Bacterial chemotaxis towards aromatic hydrocarbons in *Pseudomonas*

Jesús Lacal, Francisco Muñoz-Martínez, José-Antonio Reyes-Darias, Estrella Duque, Miguel Matilla, Ana Segura, José-J. Ortega Calvo, Celia Jiménez-Sánchez, and Juan L. Ramos

1 Consejo Superior de Investigaciones Científicas, CSIC-EEZ, Department of Environmental Protection, 18008 Granada, Spain.
2 Bio-iberis R&D, Polígono Juncaril, C/Capileira 7, 18220 Granada, Spain.
3 Consejo Superior de Investigaciones Científicas, CSIC-IFINASE, Seville, Spain.

Summary

Bacterial chemotaxis is an adaptive behavior, which requires sophisticated information-processing capabilities that cause motile bacteria to either move towards or flee from chemicals. *Pseudomonas putida* DOT-T1E exhibits the capability to move towards different aromatic hydrocarbons present at a wide range of concentrations. The chemotactic response is mediated by the McpT chemoreceptor encoded by the pGRT1 megaplasmid. Two alleles of *mcpT* are borne on this plasmid and inactivation of either one led to loss of this chemotactic phenotype. Cloning of *mcpT* into a plasmid complemented not only the *mcpT* mutants but also its transfer to other *Pseudomonas* conferred chemotactic response to high concentrations of toluene and other chemicals. Therefore, the phenomenon of chemotaxis towards toxic compounds at high concentrations is gene-dose dependent. *In vitro* experiments show that McpT is methylated by CheR and McpT net methylation was diminished in the presence of hydrocarbons, what influences chemotactic movement towards these chemicals.

Introduction

The rapid growth of the world's population has resulted in an increase in the presence of pollutants in the biosphere and among these, toxic and mutagenic xenobiotics represent a real threat to life (Kanaly and Harayama, 2010). Aromatic hydrocarbons are abundant in the biosphere due to the combustion of plant material, oil spills and the burning of petroleum derivatives. Mono-, di- and low molecular weight polycyclic aromatic hydrocarbons are easily degraded by microbes, whereas high molecular weight polycyclic aromatic hydrocarbons (PAHs) are often recalcitrant. Bioremediation uses the astonishing catabolic versatility of soil and aquatic bacteria to fight against pollutants. In addition, some strains are highly resistant to organic pollutants and have an enormous catabolic potential for biodegradation.

Although a large number of biodegradation protocols has been developed (as witnessed by some 2300 patents), the *in situ* outcomes of biodegradation are not always satisfactory. One of the reasons for the low biodegradation efficiency is the heterogeneous distribution of the pollutants or the reduced bioavailability of the target compounds (Pandey and Jain, 2002). Available information demonstrates that chemotaxis increases biodegradation efficiency in cases where the pollutant is heterogeneously distributed either in water or in soils (Max and Aitken, 2000; Bhushan et al., 2004). In bacteria sensory recognition of a chemical gradient triggers a molecular signal transduction cascade, resulting in a modulation of flagellar activity, and in the directional movement of the bacteria either towards or away from stimulatory molecules (Alexandre et al., 2004; Wadhams and Armitage, 2004; Hazelbauer et al., 2008; Osterberg et al., 2010; Lacal et al., 2010). Although chemotaxis pathways in prokaryotes are known to vary, the main components of the chemotaxis signal transduction pathway are conserved and are made up of the ternary complex between methyl-accepting chemotaxis (MCPs) proteins, the sensor CheA and the adaptor protein (CheW or CheV). Signal recognition at the chemoreceptor level modulates CheA autophosphorylation activity and in turn transphosphorylation of the CheY response regulator, which acts on the flagellar motor.

While commensal microorganisms have only a few chemoreceptors, i.e. in *E. coli* there are four chemoreceptors (Hazelbauer et al., 2008), soil and aquatic bacteria possess a high number of MCPs; for instance, *Pseudomonas* sp. and *Clostridium* sp. possess more than
20 chemoreceptors (Timmis, 2002; Hazelbauer et al., 2008). This enormous diversity of chemoreceptors is reflected in that many soil and aquatic bacteria are chemotactic to a wide range of different chemicals. Bacteria of the genus *Pseudomonas* have an enormous catabolic potential and a number of strains respond chemotactically to monocyclic and polycyclic aromatic hydrocarbons with nitro, amino or chloro substitutions (Grimm and Harwood, 1997; 1999; Parales et al., 2000; Gordillo et al., 2007; Iwaki et al., 2007; Liu and Parales, 2009), which makes this genus an ideal model system to study chemotaxis towards pollutants with the view to biotechnological exploitation.

We describe here a new form of chemotaxis through which bacteria are attracted directly to particles containing low or high concentrations of toxic compounds. The chemoreceptor involved in this phenotype in *P. putida* was identified as McpT and it is encoded on the self-transmissible pGRT1 (Rodriguez-Herva et al., 2007; Molina et al., 2011). There are two almost identical copies of *mcpT* and inactivation of either of them leads to loss of the chemotactic phenotype indicating a clear gene-dose effect. The findings reported here and the molecular mechanisms underlying the chemotactic response to high concentrations of pollutants can have important implications in the design of new strategies to combat pollutants in heterogeneously polluted sites.

