Appl Microbiol Biotechnol (2005) 66: 696–701
DOI 10.1007/s00253-004-1685-4

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Chemotaxis of *Pseudomonas stutzeri* OX1 and *Burkholderia cepacia* G4 toward chlorinated ethenes

Received: 20 November 2003 / Revised: 17 May 2004 / Accepted: 11 June 2004 / Published online: 28 July 2004

Abstract The chemotactic responses of *Pseudomonas putida* F1, *Burkholderia cepacia* G4, and *Pseudomonas stutzeri* OX1 were investigated toward toluene, trichloroethylene (TCE), tetrachloroethylene (PCE), *cis*-1,2-dichloroethylene (*cis*-DCE), *trans*-1,2-dichloroethylene (*trans*-DCE), 1,1-dichloroethylene (1,1-DCE), and vinyl chloride (VC). *P. stutzeri* OX1 and *P. putida* F1 were chemotactic toward toluene, PCE, TCE, all DCEs, and VC. *B. cepacia* G4 was chemotactic toward toluene, PCE, TCE, *cis*-DCE, 1,1-DCE, and VC. Chemotaxis of *P. stutzeri* OX1 grown on *o*-xylene vapors was much stronger than when grown on *o*-cresol vapors toward some chlorinated ethenes. Expression of toluene-*o*-xylene monooxygenase (ToMO) from *touABCDEF* appears to be required for positive chemotaxis attraction, and the attraction is stronger with the *touR* (ToMO regulatory) gene.

Introduction

The efficiency of in situ bioremediation can be increased if the bacteria and soil/groundwater contaminant are brought into close contact. Chemotaxis has an important role in enhancing the biodegradation of pollutants as it can overcome some of the limitations of in situ bioremediation such as poor bioavailability due to mass transfer limitations, low solubility, or sequestration of a chemical to a matrix surface (Parales and Harwood 2002; Samanta et al. 2002). For example, *Pseudomonas putida* G7 degrades naphthalene more rapidly than either a chemotaxis or a motility mutant (Marx and Aitken 2000).

Very little work has been done on bacterial chemotaxis toward chlorinated ethenes. *P. putida* F1 has been shown to exhibit positive chemotaxis to chlorinated ethenes, tetrachloroethylene (PCE), trichloroethylene (TCE), and *cis*-1,2-dichloroethylene (*cis*-DCE) (Parales et al. 2000). Negative chemotaxis of *P. aeruginosa* PAO1 has been observed with TCE, PCE, chloroform, and dichloromethane (Kato et al. 2001).

Chlorinated ethenes are a group of health-threatening pollutants. For example, PCE is one of 14 volatile organic compounds on the United States EPA's Priority Pollutant List (Carter and Jewell 1993). Vinyl chloride (VC) is a known human carcinogen (McCarty 1997), and both VC and *cis*-DCE are US EPA priority pollutants (Bradley and Chapelle 1998).

To accelerate aerobic attack of these pollutants, the chemotactic responses toward chlorinated ethenes as well as toluene were studied for *P. stutzeri* OX1 [(expressing toluene-*o*-xylene monooxygenase (ToMO)] and *Burkholderia cepacia* G4. *P. stutzeri* OX1 was isolated from activated sludge of a wastewater treatment plant (Baggi et al. 1987) and is the only known strain that degrades aerobically PCE (Ryoo et al. 2000); it also oxidizes TCE, 1,1-dichloroethylene (1,1-DCE), *cis*-DCE, *trans*-1,2-dichloroethylene (*trans*-DCE), VC, and chloroform (Chauhan et al. 1998; Shim and Wood 2000). ToMO consists *touARE* (a three-component hydroxylase with a catalytic oxygen-bridged dinuclear center), *TouC* (ferredoxin), *TouD* (a mediating protein), and *TouF* (NADH-ferredoxin oxidoreductase) (Bertoni et al. 1998).

B. cepacia G4 was the first pure culture that could degrade TCE (Nelson et al. 1986), and toluene *o*-monooxygenase (TOM) of *B. cepacia* G4 oxidizes TCE, 1,1-DCE, *cis*-DCE, *trans*-DCE, and VC, and converts naphthalene to naphthol (Shields et al. 1994; Shields and Francesconi 1996; Shim and Wood 2000). Six genes encode TOM (*tomA012345*), including *tomA1A3A4* (encodes a three-component hydroxylase with two catalytic

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oxygen-bridged binuclear iron centers), *tomA5* (encodes NADH oxidoreductase), and *tomA2* (encodes a protein involved in electron transfer between the hydroxylase and reductase) (Canada et al. 2002).

