with and without asterisk) indicate a significant difference at P<0.05 to the control within the same group, according to Student–Newman–Keuls post test.
Figure 3.5. Quantitative analysis of the chemotactic response of PAO1 to 2 mM catechol (open bar) and 1 mM 4CA (solid bar) in the presence of different Pi concentrations in the chemotaxis buffer using a computer-assisted capillary assay. Overnight-grown cells in 2xYT medium were cultured in T₀ or T₅ medium as indicated, and then resuspended in the chemotaxis buffer with various concentrations of Pi (KH₂PO₄). The strength of the chemotactic attraction was determined by the increase of the cell number in the analysis frame area at t₆₀ (60-s exposure) normalized to that at t₀ (N₆₀/N₀). Data are shown as mean ± SD and are derived from at least three independent experiments conducted in triplicates.
Chemotaxis is one of the most important bacterial mechanisms for sensing and responding to chemical changes in ecologically diverse environments, including toxic environments. The genus *Pseudomonas* has been shown to have a broad chemotactic capability for a wide range of compounds, which in part allows for its physiological and metabolic diversity (Spiers et al., 2000; Miller et al., 2009). Previous studies have shown that several *Pseudomonas* spp. are capable of chemotactic responses to a wide range of aromatic pollutants, but with diverse sensing specificities (Liu and Parales, 2009; Lacal et al., 2011; Parales et al., 2000; Gordillo et al., 2007; Grimm and Harwood, 1997; Grimm and Harwood, 1999; Iwaki et al., 2007). In this study, among the three well-studied, motile *Pseudomonas* spp., only PAO1 showed a positive chemotactic response to 4CA, the target pollutant.

The difference in bacterial chemo-sensing ability lies in the type and number of chemoreceptors. PAO1 has a complex chemosensory system with more than 20 chemotaxis (*che*) genes and 26 chemoreceptor genes (Kato et al., 2008), which results in its ability to thrive in various environmental niches. Chemotactic behavior is based on the recognition of environmental signals through cell surface methyl-accepting chemotaxis proteins (MCPs), of which so far the following MCPs have been identified in PAO1 for several stimuli, including PctA, PctB, and PctC for amino acids (Kuroda et al., 1995), CtpL and CtpH for inorganic phosphate (Kato et al., 1992), Aer, and Aer-2 for oxygen (Hong et al., 2004), PA2652 for malate (Alvarez-Ortega and Harwood, 2007), TlpQ for ethylene (Kim et al., 2007), and PctA for volatile chlorinated aliphatic hydrocarbons (Shitashiro et al., 2005). In this study, a role of CtpL (PA4844), a chromosomally encoded, transmembrane protein in PAO1, as a chemotactic transducer for aromatic compounds...
including 4CA and catechol was revealed. Although the precedent of chromosomally encoded chemoreceptor(s) for aromatic hydrocarbons was briefly discussed for DOT-T1E (Lacal et al., 2011), all previously reported chemoreceptor genes for aromatic pollutants so far have been plasmid-borne (Lacal et al., 2011; Grimm and Harwood, 1999; Iwaki et al., 2007). Moreover, chemoreceptor gene products in bacteria with similar chemotactic phenotypes have been reported to be close homologues. For instance, homologues of the McpT receptor with 99% sequence identity were found in a self-transmissible plasmid in DOT-T1E and the pMAQU02 plasmid of Marinobacter aquaeolei VT8, which are both responsible for the chemotaxis phenotype towards aromatic hydrocarbons (Krell et al., 2012). In contrast, the distinct chemotactic phenotype towards 4CA in the P. aeruginosa PAO1, P. putida F1 and P. fluorescens Pf01 is perhaps the result of a low sequence identity (65 – 66%) between CtpL in PAO1 and the Blast-matched MCP in F1 (YP_001265953.1) and Pf01 (YP_346355.1).

In general, compounds capable of promoting and/or inducing a positive chemotactic response serve as metabolizable nutrients. In accord, PAO1 exhibited a relatively strong attraction towards the metabolizable catechol, which it can utilize as a sole carbon source. The rapid decline in cell chemoattraction, which was observed after 45 s of catechol exposure, likely, reflected the decreased local concentration of catechol due to its uptake and metabolism. In contrast, PAO1 could only transform 4CA to 4-chloroacetanilide, which then accumulated as a dead-end product, indicating that the chemotactic response of PAO1 is independent of the cell metabolic activity to 4CA. Although the physiological sensing of bacterial chemotaxis to non-metabolizable compounds is not yet thoroughly understood, the results indicated that CtpL in PAO1 can recognize and mediate a positive chemotactic response towards metabolizable and non-metabolizable aromatic pollutants.
The capability of CtpL as a chemoreceptor for the test aromatic pollutants is proposed to be a fortuitous activity in PAO1. Since CtpL has been established as a major chemoreceptor for inorganic phosphate (Pi) taxis at low Pi concentrations, while CtpH functions for Pi taxis at high Pi concentrations (Wu et al., 2000). The expression of the ctpL gene is induced in Pi limiting conditions under the regulation of the PhoB/PhoR and PhoU proteins (Wu et al., 2000). In this study, because cells were initially cultured under Pi starvation conditions (T₀ medium) prior to the assay, a relatively high transcript expression level was detected, which correlates with the positive chemotactic response of the non-induced cells to 4CA or catechol. When cells were cultured in the presence of 5 mM Pi in the T₅ medium, ctpL transcript expression level was suppressed but could be increased by the addition of 4CA or catechol in the T₅ medium, indicating the positive inducibility of ctpL by these chemoeffectors.

The chemotactic response of PAO1 cells towards these chemoeffectors was markedly reduced to a much greater extent in the presence of high concentrations of Pi. This may suggest that these chemoeffectors and Pi share the common binding site on CtpL. The direct binding assay is required as further investigation to clarify this point. Despite the fact that the increase of ctpL expression was conducted by either a gene induction or an increase of gene-dosage in PAO1(pCTPL), the physiological chemosensing activity of PAO1 toward 4CA was not markedly enhanced. This could reflect that CtpL does not function independently as other chemoreceptors for aromatic pollutants. To acquire a full function, it may require a complex formation with other proteins in the Pst system under the regulation of Pho regulon (Wu et al., 2000).

In summary, the results of this study reveal that CtpL acts as a chemoreceptor for the positive chemotactic response of PAO1 to the non-metabolizable 4CA as well as the metabolizable catechol. The discovery of a chromosomally encoded chemoreceptor with
broader fortuitous activity towards aromatic pollutants in PAO1 expands the range of the known chemotactic transducers and their function. Future comprehensive investigation of the CtpL-protein complex formation and its regulation will be necessary to elicit or enhance the positive chemotactic response of PAO1 or other environmental bacteria towards aromatic pollutants including 4CA.
CHAPTER 4

