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**Note**

# Identification and Characterization of Methyl-Accepting Chemotaxis Proteins for Trichloroethylene in *Pseudomonas putida* F1

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Twenty seven methyl-accepting chemotaxis proteins (MCPs) from trichloroehylene (TCE)-degrading *Pseudomonas putida* F1 were examined for their ability to detect TCE in *Pseudomonas aeruginosa* PCT2 and two proteins, CttA (Pput 2149) and CttB (Pput 2348) were identified as MCPs for TCE. Chemotaxis assays revealed that CttA detected toluene as well as TCE. Quantitative reverse-transcription PCR (qRT-PCR) demonstrated that both *cttA* and *cttB* were constitutively expressed in *P. putida* F1. Overexpression of *cttA* and *cttB* enhanced positive chemotaxis to TCE in *P. putida* F1.

**Key words:** chmoetaxis, trichloroethylene, *Pseudomonas putida* F1

*Pseudomonas putida* F1 is an aerobic motile bacterium and is capable of utilizing toluene as a growth substrate via the toluene oxygenase pathway [18]. Toluene-induced cells of *P. putida* F1 show the activity to degrade the priority environmental pollutant trichloroethylene (TCE) [6,15]. Toluene dioxygenase is responsible for TCE degradation in *P. putida* F1. This TCE-degrading bacterium has been intensively studied as bioremediation agents in chloroethylene-polluted environments. One special feature of *P. putida* F1 is chemotaxis to TCE and toluene [8]. When *P. putida* F1 is grown in the presence of toluene, it shows chemotactic responses to TCE and toluene. The migration of TCE-degrading bacteria toward TCE is expected to speed the biodegradation process because it should bring the cells into contact with TCE [9]. Despite these findings, very little is known about the chemosensory protein mediating positive chemotaxis to TCE and toluene in TCE-degrading bacteria. Chemotactic ligands are detected by cell surface chemoreceptors called methyl-accepting chemotaxis proteins (MCPs). Upon binding a chemotactic ligand, a MCP generates chemotaxis signals that are communicated to the flagellar motor via a series of chemotaxis proteins. In this study, we identified and characterized MCPs for TCE in *P. putida* F1.

For chemotaxis assays, *P. putida* F1, *Pseudomonas aeruginosa* PCT2 (a *pctA pctB pctC* mutant of *P. aeruginosa* PAO1) [13], and their derivatives were grown overnight in 2x YT medium [10] with shaking. Five hundred microliters of the culture broth was inoculated into $T_0$ medium [17] in a 50-ml screw-capped vial. When cells were exposed to toluene, minimum salts basal medium [2] supplemented with 50 mM pyruvate as a solo carbon source was used for cultivation and 0.5 ml toluene was added to a small tube inside the screw-capped vial. *P. putida* and *P. aeruginosa* were cultivated at 28 and 37°C, respectively. Cells were harvested by centrifugation, washed twice with chemotaxis buffer A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid], pH 7.0), and resuspended in the same buffer. Chemotaxis was assayed by computer-assisted capillary as described previously [7]. Digital image processing was used to count the number of bacteria around the mouth of a capillary containing a known concentration of a test compound plus 1% agarose.

For quantitative reverse transcription-PCR (qRT-PCR) analysis, *P. putida* F1 was cultivated as described above. Cells were harvested by centrifugation at 4°C for 10 min. Total RNA was extracted from bacterial cells using an NucleoSpin® RNA II kit (MACHEREY-NAGEL, GmbH & Co., Düren, Germany) with a RNAprotect bacteria reagent (QIAGEN, Inc., Valencia, CA, USA) for the stabilization of RNA. RNA samples were treated with a RNase-free DNase set (MACHEREY-NAGEL) to eliminate contaminating DNA. Complete removal of DNA was confirmed by PCR. A One Step SYBR PrimeScript RT-PCR kit (Takara Bio, Inc., Shiga, Japan) was used for cDNA generation and quantitative RT-PCR. Quantitative RT-PCR was carried out using a LightCycler system (Roche Diagnostics, Tokyo, Japan) with an reverse transcription at 42°C for 15 s followed by cycles of denaturation at 95°C for 5 s, primer annealing at 57°C for 10 s, and extension at 72°C for 6 s. Standard procedures were used for plasmid DNA preparations, restriction enzyme digestions, ligations, transformations and agarose gel electrophoresis [10]. Polymerase chain reactions (PCRs)