### Results

Pseudomonas *putida* strains exhibit different chemotactic phenotypes towards toluene

A set of *Pseudomonas putida* strains (Table 1) was analysed for their chemotactic response to toluene using agarose plug chemotaxis assays. In these qualitative assays an agarose drop containing the attractant is brought into contact with a bacterial suspension and toluene diffuses into the bacterial culture creating a concentration gradient. The accumulation of bacteria around the agarose plug is a measure of chemotaxis. Two patterns of accumulation were observed: one in which cells accumulated at a distance of 1–2 mm from the toluene plug, which was termed moderate taxis (*P. putida* F1 and KT2440, Fig. 1). The second pattern was characterized by an accumulation of cells on the toluene-containing plug. This phenotype was exclusively observed for *P. putida* DOT-T1E. This phenotype was termed strong chemotaxis or hyperchemotaxis. This behaviour was observed for toluene concentrations between 0.01% (v/v) and 50% (v/v).

Chemotaxis was also studied using capillary assays. In this assay a capillary was filled with agarose containing 1% (v/v) toluene, and the open end of the capillary was submerged into a bacterial solution. We found that *P. putida* DOT-T1E cells accumulated at the entrance to the capillary, whereas *P. putida* KT2440 formed a cloud at the entrance of the capillary.

### Table 1. Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Features</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. putida</em> DOT-T1E</td>
<td>Tof&lt;sup&gt;i&lt;/sup&gt;, wild-type, Rif&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Ramos et al. (1995)</td>
</tr>
<tr>
<td><em>P. putida</em> DOT-T1E-100</td>
<td>Tof&lt;sup&gt;i&lt;/sup&gt; derivative of DOT-T1E, Rif&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Rodriguez-Herva et al. (2007)</td>
</tr>
<tr>
<td><em>P. putida</em> DOT-T1E Δc</td>
<td>Tof&lt;sup&gt;i&lt;/sup&gt; derivative</td>
<td>This laboratory</td>
</tr>
<tr>
<td><em>P. putida</em> DOT-T1E-PS28</td>
<td>Tof&lt;sup&gt;i&lt;/sup&gt;, Rif&lt;sup&gt;i&lt;/sup&gt;, pGRT1, ΔtgfV::aphA-3, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Rojas et al. (2003)</td>
</tr>
<tr>
<td><em>P. putida</em> DOT-T1E ΔmcpT1</td>
<td>Tof&lt;sup&gt;i&lt;/sup&gt;, Rif&lt;sup&gt;i&lt;/sup&gt;, Km&lt;sup&gt;r&lt;/sup&gt;, pGRT1, ΔmcpT1::pChes1, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
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<td>Tof&lt;sup&gt;i&lt;/sup&gt;, Rif&lt;sup&gt;i&lt;/sup&gt;, Km&lt;sup&gt;r&lt;/sup&gt;, pGRT1, ΔmcpT2::pChes1, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td><em>P. putida</em> KT2440 cto- (ΔtgfV::aphA-3)</td>
<td>Tof&lt;sup&gt;i&lt;/sup&gt;, Rif&lt;sup&gt;i&lt;/sup&gt;, Km&lt;sup&gt;r&lt;/sup&gt;, pGRT1, ΔmcpT1::pChes1, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Rodriguez-Herva et al. (2007)</td>
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<td><em>P. putida</em> F1</td>
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<td>This laboratory</td>
</tr>
<tr>
<td><em>P. putida</em> KT2440</td>
<td>Tof&lt;sup&gt;i&lt;/sup&gt;, Rif&lt;sup&gt;i&lt;/sup&gt;, Km&lt;sup&gt;r&lt;/sup&gt;, pGRT1, ΔmcpT2::pChes1, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Timmis (2002)</td>
</tr>
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<td><em>P. putida</em> MT53 (pWW53)</td>
<td>Tof&lt;sup&gt;i&lt;/sup&gt;, Rif&lt;sup&gt;i&lt;/sup&gt;, Km&lt;sup&gt;r&lt;/sup&gt;, pGRT1, ΔmcpT2::pChes1, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Miyakoshi et al. (2007)</td>
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<td><em>P. putida</em> pCAR1</td>
<td>Large self-transmissible plasmid</td>
<td>Rodriguez-Herva et al. (2007)</td>
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<td>pGRT1</td>
<td>pGRT1, ΔtgfV::aphA-3, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Rojas et al. (2003)</td>
</tr>
<tr>
<td>pGRT1::ΔmcpT</td>
<td>pGRT1, ΔmcpT2::pChes1, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
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<td>pGRT1::ΔmcpT2</td>
<td>mcpT gene cloned in pBBR1MCS-5, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Kovach et al. (1965)</td>
</tr>
<tr>
<td>pBBRI-MCS-5</td>
<td>mcpT gene cloned in pBBR1MCS-5, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pJL-McpT</td>
<td>mcpT, S183P cloned in pBBR1MCS-5, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Llamas et al. (2003)</td>
</tr>
<tr>
<td>pJL-McpT S183P</td>
<td>ΔmcpT, CoIE1 replicon, Km&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pCHESI</td>
<td>koai&lt;sup&gt;i&lt;/sup&gt;, IncI1 replicon, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Invitrogen</td>
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<td>pJL-McpT-Δp</td>
<td>Tof&lt;sup&gt;i&lt;/sup&gt;, lacZ::pJL-McpT promoter, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pJL-McpT::Δp</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;, protein expression vector</td>
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<tr>
<td>pJL-McpT::Δp::ΔmcpT</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;, protein expression vector containing the mcpT gene, Km&lt;sup&gt;r&lt;/sup&gt;</td>
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</table>