The chemotactic responses of *B. cepacia* G4, *P. putida* F1, and *P. stutzeri* OX1 were investigated here for TCE, PCE, *cis*-DCE, *trans*-DCE, 1,1-DCE, and VC as well as for toluene. This is the first report of chemotaxis toward VC, 1,1-DCE, and *trans*-DCE by any bacterial strain, chemotaxis with *P. stutzeri* OX1, and chemotaxis of *B. cepacia* G4 toward chlorinated ethenes. Furthermore, the involvement of *tou* genes and the *touR* (ToMO regulatory) gene in the role of positive chemotaxis toward PCE or *o*-xylene was investigated.

Materials and methods

Bacteria and growth conditions

P. stutzeri OX1 ATCC BAA-172 (Baggi et al. 1987), *P. putida* F1 (Finette et al. 1984), and *B. cepacia* G4 (Shields et al. 1989) were grown initially at 30°C and 250 rpm in 250-ml Erlenmeyer flasks containing 20 ml of Luria-Bertani (LB) (Sambrook et al. 1989) medium. After growth in LB medium, *P. stutzeri* OX1 was grown in 25 ml minimal M9 medium (Sambrook et al. 1989) supplemented with *o*-xylene or *o*-cresol vapors as the only carbon source. *P. putida* F1 and *B. cepacia* G4 were grown in M9 with toluene vapors as the only carbon source (30°C, 250 rpm) to an optical density at 600 nm (OD) of 0.5–1.0 in sealed 250-ml Erlenmeyer flasks. Dead cells for negative controls were prepared by autoclaving at 121°C for 30 min (control for non-chemotactic aggregation). To verify the involvement of the *tou* genes in chemotaxis, *P. stutzeri* M1 (Lecce et al. 1997) and *P. stutzeri* OX1 were grown only in LB medium or M9 medium containing 20 mM malate. *P. stutzeri* M1 does not grow on *o*-xylene due to an insertion in *touA* of ToMO (Lecce et al. 1997). *P. putida* PaW340 (Franklin and Williams 1980) was grown at 30°C in LB medium supplemented with streptomycin (50 μg/ml). *P. putida* PaW340 [with pKGB4213 (Arenghi et al. 1999) containing the whole ToMO locus *touABC-DEF*] was grown at 30°C in LB medium supplemented with kanamycin (30 μg/ml), and *P. putida* PaW340 [with pFP3028 (Arenghi et al. 1999), containing the *touR* (ToMO regulatory) gene, pFB1112 (Bertoni et al. 1996), containing both *touABCDEF* and *touR*, and pLAFR1 (Friedman et al. 1982), which is the control plasmid of both pFP3028 and pFB1112], were grown in LB medium supplemented with tetracycline (25 μg/ml) at 30°C to an OD of 0.5–1.0.

Chemicals

TCE, toluene, and *o*-cresol were obtained from Fisher Scientific (Pittsburgh, Pa.); 1,1-DCE, *cis*-DCE, *trans*-DCE, and *o*-xylene were obtained from Aldrich Chemical (Milwaukee, Wis.); VC was obtained from Supelco (Bellefonte, Pa.); and PCE was obtained from Sigma Chemical (St Louis, Mo.).

Chemotaxis assay

The agarose plug assay was used as described previously (Yu and Alam 1997). Plugs were prepared with 2% low-melting temperature NuSieve GTG agarose in chemotaxis buffer (Parales et al. 2000), and contained 10% (v/v) of volatile aliphatic organics (Parales et al. 2000) including toluene, TCE, PCE, *trans*-DCE, *cis*-DCE, 1,1-DCE, and VC. The temperature of the agarose was kept between 60°C and 65°C to avoid its solidification before addition of the volatile compounds. The preheated agarose mixture (10 μl) with aliphatic organics was injected between the space created by two cover glasses lying flat on the slide and separated by 1.5–2 mm on a microscope slide (kept at 60–65°C with a hot block). The agarose mixture was covered after 1 s to reduce the evaporation of volatile compounds with a third cover glass that resides on the top of the first two cover glasses and bridges the 1.5–2 mm gap. The slide was removed from the hot block, and, after 2–4 min, 100–120 μl of fresh cells harvested in the exponential growth phase in minimal medium or LB (OD between 0.5 and 1.0) was directly pipetted [without washing with chemotaxis buffer (Parales et al. 2000)] between the microscope slide and the third cover glass. For the negative controls, chemotaxis buffer was added to the agarose mixture instead of volatile compounds, and dead *P. putida* F1 and dead *P. stutzeri* OX1 were added instead of exponentially growing cells. The chemotaxis assay was performed with at least two independent cultures for each strain and for each culture, and there were at least two replicates (Tables 1, 2).