General conclusion

CAs are intermediates and building blocks of the large scale synthesis of herbicides, dye stuffs and pharmaceuticals. The environmental problems are arisen from a widespread use of these compounds in industrial production causing high spreading of free CAs in industrial effluents. Additionally, degradation of herbicides by microorganisms cause releasing and accumulation of free CAs in agricultural field. These CAs are recalcitrant in soil and aquatic environment. Toxicity not only comes from CAs itself but also from an accumulation of dead end metabolite when CAs were degraded by microorganisms. So, there are problems about toxicity of these chlorinated aromatic intermediate. From above evident, CA-utilizing bacteria are difficult to isolate and not readily isolate from existing industrial sewage treatment system. However, there are reports about isolation and identification of CA-degrading bacteria (ex. Pseudomonas, Acinetobacter, Delftia, and Comamonas strains) from soil but a few molecular information was revealed. Metabolite analysis revealed that CAs were degraded by chloroaniline dioxygenase and aniline dioxygenase which catalyze oxidative deamination to convert chloroaniline to corresponding chlorocatechol (Latorre et al., 1984; Hongsawat and Vangnai et al., 2011; Breugelmans et al., 2010). After that chlorocatechol are then metabolized by ortho or meta ring cleavage enzyme. Information on the initial step of chloroaniline dioxygenase is very limited. So other dioxygenation was concerned for usage for CAs detoxification.
In this study, I demonstrated that toluene dioxygenase can oxidize CAs including 4-chloroaniline, 2-chloroaniline, 3-chloroaniline, and 3,4-dichloroaniline. This is the first finding of CA-degradation by toluene dioxygenase. Degradation activity of CAs have been characterized in some CA-degrading bacteria and enzymes involved in CA-degradation have been named chloroaniline dioxygenase because they catalyze oxidative deamination of CAs. The \textit{dcaA1}, \textit{dcaA2}, and \textit{dcaB} genes in \textit{C. testosteroni} are only genes which have been functionally to encode chloroaniline dioxygenase. Sequence analysis reveals that chloroaniline dioxygenase in \textit{C. testosteroni} is closely related to aniline dioxygenase from aniline-degrading bacteria such as \textit{Delftia acidovorans}, \textit{D. tsuhatensis}, \textit{Frateuria} sp., and \textit{P. putida} (71-98\% identity) which oxidizes aniline via oxidative deamination. Toluene dioxygenase in \textit{P. putida} T57 shows very poor similarity with chloroaniline dioxygenase and aniline dioxygenase. But, it still not only oxidizes CAs but also catalyzes oxidative deamination. Chloroaniline dioxygenase and aniline dioxygenase have been identified in bacteria which utilize CAs or aniline as growth substrates, meanwhile \textit{P. putida} T57 cannot utilize CAs. It is rational to focus CA- or anilene-degrading microorganisms as agents for bioremediation of CA-polluted environments, but our findings suggest that toluene-oxidizing bacteria are also good candidates for a genetic source of chloroaniline degrading enzymes even though they cannot grow on CAs.

Toluene-induced \textit{P. putida} T57 cells show significant ability to degrade CAs, but it is very weak. Therefore, I conducted molecular breeding to enhance the degradation activity in \textit{P. putida} T57 and demonstrated that introduction of plasmid containing \textit{P}_{\text{kan}-todC1C2BADE} successfully enhanced (more than 250 folds) the activity to degrade 4CA in \textit{P. putida} T57 and that the recombinant strain completely degraded 2 mM 4CA (the main chloroaniline environmental pollutant) within 2 hours. The genetically-modified \textit{P.}
*P. putida* T57 could be used for remediation of polluted soils and waters in closed bioreactor systems. When using *P. putida* T57 in *in-situ* bioremediation, the strain in which the chromosomal *tod* promoter is substituted with the stronger and indigenous promoters might be better than *P. putida* T57 harboring plasmid containing the *tod* genes from regulatory point of views.

As I mentioned above, *P. putida* T57 cannot grow on 4CA even though toluene dioxygenase oxidizes these compounds. It is because *P. putida* T57 cannot utilize 4-chlorocatechol, a product of deamination of 4CA by toluene dioxygenase. At aerobic conditions, accumulated 4-chlorocatechol easily reacts with itself to form dark-reddish polymers which diminish viability of *P. putida* T57 cells. Actually, viable cells greatly decreased after 4CA degradation experiments, which means that repeated usage of the recombinant *P. putida* T57 would be quite difficult. Many microorganisms have been reported to utilize 4-chlorocatechol and genes involved in 4-chelocatechol have been also reported. Therefore, introduction of the ability to utilize 4-chlorocatechol as a growth substrate into the recombinant *P. putida* T57 strain could be solution of the problem. Alternatively, *todC1C2BA* genes from *P. putida* T57 might be introduced into 4-chlorocatechol utilizing bacteria to construct efficient chloroaniline-degrading/utilizing bacteria.

Chemotactic responses of *P. aeruginosa* PAO1 to CAs is the first finding of bacterial chemotaxis to CAs. We have also successfully identified CtpL as a chemotaxis sensor for CAs. CtpL detects not only CAs but also catechol. *P. aeruginosa* PAO1 utilizes catechol as a sole carbon source but not CAs, and it shows stronger responses to catechol than to CAs. Therefore, it is suggested that according to aromatic compounds, catechol is the original ligand for *P. aeruginosa* PAO1 and that chemotaxis to CAs is fortuitous. The
puzzle is the fact that CtpL is a chemotaxis sensor for inorganic phosphate. The *ctpL* gene is transcriptionally regulated by PhoB/PhoR and PhoU, regulatory proteins of the phosphate regulon, and its transcription is induced at conditions of phosphate-limitation. These are evidences supporting the idea that CtpL is originally a sensor for inorganic phosphate. Then, what is that reason why (and how) CtpL detects both inorganic phosphate and catechol of which chemical structures and properties are much different? As for “why”, it is quite a question to be answered. But, transcription of the *catA* gene encoding catechol 1,2-dioxygenase, a key enzyme of catechol utilization, is induced by the presence of catechol but not by phosphate limitation. It suggests that detection of catechol by CtpL might be fortuitous. To answer “how”, the single binding and competitive binding assays between phosphate and catechol, and CtpL protein (for example, using isothermal titration calorimetry) would be useful.

Chemotaxis to pollutants brings bacterial cells to areas at higher concentrations of the pollutants. Therefore, if pollutant-degrading bacteria have the ability to chemotactically respond to pollutants, bioremediation using the bacteria (especially, *in-situ* bioremediation) is expected to speed up. That is the idea why I have started this study. And, we have successfully found bacterial chemotaxis to chloroanilines and identified the chemotaxis sensor for chloroanilines in *P. aeruginosa* PAO1. Since *P. aeruginosa* is an opportunistic pathogen, it is not a good idea to confer the ability to degrade chloroanilines on *P. aeruginosa* PAO1. Actually, *P. putida* strains possess the *ctpL* homologues but *P. putida* T57 failed to respond to 4-chloroaniline even when it was grown at conditions of phosphate limitation. This is probably due to a relatively low sequence identity (65%) between CtpL in *P. aeruginosa* PAO1 and *P. putida*. Therefore, I tried to introduce *P. aeruginosa ctpL* into *P. putida* T57 to confer the ability to chemotactically respond to
chloroaniline. But, the recombinant *P. putida* T57 still failed to respond to 4CA. Additional factors might be required for detection of chloroanilines by *P. aeruginosa* PAO1 CtpL. Molecular biology of chemosensing by CtpL should be further investigated.
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This thesis is dedicated to my parents, whose constant support and encouragement made this possible.

Finally, I would like to thank for Monbukagakusho Scholarship supported by Japanese government for the valuable supporting and great opportunity to study in Hiroshima University, Japan.
(1) Degradation of chloroanilines by toluene dioxygenase from *Pseudomonas putida* T57
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(2) Identification of CtpL as a chromosomally-encoded chemoreceptor for 4-chloroaniline
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Degradation of chloroanilines by toluene dioxygenase from Pseudomonas putida T57

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In this study, we investigated the ability of Pseudomonas putida toluene dioxygenase to oxidize chloroanilines. Toluene-induced P. putida T57 cells degraded 4-chloroaniline (4CA) more rapidly than toluene-non-induced cells, suggesting that toluene dioxygenase pathway was involved in 4CA degradation. Escherichia coli harboring P. putida T57 genes encoding toluene dioxygenase complex (todC1C2BA) showed 4CA degradation activity, demonstrating that toluene dioxygenase oxidizes 4CA. Thin-layer chromatography (TLC) and mass spectrometry (MS) analyses identified 4-chlorocatechol and 2-amino-5-chlorophenol as reaction products, suggesting that toluene dioxygenase catalyzes both 1,2- and 2,3-dioxygenation of 4CA. A plasmid containing the entire tod operon (todC1C2BADE) was introduced to P. putida T57 to enhance its ability to degrade 4CA. Resulting P. putida T57 (pHK-C1C2BADE) showed 250-fold higher 4CA degradation activity than P. putida T57 parental strain. P. putida T57 (pHK-C1C2BADE) degraded 2-chloroaniline (2CA), 3-chloroaniline (3CA), and 3,4-dichloroaniline (34DCA) as well as 4CA, but not 3,5-dichloroaniline (35DCA). The order of the degradation rate was: 4CA > 3CA > 2CA > 34DCA.