Gm<sup>r</sup>, Km<sup>r</sup>, Rif<sup>i</sup> and Sm<sup>i</sup> indicate resistance to gentamicin, kanamycin, rifampicin and streptomycin respectively. In addition, Tof<sup>i</sup> indicates that the strain grows on toluene, whereas Tof<sup>i</sup> indicates that the strain cannot use toluene as the sole carbon and energy source. Tof<sup>i</sup> and Tof<sup>i</sup> stand for tolerance and sensitivity, respectively, to > 0.3% (v/v) toluene in liquid medium.
Fig. 1. Chemotactic behaviour of different strains of *P. putida* towards toluene. A. Agarose plug assays of strains F1, DOT-TIE and KT2440. Strain DOT-TIE-100 is a derivative of DOT-TIE lacking the large, self-transmissible plasmid pGRT1 (Rodríguez-Herva et al., 2007). Strain KT2440 (pGRT1::ΔtggV) has the *tggV* gene disrupted by a kanamycin cassette. Strains presenting the hyperchemotaxis phenotype are underlined. Controls show the response of strain DOT-TIE to buffer and succinate. B. Capillary assays of strains DOT-TIE, DOT-TIE-100 and KT2440 towards toluene which was immobilized in the capillary at a concentration of 10% (v/v).

Table 2. Different phenotypes observed for the chemotaxis of *P. putida* DOT-T1E towards different hydrocarbons.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Benzene derivatives</th>
<th>Toluene and singly substituted derivatives</th>
<th>Multiply substituted benzene derivatives</th>
<th>Bisaromatics</th>
</tr>
</thead>
<tbody>
<tr>
<td>None or very weak chemotaxis</td>
<td>benzene, styrene, p-xylene, p-nitrotoluene</td>
<td>toluene, o-xylene, m-xylene, o-iodotoluene</td>
<td>1,2,4-trimethylbenzene, 1,3,5-trimethylbenzene</td>
<td>naphthalene, 1,2,3,4-tetrahydroxiphthalene</td>
</tr>
<tr>
<td>Moderate chemotaxis</td>
<td>benzene derivatives: chlorobenzene, nitrobenzene, ethylbenzene</td>
<td>m-iodotoluene, p-iodotoluene, o-toluidine</td>
<td>m-fluorotoluene, p-fluorotoluene, o-fluorotoluene</td>
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<td></td>
<td>fluoroarene, benzonitrile</td>
<td>m-bromotoluene, m-chlorotoluene</td>
<td>m-nitrotoluene, o-nitrotoluene</td>
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<td></td>
<td>toluene derivatives: p-ethyltoluene, p-chlorotoluene</td>
<td>m-chlorotoluene, m-bromotoluene</td>
<td>2,3-diethylphenol</td>
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<tr>
<td></td>
<td>p-bromotoluene</td>
<td>m-fluorotoluene, m-bromotoluene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperchemotaxis</td>
<td>propylbenzene, butylbenzene</td>
<td>p-fluorotoluene, o-fluorotoluene</td>
<td>Multiply substituted benzene derivatives</td>
<td></td>
</tr>
</tbody>
</table>
taxis was observed, whereas the remaining 20% showed moderate taxis. *P. putida* DOT-T1E also showed the strong chemotactic response when the agarose plug was replaced with undiluted crude oil pellets collected from the Spanish sea shores after the sinking of the Prestige oil tanker (data not shown).

The pGRT1 plasmid confers strong chemotaxis to *Pseudomonas putida* towards toluene

We showed before that the solvent-tolerant character of the DOT-T1E strain was due to the presence of a series of efflux pumps that expel solvents to the outer medium (Rojas *et al.*, 2003). From a quantitative point of view the most relevant pump is TtgGHI, encoded on the pGRT1 self-transmissible plasmid present in this strain (Rojas *et al.*, 2003; Rodríguez-Herva *et al.*, 2007). To test whether the extrusion of the chemotacticant was the reason for the strong chemotactic phenotype, a mutant strain, DOT-T1E-PS28 (Rojas *et al.*, 2003), with a non-functional TtgGHI pump, was used. Agarose plug assays revealed that the strong chemotactic behaviour of mutant DOT-T1E-PS28 was similar to that of its parental strain, suggesting that the TtgGHI efflux pump is not the primary cause of the strong chemotaxis phenotype.

To test whether the molecular determinant(s) for strong chemotaxis was/were associated with the pGRT1 plasmid, agarose plug and qualitative capillary chemotaxis assays of a plasmid-less variant of DOT-T1E, known as DOT-T1E-100, were carried out. These assays revealed that the plasmid-less strain exhibited the moderate chemotaxis phenotype and that it behaved similarly to the KT2440 and F1 strains (Fig. 1).

Rodríguez-Herva and colleagues (2007) showed that pGRT1 can be transferred to other *Pseudomonas* strains, for example KT2440. To verify whether pGRT1 is responsible for the strong chemotaxis phenotype, *P. putida* KT2440 (pGRT1::ΔtggV) was analysed with regard to strong chemotaxis. Interestingly, this strain exhibited the same strong chemotaxis phenotype as DOT-T1E (Fig. 1), providing evidence for the role of pGRT1 borne genes in strong chemotaxis.