Microscopy and photography

After pipetting the cells, chemotactic rings toward organic pollutants were observed in 5–30 min. Slides were viewed with a microscope (Zeiss Axioskop20, Munchen-Hallbergmoos, Germany) equipped with a CCD camera (Hitachi KP-D50 Color Digital, Tokyo, Japan) and Axio-Vision software. Chemotactic responses were observed at 100× or 400× total magnification with an eyepiece magnification of 10× and objective of 10× or 40×. The size of the chemotactic ring was determined using Axio-Vision software.

Results

Agarose plug chemotaxis assay

To study chemotaxis, the agarose plug assay (Yu and Alam 1997) was used. The assay is an easy and useful technique to work with volatile compounds, since the evaporation

losses of the volatile compounds are reduced (Parales et al. 2000). The positive chemotactic cells moved toward the agarose plug containing the chlorinated aliphatic and formed a ring structure (Fig. 1). When only chemotaxis buffer was present in the agarose plug, the cells were completely insensitive, and no ring was found (Fig. 1); also, dead bacteria failed to respond.

In the plug assays, a concentration gradient of the dissolved volatile compound existed. After diffusion of the volatile compounds into the cells, positive chemotactic bacteria swam toward the plug, and accumulated near the plug without touching it. This indicated that cells responded to an optimal concentration of the volatile organic compound (Parales et al. 2000). Accumulation of cells around only half of the agarose, which was observed toward *trans*-DCE, indicated that the volatile compound *trans*-DCE might not have diffused homogeneously (Table 1). After 1–3 h, the chemotactic rings disappeared.

The strength of the bacterial chemotactic response was quantified by measuring the size of the ring (4–40 μm) in order to judge its significance for bioremediation (Table 1). (Chemotactic rings more than 20 μm thick indicate a strong response; rings less than 20 μm indicate a weak response.) Bacteria were originally observed in the microscope, and the size of the ring was determined using the Axio-Vision program. A strong response indicated that more cells accumulated near the plug containing the attractant and formed a thicker ring.

Chemotactic responses of *P. putida* F1 and *B. cepacia* G4

The chemotactic response of toluene-grown *P. putida* F1 to the chlorinated aliphatics and toluene are shown in Fig. 1 and Table 1. Previously it was observed that *P. putida* F1 grown with toluene and pyruvate showed positive chemotactic responses to PCE, TCE, and *cis*-DCE (Parales et al. 2000). Here, *P. putida* F1 was chemotactic toward the chlorinated ethenes PCE, TCE, and *cis*-DCE as well as toward *trans*-DCE, 1,1-DCE, VC, and toluene, and formed a ring structure around the agarose plug. Strong chemotaxis was observed toward toluene, TCE, *cis*-DCE, and VC, and weak chemotaxis was observed toward PCE, 1,1-DCE, and *trans*-DCE. Formation of only a half ring was observed with *trans*-DCE, which might be due to uneven diffusion of *trans*-DCE.

Previously it was shown that *B. cepacia* G4 grown with toluene and succinate was chemotactic toward toluene (Parales et al. 2000), and the same result was found here. In addition, *B. cepacia* G4 grown on toluene vapors without succinate was found by us to be chemotactic toward the chlorinated ethenes PCE, TCE, 1,1-DCE, *cis*-DCE, and VC (Fig. 1; Table 1). The chemotaxis was strongest toward PCE, TCE, and 1,1-DCE, and weakest toward toluene, *cis*-DCE, and VC. No chemotaxis was found for *trans*-DCE. The addition of succinate or pyruvate during growth was not required for positive chemotaxis attraction.