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[Key words: Chloroaniline; Biodegradation; Toluene dioxygenase; Pseudomonas putida; Oxidative deamination]

Chloroanilines are industrial chemicals widely used as intermediate compounds in the production of pesticides, polyurethanes, rubber, pharmaceutical products, and dyes (1,2). They have been registered in the high production volume chemical program of the Organization for Economic Co-operation and Development (3). In chloroaniline-polluted sites, several chloroaniline-degrading bacteria have been isolated. They include Pseudomonas sp. J12, Delftia acidovorans CA28, Comamonas testosteroni 12, Acinetobacter baumannii CA2, Pseudomonas putida CA16, Delftia tsuruhatensis H1, and Acinetobacter baylyi GF2 (6–11). Metabolite analysis, together with analogy with the reaction catalyzed by aniline dioxygenase (12), indicated that the initial step of chloroaniline degradation is an oxidative deamination by chloroaniline dioxygenase to convert chloroanilines to corresponding chlorocatechols (6,11,13). Chlorocatechols are then metabolized by ortho ring cleavage enzymes or meta ring cleavage enzymes. Although there are several reports concerning characterization of ring cleavage enzymes of chlorocatechols and their genes (6,7,9–11,14–17), information on the initial step and chloroaniline dioxygenase is very limited and only dcaA1, dcaA2, and dcbB genes in C. testosteroni have been functionally demonstrated to encode chloroaniline dioxygenase (18).

P. putida T57 was isolated from activated sludge of chemical factory as a toluene-utilizing bacterium (19). It metabolizes toluene via toluene dioxygenase pathway. In toluene dioxygenase pathway, toluene is oxidized to toluene cis-hydrodiol by toluene dioxygenase (products of the todC1C2BA genes), which is oxidized to 3-methylcatechol by toluene cis-dihydrodiol dehydrogenase (the gene product of todD), and 3-methylcatechol is further metabolized by meta-cleavage enzyme, 3-methylcatechol 2,3-dioxygenase (the gene product of todE). Amino acid sequences of the P. putida T57 todC1C2BADE products are completely identical to those of P. putida F1 counterparts. P. putida toluene dioxygenase has broad substrate specificity (20). In addition, we revealed that tod operon in both P. putida F1 and P. putida T57 was induced by 4-chloroaniline (4CA) as well as toluene (21). Therefore, we speculate that toluene...
dioxane could degrade/oxidize chloroanilines although there have been no reports about transformation of chloroanilines by toluene dioxygenase. In this study, we investigated the ability of *P. putida* toluene dioxygenase to degrade chloroanilines. We also tried enhancement of the ability of *P. putida* T57 to degrade chloroanilines using genes of toluene dioxygenase pathway.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α was used for plasmid construction and DNA manipulation. *E. coli* was grown at 37°C with shaking in Luria–Bertani (LB) medium (22) supplemented with appropriate antibiotics. For solid medium, 2% agar was added to LB medium. *P. putida* strains were grown at 28°C with shaking in 2× YT medium and MSBY medium supplemented with 0.5% ethanol (MSBYE medium). MSBY medium consisted of 4.3 g of NaH2PO4, 3.4 g of KH2PO4, 2.0 g of (NH4)2SO4, 0.34 g of MgCl2, 0.001 g of MnCl2, 4.0 g of FeSO4, 7H2O, 0.026 g of CaCl2, 2.0 g of Na2MoO4, 0.02 mg of Na2SeO4, 0.01 mg of ZnCl2, 7H2O, 0.01 mg of CoCl2, 6H2O, 0.001 mg of NiSO4, 6H2O, 0.001 mg of Na2SeO4, and 0.1 g of yeast extract per liter of deionized water. When necessary, kanamycin and ampicillin were added at 50 µg/ml and 50 µg/ml, respectively.

DNA manipulation Standard procedures were used for plasmid DNA preparations, restriction enzyme digestions, ligations, transformations, and agarose gel electrophoresis (22). **PCR reactions were carried out using KOD Plus DNA polymerase (Toyobo, Tokyo, Japan) according to the manufacturer’s instructions.** *P. putida* was transformed by electroporation as described previously (19). DNA sequencing analysis was performed for PCR products to validate accuracy of PCR amplification.

**Plasmid construction** Plasmid pHA10 is a broad-host-range vector with carbenicillin resistance gene as a selectable marker gene (23). Since *P. putida* is inherently resistant to carbenicillin, carbenicillin resistance gene was replaced with *kan* gene from pUC4K (24) to construct broad-host-range vector pHK1 for gene from pUC4K(24) to construct broad-host-range vector plasmid pHK1 for constellation. strong and constitutive promoter system chloroform/ethyl acetate:acetic acid (50:50:1 v/v/v). Metabolites were detected by ferric chloride (detection of phenolic compounds) (25). Metabolites were further analyzed by mass spectrometry (MS). Each TLC spot was scrapped off and extracted with methanol. The extract was analyzed by MS using an LTQ Orbitrap XL (Thermo Fisher Scientific Inc., MA, USA) in the flow-injection mode.

**High performance liquid chromatography analysis** Substrates and their metabolites were also analyzed by high performance liquid chromatography (HPLC) system (JASCO Co., Tokyo, Japan) with a reverse phase column (TSKgel ODs-80TM, Tosoh, Yamaguchi, Japan) at a flow rate of 0.8 ml/min. UV absorption was measured at 240 nm. The compounds were eluted by using a linear gradient of 10–70% acetonitrile-water over 30 min.

**RESULTS AND DISCUSSION**

**Toluene-induced *P. putida* T57 degraded 4CA** *P. putida* T57 possesses toluene-inducible toluene dioxygenase (Tod) pathway (21) and utilizes several aromatic compounds through Tod pathway (19). Since toluene dioxygenase has broad substrate specificity (20), we supposed that toluene-induced *P. putida* T57 could degrade 4CA. We performed resting-cell reaction to examine *P. putida* T57 for its ability to degrade 4CA. Toluene-non-induced cells showed very weak, but significant activity of 4CA degradation (Fig. 1). Toluene-induced *P. putida* T57 cells degraded 4CA more rapidly than toluene-non-induced cells. This result indicates that toluene induces the additional ability to degrade 4CA in *P. putida* T57, which supports the idea that Tod pathway is involved in 4CA degradation.

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**TABLE 1. Bacterial strains and plasmids used in this study.**

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<th>Strain or plasmid</th>
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<td>19</td>
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<tr>
<td><em>Escherichia coli</em> DH5α</td>
<td>F-, ΔlacZΔM15, ΔlacZYA-argF(U169), deoR, recA1, endA1, thi-1, M15, araD139, M15, thi-1, endA1, recA1, phoA, supE44, thi-1, M15, ΔlacZYA-argF(U169), deoR</td>
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<td>pH10</td>
<td>Escherichia –Pseudomonas shuttle vector; Cic</td>
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<td>pHK1</td>
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| pUC4K | pUC4K with a 3.7-kb PCR fragment containing todC1todC2BADE from pUC4K and C1C2BADE with a 5.5-kb BamHI–NolI fragment containing **TABLE 1. Bacterial strains and plasmids used in this study.**

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<td>This study</td>
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Ap’, ampicillin resistance; Cβ, carbenicillin resistance; Km’, kanamycin resistance.

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P. putida T57 toluene dioxygenase oxidizes 4CA  
Initial step of Tod pathway, oxidation of aromatic compounds, is catalyzed by toluene dioxygenase (19). To confirm that Tod pathway is involved in 4CA degradation, we introduced todC1C2BA genes encoding toluene dioxygenase complex (toluene dioxygenase, ferredoxin and ferredoxin reductase) into E. coli DH5α and examined the resulting recombinant E. coli strain to oxidize 4CA. Resting-cell reaction indicated that E. coli DH5α (pHK-C1C2BA) degraded 4CA (Fig. 2A). TLC followed by ferric chloride staining found three phenolic metabolites (Fig. 2B) and subsequent MS analysis identified them as 4-chlorocatechol, 2-amino-5-chlorophenol, and 5-chloropyrogallol (Fig. 2C). In the previous studies, it was reported that the initial step of 4CA degradation in chloroaniline-degrading bacteria is oxidative deamination and its product is 4-chlorocatechol (6,18). 4-Chlorocatechol formation in E. coli DH5α (pHK-C1C2BA) resting-cell reaction indicates that toluene dioxygenase catalyzes 1,2-dioxygenation of 4CA, which generates 4-chloroaniline-1,2-cis-dihydrodiol and it converts to 4-chlorocatechol by spontaneous deamination (Fig. 3A). Generation of 2-amino-5-chlorophenol suggests that toluene dioxygenase also catalyzes 2,3-dioxygenation of 4CA, which generates 4-chloroaniline-2,3-cis-dihydrodiol and it then converts to 2-amino-5-chlorophenol by spontaneous dehydration. It is supposed that 5-chloropyrogallol was generated by further dioxygenation of 4-chlorocatechol. In E. coli DH5α (pHK-C1C2BA) resting cell reaction using 4-chlorocatechol as a substrate, we also detected 5-chloropyrogallol (data not shown), supporting this idea.

