**Pseudomonas putida** F1 has multiple chemoreceptors with overlapping specificity for organic acids

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Previous studies have demonstrated that *Pseudomonas putida* strains are not only capable of growth on a wide range of organic substrates, but also chemotactic towards many of these compounds. However, in most cases the specific chemoreceptors that are involved have not been identified. The complete genome sequences of *P. putida* strains F1 and KT2440 revealed that each strain is predicted to encode 27 methyl-accepting chemotaxis proteins (MCPs) or MCP-like proteins, 25 of which are shared by both strains. It was expected that orthologous MCPs in closely related strains of the same species would be functionally equivalent. However, deletion of the gene encoding the *P. putida* F1 orthologue (locus tag Pput_4520, designated *mcfS*) of McpS, a known receptor for organic acids in *P. putida* KT2440, did not result in an obvious chemotaxis phenotype. Therefore, we constructed individual markerless MCP gene deletion mutants in *P. putida* F1 and screened for defective sensory responses to succinate, malate, fumarate and citrate. This screen resulted in the identification of a receptor, McfQ (locus tag Pput_4894), which responds to citrate and fumarate. An additional receptor, McfR (locus tag Pput_0339), which detects succinate, malate and fumarate, was found by individually expressing each of the 18 genes encoding canonical MCPs from strain F1 in a KT2440 *mcpS*-deletion mutant. Expression of *mcfS* in the same *mcpS* deletion mutant demonstrated that, like McfR, McfS responds to succinate, malate, citrate and fumarate. Therefore, at least three receptors, McfR, McfS, and McfQ, work in concert to detect organic acids in *P. putida* F1.

**INTRODUCTION**

Motile bacteria have the ability to respond to rapidly changing environmental conditions by sensing environmental cues. The general features of the chemosensory systems used by microbes to detect and respond to environmental stimuli are conserved in the Bacteria and Archaea, albeit with some variations (Szurmant & Or达尔, 2004). The basis for the prokaryotic chemosensory signal transduction process centres around a two-component system comprising a sensory histidine kinase (CheA) and a response regulator (CheY) (Wadhams & Armitage, 2004). CheA receives input signals from specific chemoreceptor proteins, and transmits signals to CheY by transferring a phosphoryl group. CheY-P interacts with the flagellar machinery to modulate rotation of the flagellar motor and thus change cell behaviour and movement. A third group of proteins allows cells to adapt to current conditions by modulating the activity of CheA (Roberts *et al*., 2010). The best-characterized mechanism of adaptation involves methylation of specific residues on membrane-bound chemoreceptors called methyl-accepting chemotaxis proteins (MCPs) (Hazelbauer *et al*., 2008; Hazelbauer & Lai, 2010; Sourjik & Armitage, 2010).

The specificity of bacterial chemotaxis systems resides in the chemoreceptors, which include MCPs and periplasmic ligand-binding proteins. While *Escherichia coli* has four...
canonical MCPs with two transmembrane domains flanking a periplasmic ligand-binding domain (Hazelbauer & Lai, 2010), many other bacteria have significantly more MCPs (Lacal et al., 2010b). For example, members of the genus Pseudomonas typically have >25 MCP-like proteins encoded in their genomes (Parales et al., 2004). A possible reason for the wealth of chemoreceptors may be related to the diverse catabolic capacity of the pseudomonads (Stanier et al., 1966) and the plethora of carbon sources in their environments. The ability to detect available carbon and energy sources allows bacteria to direct their movement to optimal environments for growth and propagation. Studies have shown that Pseudomonas putida strains are capable of chemotaxis towards a wide range of growth substrates, including aromatic compounds, amino acids and TCA cycle intermediates (Ditty et al., 2013; Grimm and Harwood, 1997; Harwood et al., 1984, 1990; Lacal et al., 2010a; Parales et al., 2000; Parales, 2004). Considering the large number of MCPs present in pseudomonads, relatively few have been functionally characterized (Alvarez-Ortega & Harwood, 2007; Grimm & Harwood, 1999; Iwaki et al., 2007; Kuroda et al., 1995; Lacal et al., 2010a, 2011; Liu et al., 2009; Oku et al., 2012; Taguchi et al., 1997; Wu et al., 2000).