Table 1 Chemotaxis of *Pseudomonas stutzeri* OX1, *Burkholderia cepacia* G4, and *Pseudomonas putida* F1 toward chlorinated ethenes

Strain	Growth medium	Compound								
		Buffer	Toluene	Tetrachloroethylene	Trichloroethylene	*cis*-1,2-Dichloroethylene	*trans*-1,2-Dichloroethylene	1,1-Dichloroethylene	Vinyl chloride
P. stutzeri OX1	*o*-Xylene	–[a]	++	++	+	+	+	++	+
P. stutzeri OX1	*o*-Cresol	–	+	++	–	–	+	++	++
B. cepacia G4	Toluene	–	+	++	++	+	–	++	+
P. putida F1	Toluene	–	++	+	++	++	+	+	++
P. putida F1[b]	Toluene + pyruvate	–	++	+	++				
Dead *P. putida* F1		–	–	–	–				
Dead *P. stutzeri* OX1		–	–	–	–				

[a] ++ Strong response (diameter of chemotactic ring 20–40 μm), + weak response (diameter of chemotactic ring 4–20 μm), – no response
[b] Data from Parales et al. (2000)

Table 2 Chemotaxis of *Pseudomonas putida* PaW340 expressing *touABCDEF* and *touR* toward *o*-xylene. Antibiotics used for growth were streptomycin at 50 μg/ml (Sm 50), tetracycline at 25 μg/ml (Tc 25), and kanamycin at 30 μg/ml (Kan 30)

Strains	Genes	Growth medium	Chemotactic ring
P. putida PaW340	Negative control	LB[a] (Sm 50)	–
P. putida PaW340/pLAFR1	Negative control	LB (Tc 25)	–
P. putida PaW340/pFP3028	*touR*	LB (Tc 25)	–
P. putida PaW340/pKGB4213[b]	*touABCDEF*	LB (Kan 30)	+
P. putida PaW340/pFB1112[b]	*touABCDEF* + *touR*	LB (Tc 25)	++

[a] *LB* Luria-Bertani
[b]Images shown in Fig. 1.

Chemotactic responses of *P. stutzeri* OX1

P. stutzeri OX1 grown on *o*-xylene or *o*-cresol vapors was chemotactic and formed a ring structure around agarose plugs with TCE, PCE, toluene, all DCEs, and VC. Chemotaxis toward TCE and *cis*-DCE of *P. stutzeri* OX1 cells grown on *o*-xylene vapors was better than that of cells grown on *o*-cresol vapors (no ring formed for *o*-cresol grown cells). On the other hand, chemotaxis toward VC was stronger with *o*-cresol-grown cells. Similar