Król et al. demonstrated that multi component chloroaniline dioxygenase encoded by dcaA1, dcaA2, and dcaB are responsible for conversion of 3-chloroaniline (3CA) to 4-chlorocatechol in C. testosteroni WDL7 (18). The dcaA1, dcaA2, and dcaB genes code for large and small subunits of chloroaniline dioxygenase, and chloroaniline dioxygenase reductase, respectively. Chloroaniline dioxygenase large subunit (DcaA1) shares high amino acid sequence identity (79–98% identity) with aniline/dichloroaniline large subunits of aniline-utilizing bacteria, D. acidovorans 7N (26), P. putida UCC22 (27), D. tsuruhatensis AD9 (28), and Frateuria sp. ANA-18 (29) while chloroaniline dioxygenase small subunit (DcaA2) shows 71–97% identity to aniline dioxygenase small subunits of these aniline-utilizing bacteria. Large (TodC1) and small subunits (TodC2) of P. putida T57 toluene dioxygenase show much lower identity to aniline dioxygenase small subunits of these aniline-utilizing bacteria. Large (TodC1) and small subunits (TodC2) of P. putida T57 toluene dioxygenase are phylogenetically distant from those of aniline/chloroaniline dioxygenases (Fig. 4). Nonetheless P. putida T57 toluene dioxygenase still catalyzes oxidative deamination from 4CA to 4-chlorocatechol. It is the first finding that toluene dioxygenase catalyzes oxidative deamination of 4CA.
Enhancement of 4CA degradation activity in *P. putida* T57 by gene dosage effect

Even after toluene induction, *P. putida* T57 showed only weak 4CA degradation ability. To enhance 4CA degradation ability of *P. putida* T57, we introduced plasmid pHK-C1C2BADE harboring the entire *tod* operon into *P. putida* T57 and resulting *P. putida* T57 (pHK-C1C2BADE) was subjected to resting cell reaction to evaluate 4CA degradation. *P. putida* T57 (pHK-C1C2BADE) showed approximately 250-fold higher 4CA degradation rate (1.5 mM/h) than toluene-induced parent strain did (5.7 mM/h) (Figs. 1 and 5A). Resting cell reaction was conducted at different initial concentrations of 4CA (1-4 mM) and it was revealed that *P. putida* T57 (pHK-C1C2BADE) completely degraded up to 2 mM 4CA (Fig. 5A). Thus, 4CA degradation ability can be enhanced by increasing gene dosage of *tod* operon in *P. putida* T57.

Resting cell reaction using *E. coli* DH5α (pHK-C1C2BA) suggests that toluene dioxygenase converts 4CA to 4-chloroaniline-1,2-cis-dihydrodiol and 4-chloroaniline-2,3-cis-dihydrodiol (Fig. 3A). Toluene-2,3-cis-dihydrodiol oxidoreductase (the gene product of *todD*) oxidizes toluene-2,3-cis-dihydrodiol to 3-methylcatechol (19). Therefore, this enzyme is supposed to convert 4-chloroaniline-2,3-cis-dihydrodiol to 3-amino-5-chlorocatechol (compound VII in Fig. 3B). *P. putida* F1, which possess identical Tod pathway, can degrade 4-chlorocatechol, but not 3-chlorocatechol (31,32). In resting cell reaction using 4-chlorocatechol as a substrate, *P. putida* T57 (pHK-C1C2BADE) completely degraded 0.5 mM 4-chlorocatechol and produced a yellow color compound (data not shown), suggesting that catechol 2,3-dioxygenase (the gene product of *todE*) catalyzes meta-cleavage of 4-chlorocatechol. Therefore, *P. putida* T57 (pHK-C1C2BADE) is supposed to degrade 4-chlorocatechol but not 3-amino-5-chlorocatechol nor 5-chloropyrogallol. From these results, the degradation pathway of 4CA in *P. putida* T57 (pHK-C1C2BADE) is proposed in Fig. 3B. It is noteworthy that *P. putida* T57 (pHK-C1C2BADE) could not utilize 4CA and 4-chlorocatechol as sole carbon and energy sources (data not shown).

**Substrate specificity** Various chloroanilines, including 2-chloroaniline (2CA), 3CA, 3,4-dichloroaniline (34DCA), and 3,5-dichloroaniline (35DCA) were tested for biodegradation by *P. putida* T57 (pHK-C1C2BADE). In resting cell reaction, degradation rates of 2CA, 3CA, 4CA, and 34DCA by *P. putida* T57 (pHK-C1C2BADE) were found to be 0.15 mM/h, 0.5 mM/h, 1.5 mM/h, and 0.1 mM/h, respectively (Fig. 5B). This strain hardly degraded 35DCA.

In conclusion, we, for the first time, demonstrated that *P. putida* toluene dioxygenase can oxidized chloroanilines. It catalyzes
1,2- and 2,3-dioxygenation of 4CA. 1,2-Dioxygenation of 4CA results in oxidative deamination of 4CA and 4-chloroacetol is generated. Introduction of *tod* operon considerably enhanced the ability of *P. putida* T57 to degrade chloroanilines. *P. putida* T57 (pHK-C1C2BADE) degraded 2CA, 3CA, and 34DCA as well as 4CA. Among chloroanilines tested, 4CA was the best substrate followed by 3CA, 2CA, and 34DCA, but it hardly degraded 35DCA. These results suggest that toluene dioxygenase pathway would be useful for bioremediation of chloroaniline-contaminated sites. However, *P. putida* T57 (pHK-C1C2BADE) could not utilize chloroanilines as sole carbon and energy source in spite of its strong activity degrade chloroanilines, indicating that it could not mineralize chloroanilines and that it would accumulate dead end products. To completely degrade chloroanilines, it is necessary to provide *P. putida* T57 recombinant strain with the ability to efficiently catabolize chlorocatechols.

**References**

Title: Identification of CtpL as a chromosomally-encoded chemoreceptor for 4-chloroaniline and catechol in *Pseudomonas aeruginosa* PAO1

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Running title: PAO1 chemoreceptor for 4-chloroaniline and catechol

Keywords: Chemotaxis, Methyl-accepting chemotaxis protein, 4-chloroaniline, *P. aeruginosa* PAO1
ABSTRACT

Bacterial chemotaxis influences their ability to survive and thrive in most environments, including polluted ones. Despite numerous reports of the phenotypic characterization of chemotactic bacteria, only a few molecular details of chemoreceptors for aromatic pollutants have been described. In this study, the molecular basis of chemotaxis towards an environmentally toxic chlorinated aromatic pollutant, 4-chloroaniline (4CA), was evaluated. Among the three Pseudomonas spp. tested, Pseudomonas aeruginosa PAO1 (PAO1) exhibited positive chemotaxis to both the non-metabolizable 4CA, where 4-chloroacetanilide was formed as a dead-end transformation product, and to the metabolizable catechol. Molecular analysis of all 26 mutants with a disrupted methyl-accepting chemotaxis gene revealed that CtpL, a chromosomally-encoded chemoreceptor, was responsible for the positive chemotactic response towards 4CA. Since CtpL has previously been described as a major chemoreceptor for inorganic phosphate at low concentrations in PAO1, this is to report a fortuitous capability of CtpL to function towards aromatic pollutants. In addition, its regulation was not only dependent on the presence of the chemoattractant inducer, but was regulated by conditions of phosphate starvation. These results expand the range of known chemotactic transducers and their function in the environmental bacterium, PAO1.