The sequence analysis of the low-copy-number (1-3 copies per cell) 133 kbp pGRT1 plasmid (Fig. S1) revealed the presence of two ORFs, which encode for methyl-accepting chemotaxis proteins (MCP), that we termed McpT1 and McpT2, and which vary in only one amino acid at position 201 (glutamic acid or alanine). To provide unequivocal evidence of the potential role of these genes in the chemotactic behaviour, mutants were generated by site-directed mutagenesis (see Experimental procedures). The inactivation of either of the alleles resulted in the loss of the strong chemotactic behaviour resulting in strains that only exhibited the moderate chemotaxis phenotype (Fig. 3). To quantify the response of the *mcpT* mutants to toluene and succinate – used as control – quantitative capillary assays were performed. To this end a modified version of the assays described by Adler (1973) was employed (see Experimental procedures). Buffer containing different concentrations of the chemoattractant is placed into the capillary, which is then brought into contact with a bacterial suspension. After and exposure of 10 min, the contents of the capillary are collected and the number of colony forming units is quantified.

In an initial series of experiments the response of strains DOT-T1E, its plasmid-free derivative DOT-T1E-100 and a DOT-T1E mutant deficient in the genes *mcpT1* and *mcpT2* were analysed. As shown in Fig. 4A, in the absence of toluene (buffer present in the capillary) these three strains were characterized by a similar motility. The DOT-T1E wild-type strain showed an initial response at 1% of toluene and a significant increase in the magnitude of response at 5% and 10% toluene was observed. For the mutant strains deficient in *mcpT1* and *mcpT2* an initial slight response was also observed at 1% toluene but the increase in taxis at 5% and 10% toluene was significantly modest as compared with the wild-type strain. Surprisingly, the DOT-T1E-100 strain, lacking in the entire plasmid, did not respond at 1% toluene and less cells accumulated in the capillary containing 5% and 10% toluene than in the buffer control. The difference in responses observed in the *mcpT* double mutant and the plasmid-less strain is likely due to the presence or, respectively, absence of the plasmid encoded TtgGHI efflux pump, which has been shown to play a key role in
conferring resistance to elevated concentrations of toluene. Although the agarose plug assays reported above demonstrated that TtgGHI is not the primary determinant of the strong chemotaxis phenotype, these quantitative measurements suggest that the resistance conferred by this efflux pump increases the magnitude of the response.

To provide further grounds on this hypothesis we carried out further assays and reasoned that the cloning of an mcpT allele in a medium-copy-number plasmid should be sufficient enough to provide the strong chemotactic behaviour to a recipient host. To test this hypothesis the mcpT1 allele was cloned into the broad-host range medium-copy-number (15-30 copies per cell) vector pBBRMC-S-5 and the resulting plasmid, pJL::mcpT was introduced into different P. putida backgrounds, namely, the plasmid-free derivative of DOT-T1E, DOT-T1E-100, the mcpT1 and mcpT2 mutants and P. putida KT2440. In all cases the acquisition of the pJL::mcpT plasmid conferred the strong chemotactic response to the host (Fig. 3). A second series of quantitative chemotaxis assays was aimed at quantifying the response of strain KT2440 and its derivative harbouring plasmid pGRT1::ttgV to 10% toluene. For these experiments a derivative of plasmid pGRT1 was used, which contained a kanamycin resistance cassette inserted into the ttgV gene. Strain KT2440 showed a motility comparable to that of the plasmid-less version of DOT-T1E (Fig. 4B). It should be noted that both strains lack the TtgGHI efflux pump. However, taxis of strain KT2440 pGRT1::ttgV was largely increased as compared with its parental strain, which illustrates that the transfer of plasmid pGRT1 to other Pseudomonas strains causes an increase in the chemotactic attraction towards toluene.

A BLAST search using the McpT as query revealed that McpT homologues (>99% sequence identity) are exclusively present as monocytoplasmid, i.e. pCAR1 of Pseudomonas resinovorans (Miyakoshi et al., 2007), the TOL plasmid pWW53 of P. putida (Yano et al., 2007) and the plasmid pMAQU02 of Marinobacter aquaeolei VT8. Interestingly, these three homologues are identical amongst them and share 98.8% sequence identity (1 amino acid change) with McpT of pGRT1.

Plasmid pCAR1 was transferred into P. putida KT2440 (Miyakoshi et al., 2007), and the resulting strain, P. putida KT2440 (pCAR1), assayed with regards to its chemotactic response to toluene. We found that KT2440 (pCAR1) exhibited only the moderate chemotaxis phenotype. The 108 kbp plasmid pWW53 is a well-studied TOL plasmid (Yano et al., 2007) present in P. putida MT53. Pseudomonas putida MT53 containing pWW53 exhibited only the moderate chemotactic response towards toluene (not shown). This set of results support that the McpT protein...
plays a key role in the chemotactic response to toluene and that its effect is gene dose-dependent.

Expression of the mcpT1 promoter is constitutive

The mcpT genes in pGRT1 are monocistronic units, and the expression of the genes was studied using a fusion of the P_mcpT promoter region to lacZ in pMP220. β-Galactosidase assays were carried out with DOT-T1E and DOT-T100 cells grown on M9 minimal medium with glucose, in the absence and in the presence of toluene. In both genetic backgrounds, the level of activity in the absence and the presence of aromatic hydrocarbons was found to be 100 ± 10 Miller units regardless of the growth phase, indicating that the mcpT genes are constitutively expressed.

Methylation of the chemoreceptor McpT by Pseudomonas putida CheR

The above data support that the mcpT gene mediates the strong chemotactic response. We have assessed whether McpT has the typical characteristics of a chemoreceptor. One of such characteristics is the capability to be methylated by a CheR methyltransferase and the capability of receptor ligands to modulate this activity. To this end, the mcpT was cloned in pET200/D-TOP0, expressed in E. coli (Fig. S1) and the corresponding membranes were purified. Control membranes devoid of McpT were also prepared using cells that only bear pET200/D-TOP0. Subsequently, methylation assays were performed using purified CheR (PP3760) from _P. putida_ DOT-T1E in the absence and in the presence of toluene.