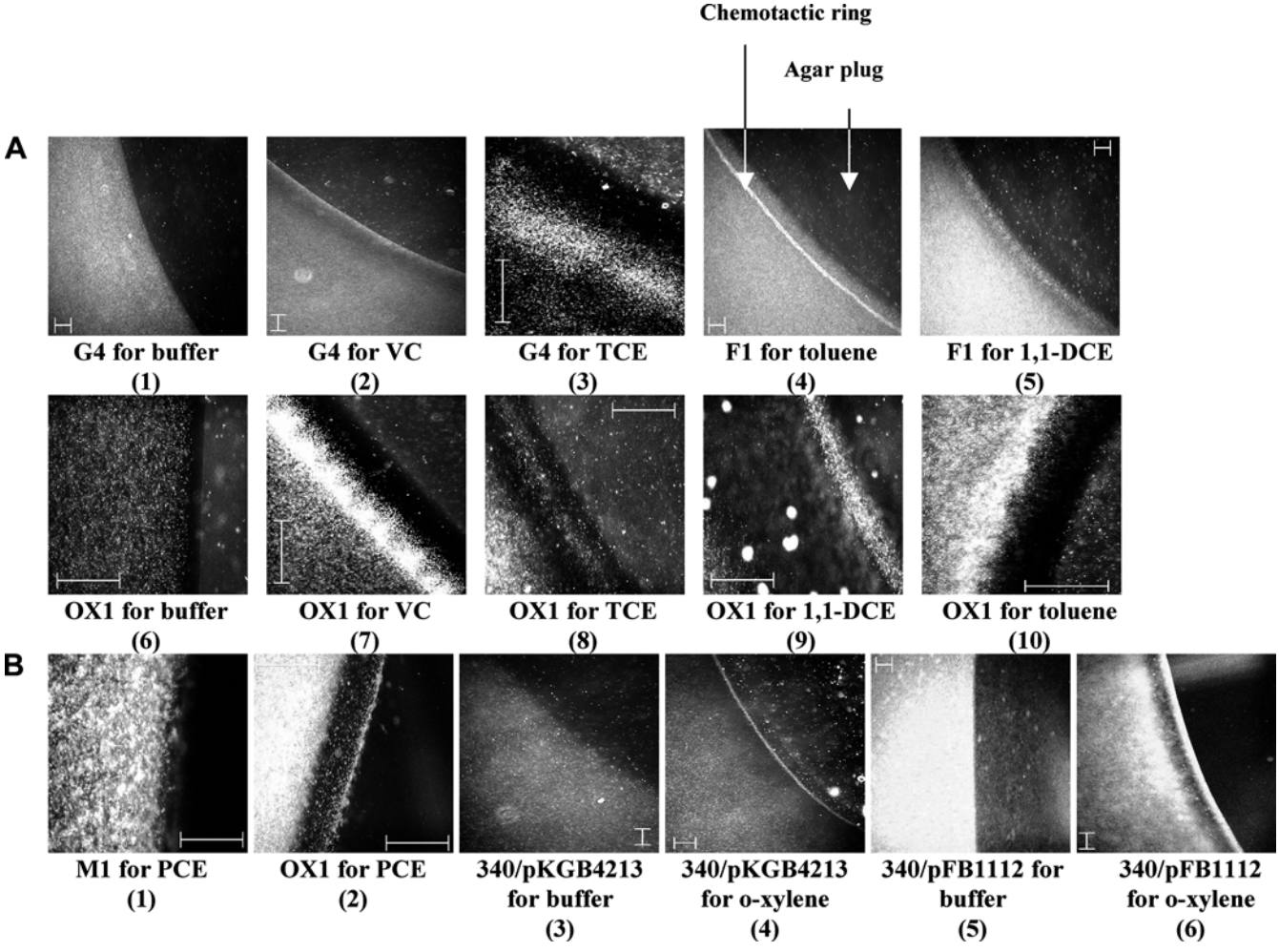

Fig. 1 Representative chemotactic responses toward the volatile organic compounds (**a**), and the role of *tou* genes and *touR* on positive chemotaxis response toward tetrachloroethylene (PCE) or *o*-xylene (**b**). Chemotaxis of toluene-grown *Burkholderia cepacia* G4 toward chemotaxis buffer (*a1*), vinyl chloride [(VC), *a2*], and trichloroethylene [(TCE), *a3*]. Chemotaxis of toluene-grown *Pseudomonas putida* F1 toward toluene (*a4*) and 1,1-dichloroethylene [(1,1-DCE), *a5*]. Chemotaxis of *o*-cresol-grown *Pseudomonas stutzeri* OX1 toward chemotaxis buffer (*a6*) and VC (*a7*), and *o*-xylene-grown *P. stutzeri* OX1 toward TCE (*a8*), 1,1-DCE (*a9*), and toluene (*a10*). Chemotaxis of malate-grown *P. stutzeri* M1 (*b1*) and malate-grown *P. stutzeri* OX1 (*b2*) toward PCE. Chemotaxis of *P. putida* PaW340/pKGB4213 (*touABCDEF*; *b3*, *b4*) and *P. putida* PaW340/pFB1112 (*touABCDEF* + *touR*; *b5*, *b6*) toward chemotaxis buffer and *o*-xylene. *Scale bar* 100 μm

chemotactic responses were observed with *o*-xylene-grown and *o*-cresol-grown *P. stutzeri* OX1 cells toward PCE, *trans*-DCE, and 1,1-DCE.

ToMO plays a role in chemotaxis

To investigate the role of *tou* genes in chemotaxis, the differences in the chemotactic responses toward PCE were checked for mutant *P. stutzeri* M1, which has an inactive *touA* (alpha fragment of ToMO) but active *touR* (Bolognese et al. 1999), and wild-type *P. stutzeri* OX1 (Fig. 1). Malate-grown M1 cells did not form a chemotactic ring around the PCE-containing agarose plug but malate-grown OX1 cells did. The same results were obtained when the strains were grown on LB; hence, *touA* is important for chemotaxis attraction, and the carbon source used during cell growth does not seem to matter for chemotaxis toward PCE.