Table 1. Oligonucleotides used in this study

| Name | Sequence (5' to 3') |
|---|---|
| PPF02f | AATTGAGCTCTCAACGCATTCCGTTCGGTTAC |
| PPF02r | TATGGGTACCAGGTCAGGCAGCGACGAGATAC |
| PPF04f | ACTTGCTCGAGCTGTCGATGCCACGACTTG |
| PPF04r | GTAAACTGCAGAAAGACTGTGAGGCTATCGAACG |
| qPFgyrBf | CAGACTTACGTTCACGGTGTTCC |
| qPFgyrBr | GATGGCTTGAAGTGGATGTGG |
| qPFtodC2f | AGCGTCCCTTCATCATCGTG |
| qPFtodC2r | GACATTCACTACTTCATGCCCATTC |
| qPF02f | ACGTGTCTTCGGGCTTTATC |
| qPF02r | TCTGGCTCTTGTTGTTCAGC |
| qPF04f | CGCCTTGATGAAGTGGTTG |
| qPF04r | TGGTATTCGCCACGGTTC |
| KmRf | GTAAGACATATGCGGGAAGATGCGTGATCTG |
| KmRr | ATGATGCATATGGGAAAGCCACGTTGTGTCTC |
| Ptaqf | ATCGATATCCAGCTTGTCTGTAAGCGGATG |
| Ptaqr | ATCGATATCCAGTAGTAGGTTGAGGCCGTTG |

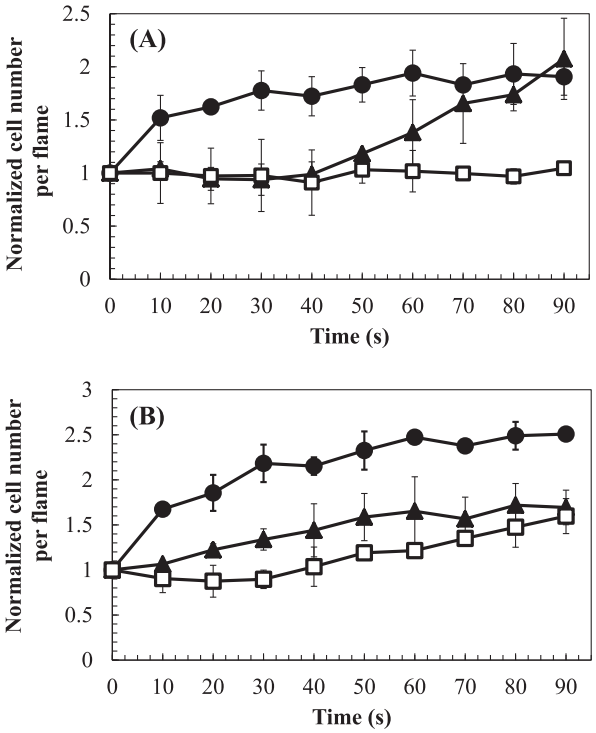


Fig. 1. Chemotaxis responses to TCE (A) and toluene (B) by *P. aeruginosa* strains. Digital image processing was used to count the number of bacteria around the mouth of a capillary containing 0.8 mM TCE and 1% agarose (A) or 0.1 mM toluene and 1% agarose (B). One videotape frame was analyzed at each time point and the normalized cell number was calculated by dividing the number of bacteria at each time point by that at the initiation of the observation. The chemotactic response is presented as the normalized cell number. PCT2 (open squares), PCT2 [pHEC02] (closed circles), PCT2 [pHEC04] (closed triangles). Vertical bars represent the standard deviations of measurements done in triplicate experiments.

were carried out using KOD plus (Toyobo, Tokyo, Japan) DNA polymerase according to the manufacturer's instructions. Oligonucleotides used for PCR and qRT-PCR are listed in Table 1. For *P. aeruginosa*, pUCP18 [12] was used as a plasmid vector. For *P. putida* F1, pUCPK18taq was used as a plasmid vector. Kanamaycin resistant gene from pUC4K [16] was introduced into pUCP18 and *lac* promoter of pUCP18 was substituted with *taq* promoter from pHA10 [1] to construct pUCPK18taq.