With McpT-free membranes, regardless of the presence of toluene and regardless of addition of CheR, low levels of methylation were found (Fig. 5). With the McpT enriched membranes, the methylation signal was high when CheR was added. Interestingly, in the same assay but in the presence of toluene, the level of methylation was 20–30% lower than in the absence of toluene. To confirm that McpT is indeed methylated by CheR, aliquots of radiolabelled methylated McpT-containing membranes were subjected to SDS-PAGE, the gels were stained with Coomassie, fixed and autoradiographed. The results unequivocally confirmed that McpT was the protein methylated by CheR (Fig. 5A).

A helix-break point mutation in the McpT ligand binding domain inactivates McpT

To further provide insights into the role of McpT in the chemotactic response a site directed mutant was constructed based on McpT sequence analysis. The analysis of the 552 amino acid sequence of McpT using DAS (Cserzó _et al._, 1997) revealed the presence of two transmembrane regions flanking the putative periplasmic ligand binding domain (LBD) that spans from residue G27 to M202 (Fig. S2). The prediction of the secondary structure of this domain using a consensus method indicated the presence of four α-helices (Deleage _et al._, 1997), which is consistent with a 4-helix bundle arrangement typical of TarH-type domains (Yeh _et al._, 1996). In TarH
the final α-helix of the LBD extends into the membrane forming the second transmembrane region. Ligand binding induces a piston-type shift of this transmembrane helix, which is thought to be the regulatory stimulus altering CheA autophosphorylation (Otte 

On the basis of the in silico data, we reasoned that a mutant of McpT in which Ser183 (an amino acid located in the middle of the final helix of the LBD) is replaced by the helix-breaking amino acid; proline could interfere with receptor functioning. The mutant variant was generated by site-directed mutagenesis, cloned in pBBMRCSS5 to yield pJL::mcpTS183P and transferred to KT2440, DOTT1E-100 and DOT-T1E (pGRT1::ΔmcpT) and chemotaxis assays were done. In contrast with the strain bearing the native McpT protein, the set of constructs with the McpTS183P mutant showed only the moderate chemotaxis phenotype. Therefore, a single point mutation, which, most likely, interrupts transmembrane signalling resulted in the inactivation of McpT and has a major phenotypic effect in the chemotactic response to toluene.

Discussion

It is generally assumed that throughout evolution microorganisms developed effective mechanisms that assist them to regulate cellular functions in response to environmental changes (Taguchi et al., 1997). Of these, chemotaxis is one of the best studied spatial-temporal behaviours required for the colonization of niches. One of the remarkable features of chemotaxis is that bacteria sense a wide range of chemicals at different concentrations and respond to them. In this regard, P. putida bearing the NAH7 plasmid responds to naphthalene using the NahY protein as a chemoreceptor (Grimm and Harwood, 1989) and P. fluorescens strain KU-7 responds to 2-nitrobenzoate through a plasmid-encoded NbaY chemoreceptor (Iwaki et al., 2007). A limited number of studies have shown that chemotactic movement enhances the mineralization rate of heterogeneously distributed toxic organic compounds (Marx and Atiken, 2000; Bhushan et al., 2004). In this study we have shown that a number of P. putida strains respond chemotactically to different aromatic hydrocarbons, and that two types of behaviour were evident in agarose plug assays; one was the formation of rings at a defined distance from the chemical, a phenomenon we term moderate chemotaxis; whereas the other is an unusual new phenotype, termed strong chemotaxis in which P. putida DOT-T1E cells are attracted to the agarose plug in which the pollutant is embedded. The strong chemotactic response was also observed with crude oil, reinforcing the potential use of bacteria displaying the strong chemotactic behaviour for the practical treatment of polluted sites.

Pseudomonas putida DOT-T1E is considered an extremophile in the sense that it grows in the presence of concentrations of solvents that impede the growth of most microbes. The strong chemotactic response adds a new remarkable property to DOT-T1E, i.e. the ability to swim towards both low and very high concentrations of pollutants. Interestingly, the key genes responsible for these two phenotypes (solvent tolerance and hyperchemotaxis) are encoded on the 133-kb self transmissible pGRT1 plasmid (Rodríguez-Hervá et al., 2007). Another relevant feature is that both solvent tolerance and chemotactic responses are gene-dose dependent. In this study we showed that two copies of the chemoreceptor mcpT gene (the two alleles differ in a single nucleotide) are required for the strong chemotactic response. These copies appear in a 4.5 kb duplicated fragment of pGRT1 (Fig. S1), and inactivation of either of these genes results in the loss of the strong chemotactic phenotype. Furthermore, cloning of either of the mcpT alleles in a medium-copy number vector is sufficient to confer the strong chemotactic phenotype to different strains of P. putida. It is also of interest to note that a plasmid-free derivative of DOT-T1E, strain DOT-T1E-100, still retained the moderate chemotactic response to toluene, which indicated that toluene and related aromatics can be also be detected by one or more chromosomally encoded chemoreceptors. A type of hyperchemotaxis response was previously described in Halobacterium salinarum and B. subtilis regarding their response to the oxygen levels in the medium (Aono et al., 2002; Kristich and Orda, 2004). Two types of chemoreceptors, HemA and HtrVIII in H. salinarum, and HemA-0s and OI3545 in B. subtilis were found to be required for this hyperchemotactic response (Brooun et al., 1998; Zhulin, 2001; Delalez and Armitage, 2009). Since McpT1 and McpT2 in P. putida differ in only one amino acid, we suggest that bacterial hyperchemotaxis is the result of the participation of more than one chemoreceptor, or mediated through a gene-dose dependent effect.