To investigate the importance of the ToMO regulatory gene *touR* for chemotaxis and confirm the necessity of the *tou* locus, the chemotactic response of *P. putida* PaW340 to *o*-xylene was investigated in the presence of both *touABCDEF* and *touR*. *P. putida* PaW340/pKGB4213 (Arenghi et al. 1999), which contains *touABCDEF*, was chemotactic toward *o*-xylene (Fig. 1; Table 2). *P. putida* PaW340 with pFP3028 (Arenghi et al. 1999), which contains the *touR* but not *touABCDEF*, was not chemotactic toward *o*-xylene. *P. putida* PaW340 with pFB1112 (Bertoni et al. 1996), which has *touABCDEF* and *touR*, showed a stronger positive chemotactic response and formed a thicker ring around the *o*-xylene-containing agarose plug (Fig. 1; Table 2). Negative chemotaxis responses were observed with *P. putida* PaW340 (Franklin and Williams 1980) cells and as well as *P. putida* PaW340 cells with pLAFR1 (control plasmid of pFP3028 and pFB1112, Friedman et al. 1982). As expected, no chemotactic ring was observed with *P. putida* PaW340 with pFB1112 for the negative control of a buffer-containing agarose plug. These results corroborate the importance of the *tou* operon for chemotaxis toward *o*-xylene, and indicate the response might be enhanced in the presence of *touR*.

Discussion

Our results of chemotaxis by *P. putida* F1 toward PCE, TCE, *cis*-DCE, and toluene and *B. cepacia* G4 toward toluene compare well to those reported previously (Parales et al. 2000). Here, energy sources such as succinate or pyruvate during cell growth in minimal media were not required for positive chemotaxis attraction. In addition to these compounds, it was also found here that *P. putida* F1 and *B. cepacia* G4 are chemotactic toward 1,1-DCE and VC, and that *P. putida* F1 is chemotactic toward *trans*-DCE; this is the first report of these compounds acting as attractants for these two strains. Since in *P. stutzeri* OX1, the carbon source used during cell growth did not affect chemotaxis toward PCE, 1,1-DCE, and *trans*-DCE but affected chemotaxis with *cis*-DCE and TCE, the growth medium might be important for some substrates.

It was reported previously that the genes required for positive chemotactic response to toluene are the regulatory proteins *TodS* and *TodT*, which regulate the *todRXFC1C2-BADEGIH* operon for toluene degradation in *P. putida* F1 (Parales et al. 2000). Similarly, *pcaK*, which has a role in the degradation of aromatic acids, has been shown to be required for chemotaxis toward 4-hydroxybenzoate by *P. putida* PRS2000 (Ditty and Harwood 2001; Parales and Harwood 2002). *nahY*, which is part of an operon that contains genes for salicylate degradation, has been shown to be required for chemotaxis toward naphthalene by *P. putida* G7 (Grimm and Harwood 1999; Parales and Harwood 2002). *tfdK*, which is located on plasmid pJP4 within the cluster of *tfd* genes that are required for 2,4-dichlorophenoxyacetate (2,4-D) degradation, has been shown to be required for chemotaxis toward 2,4-D by *Ralstonia eutropha* JMP134 (pJP4) (Hawkins and Harwood 2002; Parales and Harwood 2002). Hence, the chemotactic response appears to be coordinated with catabolic-gene expression as shown from previous studies.

The importance of the whole ToMO locus (*touABCDEF*) and the regulatory gene (*touR*) for chemotaxis was investigated in this work. These results showed the importance of the *tou* operon for chemotaxis toward *o*-xylene or PCE and indicated the response might be enhanced with *touR*. Since positive chemotaxis was also observed with *P. putida* F1 expressing toluene 2,3-dioxygenase and with *B. cepacia* G4 expressing TOM, oxygenases might be playing an important role in chemotaxis, but still more data are required to understand the role of catabolic structural and regulatory genes in the chemotactic response.

Previously, it was shown that ToMO from *P. stutzeri* OX1 degrades the chlorinated ethenes PCE, TCE, 1,1-DCE, *cis*-DCE, *trans*-DCE, and VC, individually and as mixtures (Chauhan et al. 1998; Ryoo et al. 2000; Shim and Wood 2000). It was also shown that PCE and TCE induce their own degradation in this strain (Ryoo et al. 2000, 2001). Hence, the positive chemotaxis attraction toward PCE and other less chlorinated compounds that was discovered here makes *P. stutzeri* OX1 expressing ToMO very attractive for bioremediation of chlorinated ethenes, since these pollutants induce chemotaxis, induce expression of ToMO, and are degraded by ToMO with a stoichiometric release of chloride ions.

Acknowledgements This study was supported by the US Army Research Office (award no. DAAD19-00-1-0568).

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