INTRODUCTION

Chemotaxis is one of the most important behavioral adaptations of bacteria to mediate a balance between the nutritious and toxic effects of surrounding chemicals. Bacterial chemotactic attraction to environmental pollutants can enhance biodegradation rates by increasing pollutant bioavailability, and this potentially leads to an improvement in the bioremediation efficiency (1).
Several studies have reported the isolation and characterization of bacteria with a chemotactic response towards a variety of hazardous aromatic pollutants, including pesticides (2, 3), petroleum-associated monoaromatic (4-6), polyaromatic (7, 8), and aromatic derivatives (9, 10). However, such information is relatively scarce for some of the recently recognized recalcitrant xenobiotics, such as chloro-nitroaromatics, furan, chlorinated anilines, and so on (9, 11). Despite the numerous reports of the isolation and phenotypic characterization of chemotactic bacteria, the specificity of bacterial chemotactic response to chemicals is actually determined by their chemoreceptors (12). However, so far only three chemoreceptors for aromatic pollutants have been described; the plasmid-encoded NahY in the naphthalene-degrading *Pseudomonas putida* G7 (13), the plasmid-encoded NbaY in the 2-nitrobenzoate-degrading *P. fluorescens* KU-7 (14), and the plasmid-encoded McpT in the toluene, benzene, and ethylbenzene-degrading *P. putida* DOT-T1E (DOT-T1E) (5, 12).

4-Chloroaniline (4CA) is a chlorinated aromatic amine (i.e. a chlorinate aniline), that is now ubiquitous in the environment (15) because it has not only been intensively used in and released from chemical industries, but it is also a key intermediate in the natural transformation of pesticides (16). Due to its toxicity, it has been recognized as one of the priority hazardous substances and is subject to cleanup using biological treatment (17). Biodegradation of the pollutant can be enhanced using motile bacteria with suitable chemotactic capabilities to target the pollutant chemical(s), but for 4CA chemotaxis has yet to be reported.

In this study, the potential chemotactic response to 4CA was initially examined in three well characterized chemotactic *Pseudomonas* species (*P. putida* F1, *P. fluorescens* Pf01 and *P. aeruginosa* PAO1 (PAO1)). The positive chemotactic response of PAO1 to 4CA led to further investigations on the quantitative analysis of bacterial chemotaxis and the biotransformation of
4CA by this bacterium. Molecular analysis revealed that CtpL is the chemoreceptor responsible for chemoattraction of PAO1 to the non-metabolizable 4CA as well as to the metabolizable catechol. The highlight of the work is to report a chromosomally encoded chemoreceptor responsible for chemoattraction of chlorinated aromatic pollutants and to describe a fortuitous capability of CtpL, which has previously been reported to serve as a major chemoreceptor for inorganic phosphate (Pi) at low concentrations in PAO1 (18).

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions and culture media. The bacterial strains, mutants, and plasmids used in this study are listed in Table 1 (19-26). Each strain of the three Pseudomonas species, and the series of PAO1 mutants, was initially cultured in 2xYT medium (1.6% (w/v) tryptone, 1.0% (w/v) yeast extract, 0.5% (w/v) NaCl) at 28 °C for 18 h. For the chemotaxis assay, a 2% (v/v) culture inoculum was then transferred to the Pi-free T₀ medium (g/liter: Tris-HCl (pH 7.6) 10, glucose 2, NaCl 2, NH₄Cl 1, KCl 0.1, Na₂SO₄ 0.1, CaCl₂⋅2H₂O 0.01, MgCl₂⋅6H₂O 0.01, FeCl₃ 0.001) (18), or the 5 mM-Pi-containing T₅ medium (as indicated), with or without the addition of the target chemical (either 4CA or catechol). After 6 h of growth, cells were harvested, washed twice at room temperature and re-suspended in chemotaxis buffer (10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 7.0) (18).

Quantitative chemotaxis assay. Computer-assisted capillary assays were conducted as described previously (27). Cell movement was observed under an inverted microscope within the area of approximately 0.8 x 0.8 mm² around the capillary orifice and was recorded as a MP4 file. Still digital images captured in each frame were used to count the number of bacterial cells at the initial time (N₀) and at each given time interval (Nₜ) around the mouth of a capillary tube.
containing a known concentration of the test compound solidified with 1% (w/v) agarose. Casamino acid at 2% (w/v) was used as a positive control. The strength of the chemoefector was determined in terms of the normalized cell number per frame \( \frac{N_t}{N_0} \) as a measure of the magnitude of cell chemotactic response toward the test chemical.

**DNA manipulation, plasmid construction and electroporation.** Standard techniques for DNA preparation, plasmid construction, and cloning were used (24). The enzymes were all from Toyobo, Tokyo, Japan. PAO1 was transformed by electroporation as described previously (18).

**RNA extraction and quantitative real time reverse transcriptase PCR (qrtRT-PCR).** Overnight-grown cells in 2xYT medium were used as an inoculum for cell growth in the following media: 11-mM glucose-containing T₀ or T₅ medium, or T medium, each with or without 4CA (1 mM). After 6 h incubation, cells were harvested and the total RNA from them using NucleoSpin® RNA II kit (Macherey-Nagel, PA, USA) according to the manufacturer’s instructions. Two-stage quantitative real time (qrt)RT-PCR was performed by first using RT-PCR to generate the cDNA from the total RNA using the One-Step SYBR PrimeScript RT-PCR kit (Takara Bio Inc., Japan), and then using this cDNA for the qrt-PCR stage on a LightCycler 1.5 thermocycler (Roche Diagnostics, USA). Thermal cycling for the qrt-PCR was performed at 42 °C (5 s) followed by 40 cycles of 95 °C (5 s), 57 °C (10 s), and 72 °C (6 s), using the ctpL or gyrB primers. The gene expression data were normalized to the expression of the endogenous reference gyrB gene, and reported as the relative value. The primers for ctpL, which were designed between transmembrane regions 1 and 2 to give an expected product size of 119 bp, were 5’-GGAGTTGCGCGAATTCAG-3’ and 5’-CGGACCATTGTTCCAGGTT-3’. The primers for gyrB were 5’-TGCTGCTGACCATTCTTCTTCC-3’ and 5’-CTTGCTTGCCTTTCTTGACCTT-3’ giving an expected product size of 98 bp. Subsequently,
gene expression data was subjected to one-way ANOVA analysis with Student–Newman–Keuls post test using GraphPad InStat version 3.00 (GraphPad, SD, USA). Data were means of at least three independent experiments with standard deviation. P-values < 0.05 were considered significant.

4CA Biotransformation. 4CA transformation was conducted using a resting-cell suspension of PAO1. Cells were grown at 28 °C, 150 rpm, for 14 h in mineral salt basal (MSB) medium (4.3 g of K$_2$HPO$_4$, 3.4 g of KH$_2$PO$_4$, 2.0 g of (NH$_4$)$_2$SO$_4$, 0.34 g of MgCl$_2$.6H$_2$O, 1 mg of MnCl$_2$.4H$_2$O, 6 mg of FeSO$_4$.7H$_2$O, 26 mg of CaCl$_2$.2H$_2$O, 0.02 mg of Na$_2$MoO$_4$.2H$_2$O, 0.01 mg of ZnCl$_2$.7H$_2$O, 0.01 mg of CoCl$_2$.6H$_2$O, 0.01 mg of CuSO$_4$, 0.001 mg of NiSO$_4$.6H$_2$O and 1 µg of Na$_2$SeO$_4$, per liter of deionized water) supplemented with 0.1g/L of yeast extract (MSBY). The overnight-grown cells were then harvested. The cell pellet was washed twice with MSB medium and re-suspended in MSBY to the optical density of 5. Then, 4CA was added at 2 mM into the suspension culture and incubated at 28 °C and 150 rpm for 30 h. At the specified time interval, the samples were taken for chromatography analysis.