In bacterial chemotaxis, the chemoreceptor proteins or MCPs are reversibly methylated as a way of receptor adaptation to varying concentrations of chemoattractants. In Escherichia coli and Salmonella typhimurium, the enzyme responsible for this modification is CheR (Boukhvalova et al., 2002), which catalyses the transfer of methyl groups from S-adenosylmethionine (SAM) to specific glutaryl residues in the cytosolic domain of MCPs. Binding of chemoattractants at the periplasmic domain triggers a conformational changes, which modulates CheA activity (Chervitz and Falke, 1996) and which in parallel alters receptor methylation by CheR (Brooun et al., 1998). Receptor methylation is thought to generate a molecular stimulus that travels back to the periplasmic domain and resets the receptor complex to its null state by...
reversal of the conformational change generated by ligand binding (Lai et al., 2006), which is a mechanism leading to receptor adaptation. It is known that chemoattractants in *E. coli* cause an increase in net methylation whereas in *Bacillus subtilis* attractants cause net reduction of methylation (Alexander and Zhulin, 2007). Here we show that McpT in *P. putida* is specifically methylated by CheR and that amount of methylation of McpT was moderately but reproducibly reduced in the presence of toluene. This supports that toluene interacts with McpT and indicates that the methylation mechanism in *P. putida* might resemble that described for *B. subtilis*. Inhibition of McpT methylation could affect the autophosphorylation of CheA that in turn modulates CheY phosphorylation level and subsequently the functioning of the flagella motor.

To place these observations in a large context we should note that when bacteria are exposed to compounds which are toxic but which at the same time can be used as a carbon source, microorganisms face the obvious dilemma as to flee from these compounds or to fight and use them. The decision-making process is based on the action of different signal transduction proteins. Some bacteria have evolved to flee whereas others have evolved to resist and to use the toxic compounds. Among the last group of strains *P. putida* DOT-T1E is a well characterized example and the data reported here largely contribute to the mechanisms which form the basis for this ‘decision’. Strain DOT-T1E can use toluene as its sole carbon source, which is due to the action of the TOD pathway which is under the control of the TodS/TodT two-component system. The sensor kinase TodS was found to bind toluene and upregulate gene expression accordingly (Lacal et al., 2006; Busch et al., 2007; 2009).

In addition this strain possesses the TtgGH-I efflux pump which expels toluene (Rojas et al., 2003). The action of this pump was found to be the ultimate determinant for solvent tolerance in this microorganism. The TtgV repressor binds toluene and upregulates pump expression accordingly (Guazzaroni et al., 2005). Both toluene sensor proteins are present in the cytosol, but the affinity of TodS for toluene is around 2 orders of magnitude higher than that of TtgV. This is consistent with the notion that at low toluene concentration the bacteria degrades toluene and only at high toxic concentration the TtgV mediated upregulation of the ttgGH-I operon.

In the present work we have characterized the primary player which can be associated to the flee-part of the dilemma. Aromatic hydrocarbons are for some bacteria chemoattractants, whereas for others chemorepellents. McpT was shown to mediate a strong chemoattractant response. Interestingly, two of the three toluene binding signal transduction proteins (McpT and TtgV-TtgGHI) are present on the plasmid pGRT1. This might indicate that the highchemotaxis reported here requires mechanisms which guarantee the survival of the bacteria in the presence of the chemoattractant.

Regardless of the molecular mechanism of McpT functioning, it is interesting to note that McpT responds to a wide range of chemicals, that the mcpt genes are plasmid borne and they can be horizontally transferred to other microbes. Genome analysis reveals that almost identical McpT homologues are present in a series of other bacterial plasmids, which are present in strains that are able to degrade aromatic hydrocarbons. This is consistent with the notion that the strong chemotaxis phenotype is not restricted to strain DOT-T1E but might have co-evolved in several bacteria with the capacity to degrade aromatic hydrocarbons. From a biotechnological perspective in bioremediation strong chemotaxis can be exploited to allow bacteria to move towards pollutants and achieve a more efficient bioremediation outcome in heterogeneously polluted sites.

**Experimental procedures**

**Growth of bacterial strains**

*Pseudomonas putida* wild-type and mutant strains were grown in M9 minimal medium supplemented with 10 mM succinate, in the absence (non-induced conditions) or in the presence of 1.5 mM toluene (induced). Appropriate antibiotics were added when required. Bacteria and plasmids used are listed in Table 1. Antibiotics were added to the culture medium to reach 50 μg ml⁻¹ Gm or Km and 10 μg ml⁻¹ in the case of Rif, Tc and Sm.

**Chemotaxis assays**

Agarose plug assays were carried out as previously described (Parales et al., 2000). In short, the mixture for generation of the agarose plug contained 2% (w/v) low-melting-temperature Omnipur agarose (EMD), chemotaxis buffer (40 mM K₂HPO₄/KH₂PO₄, 0.05% (w/v) glycerol, 10 mM EDTA, pH 7.0), the chemoattractant at several concentrations ranging from 0.01% to 50% (v/v) and trace amounts of Coomassie blue to provide contrast. A 5 μl drop of this mixture was placed on a microscope slide on top of a coverslip supported by two plastic strips and placed to form a chamber. Cells grown as described above were harvested when cell density was 0.3–0.7 at 660 nm, resuspended in chemotaxis buffer to reach an OD₆₀₀ of 0.7, and then introduced into the chemotaxis chamber to surround the agarose plug. As control for this series of assays, plugs without chemicals were used as negative control, whereas plugs with 6 mM succinate were used as a positive control, because *P. putida* responds chemotactically to this dicarboxylic acid (Lacal et al., 2010b).