Genome sequence analysis has revealed that Pseudomonas strains of the same species share conserved sets of orthologous MCPs. For example, Pseudomonas putida strains KT2440 and F1 share 25 out of 27 MCP-like proteins; each orthologue pair is >95% identical in amino acid sequence (Table S1, available in Microbiology Online). In P. putida KT2440, a single receptor designated MpSp was shown to mediate chemotaxis to six organic acids: succinate, malate, fumarate, oxaloacetate, citrate, isocitrate and butyrate (Lacal et al., 2010a). We expected that the presence or absence of functionally equivalent MCPs in closely related strains would result in similar chemotaxis phenotypes, but we demonstrate here that this is not the case. Surprisingly, deletion of the gene (locus tag Pput_4520) encoding the P. putida F1 orthologue of MpSp did not result in an obvious chemotaxis phenotype. Further investigation demonstrated that at least three additional receptors participate in chemotaxis to these organic acids in P. putida F1.

**METHODS**

**Bacterial strains.** The strains used in this study are shown in Table 1. E. coli strains DH5α and DH5α (pir) were used as host strains for cloned genes, and HB101 (pRK2013) was used as a helper strain in bacterial conjugations. E. coli was cultured in lysogeny broth (LB) (Davis et al., 1980) at 37°C. P. putida strains were grown at 30°C in LB or minimal medium (MSB) (Stanier et al., 1966) containing 10 mM succinate. MSB plates were solidified with 1.8% Noble agar. For growth studies, P. putida strains were grown in MSB containing 10 mM succinate, fumarate, malate or citrate. Ampicillin, kanamycin and tetracycline were used at 150, 100 and 20 μg ml⁻¹, respectively, for E. coli strains, and kanamycin and tetracycline were used at 50 and 20 μg ml⁻¹, respectively, for P. putida strains.

**DNA methods.** Plasmids were purified using a QIAprep Miniprep kit (Qiagen), and DNA fragments and PCR products were purified with a QIAquick Gel Extraction kit (Qiagen). Standard methods were used for the manipulation of plasmids (Ausubel et al., 1993). Genomic DNA from strain F1 was purified using the Puregene DNA Isolation kit (Gentra Systems). Fluorescent automated DNA sequencing was carried out at the University of California, Davis sequencing facility using an Applied Biosystems 3730 automated sequencer.

**Construction of MCP gene deletion mutants.** Each of the 18 genes predicted to encode MCPs with standard topology in P. putida F1 (locus tags Pput_0339, Pput_0342, Pput_0623, Pput_2091, Pput_2149, Pput_2828, Pput_3459, Pput_3489, Pput_3621, Pput_3892, Pput_4234, Pput_4352, Pput_4520, Pput_4764, Pput_4863, Pput_4894 and Pput_4895) was independently deleted (Liu et al., 2009; Liu et al., 2009) using the suicide vector pAW19, which carries a kanamycin-resistance gene and the sacB gene that confers sucrose sensitivity (White & Metcalf, 2004) (Table 1). For simplicity, only deletions of the MCP-encoding genes Pput_0339, Pput_4520 and Pput_4894, for which we identified functions in this study, are reported here. Pput_0339, Pput_4520 and Pput_4894 were designated mcpR, mcpS and mcpQ (for methyl-accepting chemotaxis protein from strain F1), respectively. To generate deletion constructs, 1 kb regions upstream and downstream of each MCP gene were amplified by PCR using primers listed in Table S2. The resulting PCR fragments were fused by overlap extension PCR (Horton et al., 1993) or blunt-end ligation (Adereth et al., 2005). Each product was further amplified by primerless PCR, resulting in a 2 kb fragment with an in-frame deletion of the MCP gene. Each 2 kb DNA fragment was digested with appropriate restriction enzyme(s), inserted into SpeI-SadI-digested pAW19 and sequenced. The resulting plasmids (Table 1) were introduced into E. coli DH5α (pir) and mated into P. putida F1 by conjugation in the presence of E. coli HB101 (pRK2013) (Simon et al., 1983). Kanamycin-resistant F1 exconjugants were subjected to counterselection in MSB containing 10 mM succinate and 20% sucrose. Deletions in kanamycin-sensitive strains were verified by PCR. Multiply mutated strains were constructed by repeating the process in the appropriate deletion mutant backgrounds. The mcpS gene in P. putida KT2701 (locus tag PP_4658, which is 98% identical to the gene at locus tag Pput_4520 from P. putida F1 and 100% identical to the gene in KT2440) was deleted in the same way, using the Pput_4520 primers (Table S2).