Chromatography analysis. The transformation of 4CA was analyzed by high performance liquid chromatography (HPLC) (JASCO Co., Tokyo, Japan) with a reverse phase column (TSKgel ODS-80TM, Tosoh, Yamaguchi, Japan) at a flow rate of 0.8 mL/min. UV absorption was measured at 240 nm. The compounds were eluted using a linear gradient of 10 - 70% (v/v) acetonitrile-water over 30 min. Qualitative and quantitative analyses were performed using a range of standard concentrations of 4CA (Nacalai Tesque, Japan) for comparative peak area evaluation, which had a HPLC retention time of 19.18 min under these test conditions. The transformation intermediate formed during the reaction was further analyzed using a p-chloroacetanilide (4CD) standard (Sigma-Aldrich, USA), which had two HPLC retention times...
of 14.50 and 14.75 min under these test conditions. The sample fraction collected from the
transformation reaction was then extracted and concentrated with ethyl acetate, and dried over
anhydrous sodium sulfate. The dried sample was re-dissolved with ethyl acetate prior to
identification using liquid chromatography-mass spectroscopy (LC-MS) on a LTQ Orbitrap XL
(J108) instrument (Thermo Fischer Scientific Inc., MA, USA) in flow-injection mode. Methanol
and ethyl acetate (Riedel-de Haen) were used as controls. The mass spectrum was recorded by
monoisotopic negative mode (MS2 of [M-H]) and then the expected mass of each intermediate
were determined with the Xcalibur program (Thermo Scientific, USA).

RESULTS

Chemotactic response of Pseudomonas strains to 4CA. Members of the genus Pseudomonas
are ubiquitous in a large variety of natural environments, which reflects not only that they are
considered one of the most metabolically diverse microorganisms, but also that they have a range
of physiological and genetic adaptabilities, such as chemotactic responses to surrounding
chemicals, for their growth and survival (28). Several Pseudomonas spp. have been reported to
chemotactically respond to aromatic hydrocarbons and their substituted derivatives, but their
sensing capabilities to each of these chemoeffectors are varied, presumably due to their
chemoreceptor diversity (3, 5-8, 13, 14). In this study, the well-studied motile P. putida F1 (F1),
P. fluorescens Pf01 (Pf01) and P. aeruginosa PAO1 (PAO1) (Table 1), were grown without
chemical induction and analyzed for their chemotactic response to 4CA using a computer-
assisted capillary assay. All strains showed normal motility and a positive chemotactic response
to 2% (w/v) casamino acid, the positive control, but they responded differently to 4CA. Under
the test conditions, F1 and Pf01 exhibited very weak chemotactic responses to 4CA with the
N\textsubscript{t}\textsubscript{60}/N\textsubscript{t}\textsubscript{0} value ranging from 1 – 1.2 (data not shown), while PAO1 showed a stronger chemotactic attraction (Fig. 1A). This result is in agreement with the fact that the different *Pseudomonas* species/strains can respond differently to the same chemoattractant (11). Since PAO1, one of the bacteria with well characterized chemosensory system (21), positively responded to the environmental pollutant 4CA, PAO1 was selected for further studies.

**Chemotactic response and metabolic activity of PAO1 to 4CA and aromatic analogues.**

As chemotactic responses of bacteria allow them to move toward a microenvironment that is optimal for their growth and survival (or away from detrimental ones), several bacterial growth-supporting chemicals generally act as their chemoattractants (29). To determine the metabolic activity and chemotactic behavioral responses of PAO1 to 4CA and other aromatic analogues, cell growth was determined in MSB medium supplemented with each chemical tested as a sole carbon source, while the chemotactic response was examined from cells grown under a non-induced condition. The results revealed that PAO1 was chemotactically attracted by almost all of the metabolizable, growth-supporting test chemicals, the exception being 4-hydroxybenzoic acid (Table 2). Of the four growth-supporting aromatics tested, catechol was the strongest attractant for PAO1. In addition, PAO1 showed positive chemotactic responses to some non-metabolizable chemicals, such as 3-chloroaniline (3CA), 4CA, 34DCA, and 4-aminobenzoic acid. Further investigation showed that although PAO1 was not able to utilize 4CA as a carbon source, it could stoichiometrically transform 4CA to 4CD (Fig. 2A and 2B), which appeared in PAO1 to be a dead end product in this degradation pathway (Fig. 2C). Chemotaxis assays also revealed that 4CD was not a chemoeffector for PAO1 (Table 2). Thus, the motility behavior of PAO1 towards 4CA was solely a positive chemotactic response of cells to 4CA.
The quantitative analysis of PAO1 chemotaxis with various concentrations of the non-
metabolizable 4CA and the metabolizable catechol were expressed as bell-shaped concentration
response curves, which indicated that cell chemotactic response was dependent on the
chemoeffector concentration (Fig. 1A - C). The strength of the chemotactic response of PAO1 to
both 4CA and catechol increased as the concentration increased up to the optimal concentration
at 1 mM for 4CA and 2 mM for catechol and then decreased at higher concentrations (Fig. 1C).
The difference in the cell chemotactic behavior towards the non-metabolizable 4CA and the
metabolizable catechol was that the cell chemoattraction to catechol rapidly declined after 45 s of
exposure (Fig. 1B), whereas it tended towards reaching a plateau for 4CA (Fig. 1A).
The above results indicated that the chemotactic response of PAO1 to 4CA was constitutive
under the conditions tested. Nevertheless, generally this natural ability can be enhanced if it is
inducible (9). Therefore, an induction test was conducted by growing cells in the presence and
absence of its chemoeffector as an inducer (1 mM of either 4CA or catechol) for 6 h prior to
chemotaxis assay with each corresponding chemoeffector. The results indicated that there was no
noticeable difference in the apparent chemotaxis strength towards 4CA among non-induced cells,
4CA-induced cells, and catechol-induced cells (Fig. 1D).

**Identification of the che gene involved in chemotactic response of PAO1 to 4CA.** PAO1
has a complex chemosensory system with several known chemotaxis (che) genes (21, 30, 31). To
identify the che gene(s) potentially involved in the chemoattraction of PAO1 to 4CA, five Che-
cluster defective mutants (Table 1) were examined for their responses to 4CA. All of these
mutants were confirmed to be fully motile. Upon testing with 4CA (1 mM) using the computer-
assisted capillary assay, the ΔCHE1 mutant, which has a deletion of the che cluster 1 (cheY cheZ
cheA cheB motA2 motB2 cheW), and PC4, a cheR mutant, completely lost chemoattraction to
4CA, while the chemotactic behavior of ΔCHE3 (ΔPil-Chp cluster), ΔCHE4 (ΔChe2 cluster), and ΔCHE5 (ΔWsp cluster) remained normal and similar to the response of the wildtype (PAO1-WT) (Fig. 3A). The results indicated that chemotaxis of PAO1 to 4CA appears to essentially involve the action of the che cluster 1 and cheR. This indicated that PAO1 chemotaxis to 4CA is in fact methyl-accepting chemotaxis protein (MCP)-dependent.

CtpL was identified as a chemotactic transducer for 4CA and catechol in PAO1. Bacterial cells detect chemoeffectors using cell surface MCP chemoreceptors. PAO1 contains 26 known-mcp-like genes in its genome (21, 30, 31). To identify which mcp gene(s) was/were potentially involved in the 4CA chemoattraction, a series of 26 mcp mutants, which had previously been generated by either deletion or a kanamycin-cassette insertion of an individual mcp-like gene in the PAO1 genome (18, 32, 33), were each examined for their chemotactic behavior towards 4CA. Twenty-five of these mcp mutants exhibited a normal chemoattractive behavior towards 4CA that was similar to that of the PAO1-WT. The responses of the \textit{tlpQ} (PA2573)- and \textit{pctA} (PA4309)-deficient mutants are shown as examples (Fig. 3B). In contrast, the ctpL-deficient mutant (ΔCTPL) showed no detectable chemotactic ability to 4CA (Fig. 3B). To elucidate the potential role of CtpL in the chemoattraction in PAO1 to 4CA, gene complementation was conducted prior to assaying the transformant for chemotactic ability. A plasmid harboring a functional copy of \textit{ctpL} (pCTPL) was constructed (Table 1) and introduced into the corresponding ΔctpL single mutant. Positive chemotaxis to 4CA was fully restored in the transformed ΔCTPL(pCTPL) (Fig. 3B). Furthermore, the chemotaxis response of the ctpL-deficient mutant with and without ctpL complementation to catechol (2 mM) revealed similar results; ΔCTPL showed no positive response to catechol, while the ΔCTPL(pCTPL) transformant
fully regained normal chemoattraction (data not shown). This result suggests that CtpL is responsible for the positive chemotaxis towards 4CA and catechol in PAO1.