For qualitative capillary assays, each capillary was filled with 1% (w/v) low-melting-temperature agarose in chemotaxis buffer and 2.5–10% (v/v) of the attractant. Freshly grown cells were resuspended in chemotaxis buffer to an OD₆₀₀ of 0.1 ± 0.05 and placed into an observation chamber formed by a microscope slide and a coverslip. The capillary contain-
ing the attractant was then inserted into the cell suspension. Cells were observed under 400× magnification in a Zeiss microscope coupled to a photographic camera or a video camera.

Quantitative capillary assay

A modified version of the capillary test described earlier (Acier, 1973) was used to quantify chemotaxis. In short, cells were grown in minimal medium (Iwaki et al., 2007) to a turbidity, at 600 nm (OD0.6) of 0.3–0.8. Subsequently, cells were diluted in the same medium to an OD0.6 of approximately 0.08. About 0.1 ml of this suspension was placed into a small chamber formed by placing two capillary tubes (volume 1 µl) (Microcaps, Drummond, Bromall, PA, USA) in parallel on a microscope slide. Another capillary tube, heat-sealed at one end, containing the chemoeffector solution, was immersed in the cell suspension at its open end. The system was then closed with a glass coverslip avoiding any formation of air bubbles in the chamber. The chemoeffector solution in the capillary contained minimal medium supplemented with different concentrations of toluene [1, 5 and 10% (v/v)]. After 10 min incubation, the number of cells in the capillary was quantified by colony-forming units on minimal medium M9 supplemented with 10 mM of succinate as a carbon source.

Verification of the presence of pGRT1 by PCR

The presence or absence of pGRT1 in different bacterial strains was verified by polymerase chain reaction (PCR) using the oligonucleotides 5'-CTGATCAGGCTGTTAAGGTGGCTGG-3' and 5'-GCCCTGCGTTTTAGCGATGGCTTC-3', which are fragments of the plasmid-encoded genes IspA and orf156 respectively (Rodriguez-Herva et al., 2007). A large bacterial colony was resuspended in 10 µl of water, heated at 100°C for 10 min and centrifuged at 20,000 g for 2 min. Three micro litres of the resulting supernatant was used as template for PCR amplifications using standard conditions. Reaction products were analysed on 8.0% (w/v) agarose gels. The amplified product from a pGRT1 containing strain is 2314 bp in length.

Construction of the PmcpT promoter fusion and determination of β-galactosidase activity

The mcpT promoter was amplified using MspTP1 (5' ACCTGACGGAAATTCAAGTCTTTGATT-3') and MspTP2 (5' TGTCACCTCAGTCGAATATCGTTCG-3') primers containing EcoRI and PstI sites respectively. The 153 bp PCR product was digested with EcoRI and PstI and cloned into likewise digested pMP220. As a result, the PmcpT promoter was fused to lacZ in pMP220 to produce pJL::PmcpT. To measure β-galactosidase activity, P. putida DOT-T1E cells harbouring pJL::PmcpT were grown overnight at 30°C in LB and 10 µg ml⁻¹ tetracycline. Cultures were then diluted 100-fold in the same medium supplemented or not with 1.5 mM toluene. When the cultures reached a turbidity of 0.8 ± 0.05 at 600 nm, β-galactosidase activity was determined in permeabilized whole cells, and activity was expressed in Miller units.

Generation of the mcpT mutant in pGRT1

The mcpT-1 mutant (ORF72::ΩKm) was generated by amplification of a 615 bp fragment from P. putida DOT-T1E using oligonucleotides Mcp-1 and Msp-2a5 described by Molina et al. (2011). DNA was extracted from the gel (Qiagen gel extraction kit, Qiagen) and ligated into pMBL-T plasmid (Dominion MBL). The resulting plasmid was cut with KpnI and, and the insert was extracted from the gel and ligated into the pCHESI vector (Llamas et al., 2003), which had been previously digested with KpnI and dephosphorylated. The plasmid was subsequently electroporated into P. putida DOT-T1E and transconjugants selected on LB with kanamycin. For construction of the mcpT2 mutant (ORF97::ΩKm) oligonucleotides MCP-Eco and MCP-Bam were used for amplification. The amplicon was cut with EcoRI and BamHI and ligated into the pCHESI vector previously cut with the same enzymes; and the resulting plasmid was electroporated into P. putida DOT-T1E or the mcpT1 mutant. Resulting clones were checked for the appropriate insertion by Southern blot.

Generation of plasmid pJL-McpT and complementation of the mutant

To generate plasmid pJL-McpT, the mcpT ORF-1 (the mcpT that possesses the GAG codon for E) was amplified from P. putida DOT-T1E using mcpTF (5'-CACATTGAATTCTTAGAT AATGAGGTGACAC-3') and mcpTR (5'-CTCAGAGGACCCCTAAAGCGGAGCATG-3') primers, containing EcoRI and BamHI sites respectively (underlined). The mcpTF primer also contains a stop codon for the termination of the plasmid upstream encoded protein translation. The PCR was performed with EuroTag polymerase (EuroTag clone). The desired PCR product (1703 bp) was digested with EcoRI and BamHI and then cloned into the broad-host-range plasmid pBBRMCS-5 cleaved using the same enzymes, resulting in the pJL-mcpT plasmid. The plasmid was sequenced to confirm the presence of the desired DNA.