Genome annotation has revealed that *P. putida* F1 possesses 27 putative MCP genes (locus tags: Pput 0339, Pput 0342, Pput 0601, Pput 0623, Pput 0804, Pput 1257, Pput 1390, Pput 1872, Pput 2091, Pput 2149, Pput 2217, Pput 2348, Pput 2731, Pput 2828, Pput 3459, Pput 3481, Pput 3489, Pput 3621, Pput 3628, Pput 3892, Pput 4234, Pput 4352, Pput 4520, Pput 4764, Pput 4863, Pput 4894, and Pput 4895 [genome annotation data: NC 009512]). These genes were PCR amplified from the *P. putida* F1 genomic DNA and amplified products were cloned into pUCP18. The resulting plasmids were introduced to *P. aeruginosa* PCT2 and transformants were examined for their ability to respond to TCE. *P. aeruginosa* PCT2 is a *pctA pctB pctC* triple mutant of *P. aeruginosa* PAO1 [13]. The *pctA*, *pctB*, and *pctC* genes encode MCPs for negative chemotaxis to TCE [11]. *P. aeruginosa* PCT2 is a better host for screening a gene encoding MCP for TCE than *P. aeruginosa* PAO1 because it shows much decreased repelled responses to TCE. Chemotaxis assays revealed that two transformants harboring pHEC02 and pHEC04 were significantly attracted by TCE (Fig. 1). Plasmids pHEC02 and pHEC04 contain genes encoding proteins Pput 2149 and Pput 2348, respectively. Based on these results, the Pput 2149 and Pput 2348 genes were designated *cttA* and *cttB* (*ctt* stands for chemotactic transducer for TCE).

Typical MCPs are membrane-spanning homodimers and their features are as follows: a positively 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 [3]. 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. CttA has features of typical MCP (Fig. 2) and Conserved Domain-Search (CD-Search) program (National Center of Biotechnology Information) found HAMP and TarH signal domains, typical domains of MCP, in its C-terminal region. Blastp search using amino acid sequence of the putative periplasmic domain of CttA (residues 38 to 189) as a query sequence revealed that *P. aeruginosa* PAO1 does not possess a homologue of CttA. CttB seems to be atypical MCP. Hydropathy plot found no transmembrane region in CttB and PSORT program [5] predicted that it is a cytoplasmic protein. CD-Search found two PAS domains as well as MCP signal domain (TarH) in CttB (Fig. 2). The PAS motif is known to comprise a

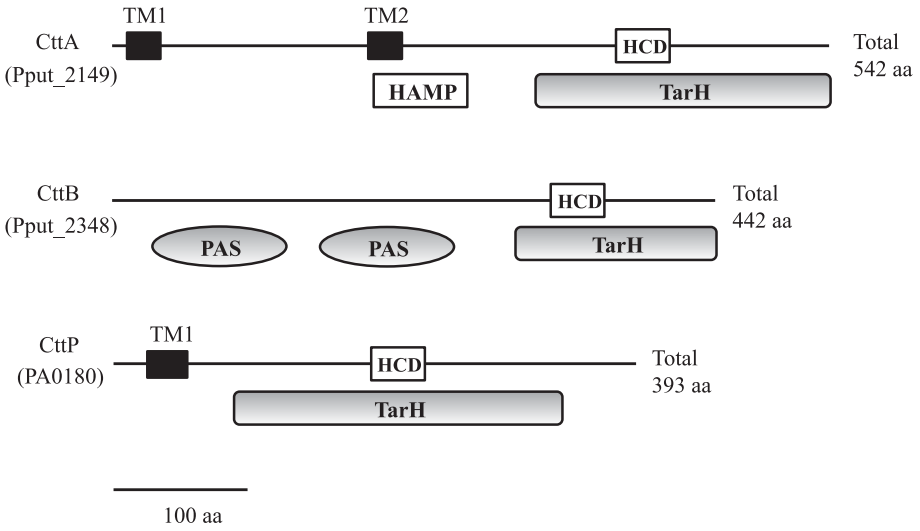


Fig. 2. Domain organization of CttA and CttB from *P. putida* F1 and CttP from *P. aeruginosa* PAO1 [2]. TM1 and TM2, transmembrane regions 1 and 2; HAMP, conserved signal transduction domain in histidine kinase, adenylate cyclase, methyl-accepting chemotaxis proteins, and phosphatases; PAS, acronym formed from names of the proteins in which imperfectrepeat sequences wre first recognized (the *Drosophia* period clock protein [PER], the vertebrate aryl hydrocarbon receptor nucler translocator [ARNT], and the *Drosophia* single-minded protein [SIM]); TarH (TarH superfamily), methyl-accepting chemotaxis protein signaling domain; HCD, highly conserved signaling domain consisted of 44 amino acid residues in methyl-accepting chemotaxis protein [11].