In addition, to further examine the gene-dosage dependency in the chemotactic response, the chemotaxis assay with 4CA was conducted with the ctpL-over-expressing PAO1 transformant (PAO1(pCTPL)). Chemoattraction to 4CA in the ctpL overexpressing cells was not significantly increased (data not shown), suggesting that additional of ctpL gene copies did not markedly enhance the positive chemotactic response in PAO1.

**Expression of ctpL and the cell response towards 4CA and catechol in the presence of inorganic phosphate (Pi).** CtpL has previously been identified as a chemoreceptor in PAO1 for the chemotaxis to Pi at low Pi concentrations (18). The synthesis of CtpL is transcriptionally regulated by the phosphate starvation regulon (Pho regulon) under Pi limitation, but not in conditions of excess Pi (18, 34). In this study, the effect of the chemoeffectors (4CA and catechol) was investigated on ctpL expression using qrt-RT-PCR analysis, and on the cell chemotactic response under condition of Pi starvation (T₀ medium) or excess Pi (T₅ medium). Cultures were initially grown in 2xYT medium with excess Pi to suppress expression of ctpL and then cells were re-inoculated and grown for 6 h in 11 mM glucose-containing T₀ medium to induce ctpL expression, or in T₅ medium to keep the ctpL levels suppressed. In both media, 1 mM 4CA or 2 mM catechol was added at the previously determined optimal concentration for cell chemoattraction. In the Pi-deficient T₀ medium, the ctpL transcript level was relatively high as expected and also positively detectable in the presence of catechol or 4CA (although the expression level was 3.5- and 2.5 fold lower than that in the absence of the test chemoeffector) (Fig. 4). In the Pi-rich T₅ medium, the ctpL transcript level was very low as expected (six-fold...
Analysis of the chemotactic responses of PAO1 towards 4CA and catechol in the presence of Pi at 0, 1, 10 and 100 µM in chemotaxis buffer revealed a positive chemoattraction of cells towards 4CA and catechol at low (0 and 1µM) Pi concentrations (Fig. 5). However, essentially no chemoattraction to the chemoeffector was noted at the higher Pi concentrations of 10-µM and 100-µM (Fig. 5).

**DISCUSSION**

Chemotaxis is one of the most important bacterial mechanisms for sensing and responding to chemical changes in ecologically diverse environments, including toxic environments. The genus *Pseudomonas* has been shown to have a broad chemotactic capability for a wide range of compounds, which in part allows for its physiological and metabolic diversity (28, 35). Previous studies have shown that several *Pseudomonas* spp. are capable of chemotactic responses to a wide range of aromatic pollutants, but with diverse sensing specificities (3, 5-8, 13, 14). In this study, among the three well-studied, motile *Pseudomonas* spp., only PAO1 showed a positive chemotactic response to 4CA, the target pollutant.

The difference in bacterial chemo-sensing ability lies in the type and number of chemoreceptors. PAO1 has a complex chemosensory system with more than 20 chemotaxis (che) genes and 26 chemoreceptor genes (21), which results in its ability to thrive in various environmental niches. Chemotactic behavior is based on the recognition of environmental signals through cell surface methyl-accepting chemotaxis proteins (MCPs), of which so far the following MCPs have been identified in PAO1 for several stimuli, including PctA, PctB, and PctC for...
274 amino acids (36), CtpL and CtpH for inorganic phosphate (37), Aer, and Aer-2 for oxygen (32),
275 PA2652 for malate (38), TlpQ for ethylene (39), and PctA for volatile chlorinated aliphatic
276 hydrocarbons (40). In this study, a role of CtpL (PA4844), a chromosomally encoded,
277 transmembrane protein in PAO1, as a chemotactic transducer for aromatic compounds including
278 4CA and catechol was revealed. Although the precedent of chromosomally encoded
279 chemoreceptor(s) for aromatic hydrocarbons was briefly discussed for DOT-T1E (5), all
280 previously reported chemoreceptor genes for aromatic pollutants so far have been plasmid-borne
281 (5, 13, 14). Moreover, chemoreceptor gene products in bacteria with similar chemotactic
282 phenotypes have been reported to be close homologues. For instance, homologues of the McpT
283 receptor with 99% sequence identity were found in a self-transmissible plasmid in DOT-T1E and
284 the pMAQU02 plasmid of *Marinobacter aquaeolei* VT8, which are both responsible for the
285 chemotaxis phenotype towards aromatic hydrocarbons (41). In contrast, the distinct chemotactic
286 phenotype towards 4CA in the *P. aeruginosa* PAO1, *P. putida* F1 and *P. fluorescens* Pf01 is
287 perhaps the result of a low sequence identity (65 – 66%) between CtpL in PAO1 and the Blast-
288 matched MCP in F1 (YP_001265953.1) and Pf01 (YP_346355.1).
289 In general, compounds capable of promoting and/or inducing a positive chemotactic response
290 serve as metabolizable nutrients. In accord, PAO1 exhibited a relatively strong attraction towards
291 the metabolizable catechol, which it can utilize as a sole carbon source. The rapid decline in cell
292 chemoattraction, which was observed after 45 s of catechol exposure, likely, reflected the
293 decreased local concentration of catechol due to its uptake and metabolism. In contrast, PAO1
294 could only transform 4CA to 4-chloroacetanilide, which then accumulated as a dead-end product,
295 indicating that the chemotactic response of PAO1 is independent of the cell metabolic activity to
296 4CA. Although the physiological sensing of bacterial chemotaxis to non-metabolizable
compounds is not yet thoroughly understood, the results indicated that CtpL in PAO1 can recognize and mediate a positive chemotactic response towards metabolizable and non-metabolizable aromatic pollutants.

The capability of CtpL as a chemoreceptor for the test aromatic pollutants is proposed to be a fortuitous activity in PAO1. Since CtpL has been established as a major chemoreceptor for inorganic phosphate (Pi) taxis at low Pi concentrations, while CtpH functions for Pi taxis at high Pi concentrations (18). The expression of the ctpL gene is induced in Pi limiting conditions under the regulation of the PhoB and PhoU proteins (18). In this study, because cells were initially cultured under Pi starvation conditions (T₀ medium) prior to the assay, a relatively high transcript expression level was detected, which correlates with the positive chemotactic response of the non-induced cells to 4CA or catechol. When cells were cultured in the presence of 5 mM Pi in the T₅ medium, ctpL transcript expression level was suppressed but could be significantly increased by the addition of 4CA or catechol in the T₅ medium, indicating the positive inducibility of ctpL by these chemoeffectors. Nevertheless, the physiologically chemotactic response of PAO1 cells towards these chemoeffectors was reduced to a much greater extent in the presence of high concentrations of Pi, probably due to the toxicity effect of the pollutant. These results suggest that both Pi limitation and the presence of aromatic chemoeffector (catechol or 4CA) are the inducers for ctpL transcript expression, but the response is not cumulative. In addition, this may suggest that these chemoeffectors and Pi share the common binding site on CtpL. The direct binding assay is required as further investigation to clarify this point. Despite the fact that the increase of ctpL expression was conducted by either a gene induction or an increase of gene-dosage in PAO1(pCTPL), the physiological chemo-sensing activity of PAO1 toward 4CA was not markedly enhanced. This could reflect that CtpL does not
function independently as other chemoreceptors for aromatic pollutants. To acquire a full function, it may require a complex formation with other proteins in the Pst system under the regulation of Pho regulon (18).

In summary, the results of this study reveal that CtpL acts as a chemoreceptor for the positive chemotactic response of PAO1 to the non-metabolizable 4CA as well as the metabolizable catechol. The discovery of a chromosomally encoded chemoreceptor with broader fortuitous activity towards aromatic pollutants in PAO1 expands the range of the known chemotactic transducers and their function. Future comprehensive investigation of the CtpL-protein complex formation and its regulation will be necessary to elicit or enhance the positive chemotactic response of PAO1 or other environmental bacteria towards aromatic pollutants including 4CA.