To express mcpT in E. coli BL21 Star™ the mcpT gene was cloned in pET20D-T7p0 (Invitrogen). McpT expression was induced by adding 0.1 mM IPTG to the bacterial culture at an OD = 0.6. Induced bacterial cultures were allowed to grow at 18°C overnight and cells were harvested by centrifugation at 4000 g for 20 min at 4°C and frozen at −80°C. Crude McpT-enriched membranes were prepared by thawing cell pellets on ice, resuspending them in ice cold Hapes buffer 30 mM pH 7, 100 mM NaCl containing EDTA-free protease inhibitor cocktail (Roche) and 100 µM benzamidine (Roche), followed by French press treatment (1100 p.s.i., three passages per sample). The homogenized cells were first centrifuged at 4000 g for 15 min at 4°C, the pellet discarded and the supernatant centrifuged at 50,000 g for 1 h at 4°C. The resulting pellet of crude McpT-enriched membranes was resuspended in small aliquots of Hapes buffer 10% sucrose, flash frozen in liquid nitrogen and stored at −80°C. Aliquots of McpT-enriched membranes were thawed just before each methylation experiments. To generate mock membranes which do not contain McpT, this procedure was repeated except that an expression plasmid which does not contain the mcpT gene was used.
Site-directed mutagenesis to generate a single point mutant of mcpT

We generated a mutant McpT protein with a single amino acid substitution (S183P) affecting the fourth alpha-helix of the McpT-LBD using the QuikChange Mutagenesis Kit (Stratagene) following the manufacturer's protocol. The introduced mutation changed the mcpT sequence from 5'-GAGGC GTCGGCAGCT-3' to 5'-GAGGC GTCGGCAGCT-3'. The mutant allele was cloned into plasmid pJL-McpTPS183P and sequenced to verify the change (see Table 1).

Methylation assays

The coding sequence of ORF PP3750 coding for CheR was cloned into the expression plasmid pET28b (Novagen). The recombinant protein was expressed with an N-terminal His tag and purified by affinity chromatography using a 5 ml HisTrap HP column (GE Healthcare). CheR methyltransferase activity was assayed as described by Stock (1984). The assays and were conducted with McpT containing membranes and mock membranes. Briefly, equal amounts of membranes were incubated with 4 µM of purified CheR, 100 µM S-adenosyl-[methyl-3H]-methionine (H-SAM: 0.83 µCi per sample), an aliquot of crude cytosolic extract of P. putida KT2440 (final protein concentration 5 mg ml–1), and toluene (final concentration of 1 mM). The final mix volume per sample was 100 µl in buffer Hapes 10 mM, NaCl 100 mM. This mix was incubated at 30°C and aliquots were removed at regular intervals. The reaction was stopped by adding 500 µl ice-cold 10% (v/v) acetic acid.

In order to quantify protein methylation in these samples, it was applied the vapour-phase equilibrium procedure described by Campillo and Ashcroft (1982). Samples were washed twice with ice-cold 10% (v/v) acetic acid, and the pellet resuspended in 200 µl 1 N NaOH. Each sample-containing tube was placed opened into a 7 ml liquid scintillation vial containing 2.4 ml of scintillation fluid. The vial was closed and incubated overnight at 37°C without shaking. The average recovery of 3H-methanol was more than 90%, and was quantified the following day using a scintillation counter. Methylation assays of mock membranes revealed a very minor activity and all measurement of McpT containing membranes were corrected with these controls.

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References


Supporting information
Additional Supporting Information may be found in the online version of this article.

Fig. S1. Physical map of the pGRT1 plasmid. The entire sequence of pGRT1 was determined by shotgun sequencing of the plasmid using the 454 technology. Sequence information can be accessed at GenBank (Molina et al., 2011).

Fig. S2. Supplementary information on McpT.
A. Protein sequence.
B. Topology and domain annotation of McpT. Analysis of the McpT sequence by DAS (Cserzo et al., 1997) revealed clearly the presence of two transmembrane regions, Tm1 and Tm2. The domain which is flanked by both Tm regions is therefore located in the periplasm. This domain, which remains unannotated in InterPro, is likely to correspond to the sensor domain of McpT. Analysis of the McpT sequence by InterPro shows that the cytoplasmic part of McpT is composed of a HAMP linker domain (InterPro IPR003660) and a MCP signal domain (InterPro IPR004089).
C. Secondary structure prediction of the probable sensor domain of McpT. Prediction was carried out using the consensus method (Deleage et al., 1997). Shown is the secondary structure prediction by four individual programs (DSC, GOR4, PHD and SOPMA) as well as the consensus: h: α-helix, c: coil, t: turn, e: β-strand. This prediction reveals that this domain is likely to be composed of a series of α-helices. Ser183, which was subjected to site-directed mutagenesis, is located in the final helix of the domain and is highlighted in green.
D. Three-dimensional model of the probable sensor domain of McpT. The model was created using the CPH models algorithm (Nielsen et al., 2010) and the structure of the ligand binding domain of the Tar chemoreceptor (pdb: 2D4U, unpublished) as template. The Tar sensor domain belongs to the TarH (InterPro IPR003122) family of proteins. The model is composed of 4 helices which are coloured differently. The sequence similarity of McpT to the Tar sensor domain and the 4-helix bundle arrangement of the model indicate that the McpT periplasmic domain is likely to be a TarH domain. It has been mentioned previously that existing bioinformatic tools to annotate TarH domains are little efficient due to the large sequence diversity within this family of proteins (Ulrich and Zhuin, 2005).