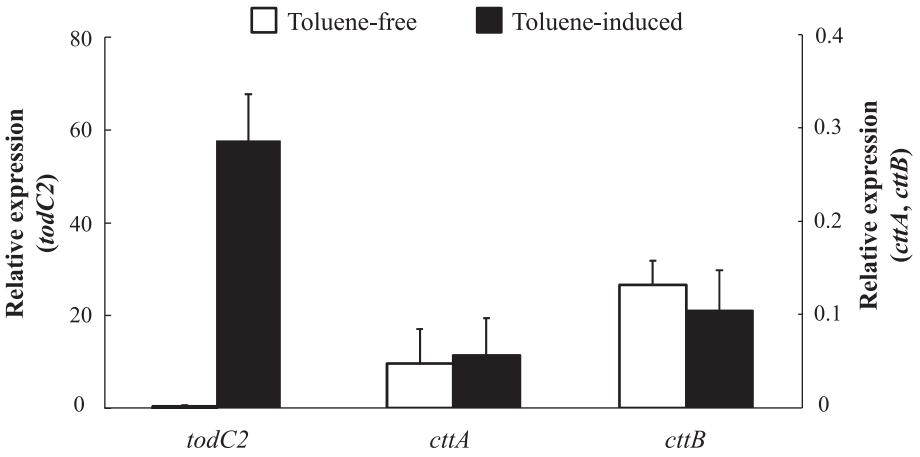


Fig. 3. Expression levels of *todC2*, *cttA* and *cttB* in *P. putida* F1.Total RNA was extracted from *P. putida* F1 cells grown in the presence of toluene (closed bars) and in the absence of toluene (open bars), and subjected to qRT-PCR. Relative expression was calculated based on *gyrB* expression as internal control gene. Vertical bars represent the standard deviations of measurements done in triplicate experiments.

binding pocket for a prosthetic group [13]. Blastp search using N-terminal amino acid sequence of CttB (residues 30 to 240) as a query reveled that CttB is 49% and 46% identical to *P. aeruginosa* PAO1 MCPs BdlA (PA1423) and PA1930, respectively, but it shows no significant similarity to CttP (PA0180) which is MCP for positive chemotaxis to TCE in *P. aeruginosa* PAO1 [4].

Parales *et al.* reported that toluene induced chemotaxis to TCE from *P. putida* F1 [8]. To investigate whether expression of *cttA* and *cttB* is induced by toluene, RNA was extracted from *P. putida* F1 cells grown in the presence and absence of toluene and subjected to qRT-PCR analysis. Although expression of toluene-inducible toluene dioxygenase gene (*todC2*) [18] increased in cells grown in the presence of toluene, *cttA* and *cttB* were constitutively expressed (Fig. 3). It is unlikely that CttA and CttB are responsible for toluene-inducible chemotaxis to TCE in *P. putida* F1 although they serve as MCPs for TCE. In this study, a MCP gene responsible for toluene-inducible chemotaxis to TCE could not be obtained probably because that gene was not functionally expressed in *P. aeruginosa* PCT2. Alternatively, an additional factor may be required for the MCP for inducible chemotaxis to TCE to detect TCE. For example, phosphate specific transportation system is essential for *P. aeruginosa* CtpL to act as a MCP for phosphate taxis [17].

Parales *et al.* demonstrated that toluene also induced chemotaxis to toluene from *P. putida* F1 [8]. Although CttA and CttB are not MCPs for inducible chemotaxis to TCE, it is interesting to investigate whether these MCPs detect toluene or not. So, we examined *P. aeruginosa* PCT2 har-