ACKNOWLEDGEMENTS

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REFERENCES


Table legends

Table 1 Bacterial strains and plasmids used in this study

Table 2 Chemotaxis and growth of PAO1 and the ΔCTPL on chloroanilines and aromatic analogues

Figure legends

FIG. 1 Quantitative analysis and induction of the chemotactic response of PAO1 to 4CA and catechol. (A, B) Non-induced PAO1 cells were prepared in T0 medium (as described in Materials and methods) prior to exposure to (A) 4CA at (▲) 0.5, (■) 1, (●) 2, (♦) 4 mM, or (B) catechol at (▲) 1, (■) 2, (●) 10 mM, solidified with 1% (w/v) agarose in a capillary tube. The results were expressed as the normalized cell number (Nt/Nt0). The chemotaxis buffer (10 mM HEPES) in agarose (∗, dashed line) was the negative control. Casamino acid was the positive control giving the Nt60/Nt0 value of approximately 4. (C) Concentration response curve for the chemotaxis of non-induced PAO1 cells to 4CA (■) or catechol (●) measure at 60 s of exposure and expressed as Nt60/Nt0. (D) Induction of PAO1 chemotaxis to 4CA. Cells were cultured overnight in 2xYT medium, and then in 11 mM glucose-containing T0 medium, without (Non-ind) or with induction by either 1 mM 4CA (4CA-ind) or 1 mM catechol (Cat-ind). Chemotaxis, using a computer-assisted capillary assay, was then performed with 1 mM 4CA or catechol. The strength of chemotactic attraction is shown as the normalized Nt60/Nt0 cell ratio. In A–D, the data are shown as mean ± SD and are derived from at least three independent experiments conducted in triplicates.
FIG. 2 Biotransformation of 4CA and the formation of transformation product in PAO1. (A) Biotransformation of 4CA (■) (initial concentration of 2 mM) by PAO1 using a resting-cell technique, and the formation of 4-chloroacetanilide (4CD, ○) as a transformation product. (B) LC-MS spectra of the transformation product obtained from the PAO1 culture supernatant of media containing 4CA after 15-h of incubation. The m/z value was 168.02, which indicates 4CD (C8H7ONCl) as the transformation product. (C) Biotransformation of 4CD (initial concentration of 2 mM) (○) by PAO1 using a resting-cell technique.

FIG. 3 Chemotactic response of PAO1 mutants to 4CA. Non-induced PAO1 mutants and the wildtype (PAO1-WT) were prepared prior to the exposure to 4CA (1 mM) in a computer-assisted capillary assay. Chemotactic response of PAO1-WT to 4CA was used as the reference positive control. The results are expressed as the normalized cell number (Nt/Nt0). (A) Chemotactic behavior of the che mutants: ΔCHE1, ΔCHE3, ΔCHE4, ΔCHE5, and PC4. Response of the PAO1-WT to chemotaxis buffer (HEPES)-containing agar was the negative control. (B) Chemotactic behavior of the single Δmcp mutants. Responses of tlpQ-deficient mutant (ΔTLPQ) and pctA-deficient mutant (ΔPCTA) are shown as representative example of the mcp mutants exhibiting a similar chemotaxis to that of PAO1-WT, while no chemotactic response to 4CA was found in the ctpL-deficient mutant (ΔCTPL), but was restored by ctpL gene complementation, ΔCTPL(pCTPL). In A and B, the data are shown as mean ± SD and are derived from at least three independent experiments conducted in triplicates.
FIG. 4 Quantitative real time RT-PCR analysis of ctpL mRNA expression in PAO1. Overnight-grown cells in 2xYT medium were used as an inoculum for cell growth in 11 mM glucose-containing T0 (open bar) or T5 (solid bar) medium, each in the presence of the solvent only control (None), catechol (2 mM) or 4CA (1 mM). After 6 h incubation, total RNA was isolated and qRT-PCR analysis of the ctpL and gyrB transcript levels was performed. The results are expressed as the relative ctpL gene expression value after normalizing to the gyrB transcript level. Data are shown as mean ± 1SD and are derived from at least three independent experiments. Italic letters (with and without asterisk) indicate a significant difference at $P<0.05$ to the control within the same group, according to Student–Newman–Keuls post test.

FIG. 5 Quantitative analysis of the chemotactic response of PAO1 to 2 mM catechol (open bar) and 1 mM 4CA (solid bar) in the presence of different Pi concentrations in the chemotaxis buffer using a computer-assisted capillary assay. Overnight-grown cells in 2xYT medium were cultured in T0 or T5 medium as indicated, and then resuspended in the chemotaxis buffer with various concentrations of Pi (KH2PO4). The strength of the chemotactic attraction was determined by the increase of the cell number in the analysis frame area at $t_60$ (60-s exposure) normalized to that at $t_0$ ($N_{t_{60}}/N_{t_0}$). Data are shown as mean ± SD and are derived from at least three independent experiments conducted in triplicates.
### TABLE 1  Bacterial strains and plasmids used in this study

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<td><em>Pseudomonas fluorescens</em> strain PfO1</td>
<td>Wild type strain</td>
<td>(19)</td>
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<tr>
<td><em>Pseudomonas aeruginosa</em> PAO1</td>
<td>Wild type strain</td>
<td>(23)</td>
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<tr>
<td>mcp-disrupted PAO1 mutants</td>
<td>PAO1 derivatives</td>
<td>(18, 20, 27, 36)</td>
</tr>
<tr>
<td>∆CTPL (previously known as PP2)</td>
<td>PAO1 derivative, ctpL (PA4844)::kan′</td>
<td>(18)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> JM109</td>
<td>recA1, endA1, gyrA96, thi-1, hsdR17(rK-mS′), relA1, Δ(lac-proAB)F′[traD36, proAB, lacIq, lacZΔM15]</td>
<td>(24)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUCP18</td>
<td><em>Escherichia coli</em>-<em>Pseudomonas</em> shuttle vector; Ch′</td>
<td>(25)</td>
</tr>
<tr>
<td>pCTPL</td>
<td>pUCP18 containing a 1.974-kb PCR fragment of ctpL (PA4844); Ch′</td>
<td>This study</td>
</tr>
</tbody>
</table>
### TABLE 2

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Chemotactic response</th>
<th>Cell growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PAO1</td>
<td>∆CTPL</td>
</tr>
<tr>
<td>Aniline</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2-Chloroaniline (2CA)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3-Chloroaniline (3CA)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4-Chloroaniline (4CA)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2,4-Dichloroaniline (24DCA)</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>3,4-Dichloroaniline (34DCA)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4-Chloroacetanilide</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Catechol</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>4-Chlorocatechol</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sodium salicylate</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Anthranilic acid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4-Aminobenzoic acid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sodium benzoate</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Nitrobenzene</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>4-Chloronitrobenzene</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Benzene</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Toluene</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Casamino acid (positive control)</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>HEPES (10 mM) (negative control)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H2O (negative control)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Concentration of the test compound was at 1 mM for chemotaxis test and 10 mM for growth determination.

\(^b\) Chemotactic response was measured by a computer-assisted capillary assay as described in Materials and methods. Cell numbers (N) in the analysis frame area were captured at a starting time (t₀) and a 60-second chemical exposure time (t₆₀). The strength of chemotactic attraction is shown by the increase of cell number in the analysis frame area at t₆₀ after normalized with those at t₀ (N₆₀/N₀). The value of the normalized cell number is represented by the + symbol: 4 ≥ +++ > 3; 3 ≥ ++ > 2; 2 ≥ + > 1.2; 1.2 ≥ - > 0; ND, not determined.

\(^c\) Cell growth in MSB medium was determined as optical density of cells at 600 nm (OD₆₀₀) after 24h of incubation. The cell optical density value is represented by the + symbol: ++ > 0.6; 0.6 ≥ + > 0.1; 0.1 ≥ - > 0 (no growth); ND, not determined.
Fig. 2

(A) Graph showing the change in 4CA remaining (mM) and 4CD formation (mM) over time (h).

(B) Mass spectrum with peaks at m/z values indicated.

(C) Graph showing the change in 4CD remaining (mM) over time (h).
Fig. 3

A

Normalized cell number per frame vs. Time (s)

- PAO1-WT
- (delta)TLPQ
- (delta)PCTA
- (delta)CTPL
- (delta)CTPL(pCTPL)

B

Normalized cell number per frame vs. Time (s)

- HEPES
- PAO1-WT
- (delta)CHE1
- (delta)CHE3
- (delta)CHE4
- (delta)CHE5
- PC4
Fig. 4
Fig. 5

![Graph showing normalized cells number per frame against phosphate concentration (µM).]