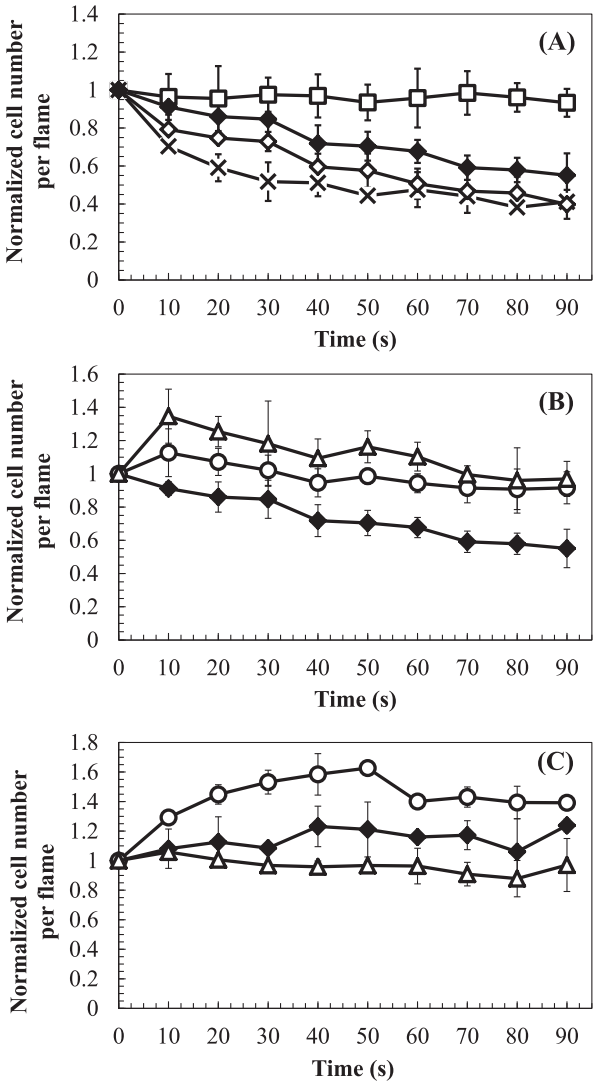


Fig. 4. Chemotaxis responses toward TCE by *P. putida* F1 strains. (A) chemotactic responses by *P. putida* F1 wild type strain grown in the absence of toluene. $H_2O$ (negative control), open squares; 0.8 mM TCE, closed diamonds; 2 mM TCE, open diamonds; 4 mM TCE, crosses. (B) chemotactic responses to 0.8 mM TCE by *P. putida* F1 (closed diamonds), F1 [pHEF02] (open circles), and F1 [pMEF04] (open triangles) grown in the absence of toluene. (C) chemotactic responses to 0.8 mM TCE by *P. putida* F1 (closed diamonds), F1 [pHEF02] (open circles), and F1 [pMEF04] (open triangles) grown in the presence of toluene. Vertical bars represent the standard deviations of measurements done in triplicate experiments.

boring pHEC02 and pHEC04 for their ability to respond to toluene. Chemotaxis assays showed that *P. aeruginosa* PCT2 [pHEC02] was attracted by toluene but not *P. aeruginosa* PCT2 [pHEC04] (Fig. 1), suggesting that CttA detects toluene as well as TCE.

To test whether chemotaxis to TCE could be enhanced by introducing the *cttA* and *cttB* genes into *P. putida* F1, we cloned *cttA* and *cttB* into pUCPK18taq to construct pMEF02 and pMEF04. The resulting plasmids were introduced to *P. putida* F1 and the transformants were examined for chemotaxis to TCE. *P. putida* F1 wild-type cells grown in the absence of showed repelled responses to 0.8–4 mM TCE (Fig. 4). *P. putida* F1 [pMEF02] and *P. putida* F1 [pMEF04] did not show repelled responses to TCE, rather was attracted by 0.8 mM TCE. *P. putida* F1 wild-type cells grown in the presence of toluene showed weak positive chemotaxis to TCE, while *P. putida* [pHEF02] showed increased responses to TCE. *P. putida* [pHEF04] did not show any responses to TCE because this strain showed very poor mobility in this cultivation condition. From these results, we can conclude that introduction of the *cttA* and *cttB* genes enhanced positive chemotaxis to TCE by *P. putida* F1.

In conclusion, two MCPs for TCE, CttA and CttB, were identified. CttA detected toluene as well as TCE. Both *cttA* and *cttB* were constitutively expressed in *P. putida* F1. Overexpression of *cttA* and *cttB* enhanced positive chemotaxis to TCE by *P. putida* F1. We are now searching MCP responsible for inducible chemotaxis to TCE in *P. putida* F1.

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