**Cloning MCP genes from P. putida F1 and KT2440.** To screen for MCP function in the KT2440/mcsp5 background (strain RPK001), each of the 18 MCP genes from strain F1 was PCR amplified and cloned into pRK415Km. For simplicity, we only report primers and plasmid construction details for those MCP genes that were identified to function in organic acid chemotaxis. Genes mcpR, mcpS and mcpQ were PCR amplified from P. putida F1 chromosomal DNA using the primers listed in Table S2 and cloned into pRK415Km, resulting in plasmids pGCF101, pGCF123, pGCF126, pGCF127 and pGCF128. The KT2440 orthologues mcpR and mcpQ were similarly cloned after amplification using the same primer sets listed above, forming plasmids pGCK101 and pGCK126 (the KT2440 genes at locus tag PP_0317 and PP_0502 were designated mcpR and mcpQ, respectively). Genes mcpS and mcpF were also amplified using 4520BamHI-for and 4520Sall-rev (Table S2) and cloned into pSRK-Km (Khan et al., 2008), generating plasmids pGCK223, pGCF225 and pGCF223, respectively. Plasmids in DH5α were transformed into the appropriate P. putida strains by triparental matings with E. coli HB101 (pRK2013) (Simon et al., 1983).

**Chemotaxis assays.** The qualitative capillary assay was carried out as previously described (Grimm & Harwood, 1997) with slight modifications. Bacterial cells were harvested in mid-exponential phase (optical density at 660 nm [OD660] 0.3–0.45) by centrifugation at 4500 r.p.m. for 5 min and washed once with chemotaxis buffer (CB;
were inserted into the pool of bacterial cells. All of the organic acids melting-temperature agarose (NuSieve GTG, Lonza) dissolved in CB Petri dish. Microcapillaries (1 chamber formed by a coverslip, a glass U-tube and the bottom of a

\[ 50 \text{ mM potassium phosphate buffer \[ \text{pH 7.0} \] , 10 \mu M disodium EDTA, 0.05 \% glycerol} \] (Parales et al., 2000). Washed cells were suspended in CB to an OD\text{_{600}} of approximately 0.10, placed in a chamber formed by a coverslip, a glass U-tube and the bottom of a Petri dish. Microcapillaries (1 \mu l) containing attractants in 2 % low-melting-temperature agarose (NuSieve GTG, Lonza) dissolved in CB were inserted into the pool of bacterial cells. All of the organic acids were initially tested at 10 mM. If no response was detected, compounds were tested at 50 mM. In all experiments, negative (CB) and positive (0.2 % Difco Casamino acids [BD Biosciences]) controls were included. The response was visualized at \times 40 magnification on a Nikon Eclipse TE2000 S microscope and photographed using an Evolution MicroPublisher 3.3 RTV camera and Evolution MP/QImaging software (Media Cybernetics Inc.).

Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics*</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td>E. coli DH5α</td>
<td>Cloning host</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>E. coli DH5αΔpir</td>
<td>Cloning host</td>
<td>William W. Metcalf</td>
</tr>
<tr>
<td>HB101</td>
<td>Host for plasmid pRK2013 for plasmid mobilization</td>
<td>Sambrook et al. (1989)</td>
</tr>
<tr>
<td>P. putida F1</td>
<td>Wild-type</td>
<td>Finette et al. (1984); Gibson et al. (1970)</td>
</tr>
<tr>
<td>GC001</td>
<td>F1 Δ aer2 Δ mcfS (ΔPput_3628 Δ Pput_4520)</td>
<td>This study</td>
</tr>
<tr>
<td>GC017</td>
<td>F1 Δ aer2 Δ mcfQ (ΔPput_3628 Δ Pput_4894)</td>
<td>This study</td>
</tr>
<tr>
<td>GC021</td>
<td>F1 Δ aer2 Δ mcfR (ΔPput_3628 Δ Pput_0339)</td>
<td>This study</td>
</tr>
<tr>
<td>GC023</td>
<td>F1 Δ mcfS Δ mcfQ (ΔPput_4520 Δ Pput_4894)</td>
<td>This study</td>
</tr>
<tr>
<td>GC103</td>
<td>F1 Δ aer2 Δ mcfS Δ mcfQ (ΔPput_3628 Δ Pput_4520 Δ Pput_4894)</td>
<td>This study</td>
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<tr>
<td>KT2701</td>
<td>Sm\textsuperscript{r} derivative of KT2440</td>
<td>Franklin et al. (1981); Sarand et al. (2008)</td>
</tr>
<tr>
<td>RPF003</td>
<td>F1 Δ aer2 Δ mcfR Δ mcfQ (ΔPput_3628 Δ Pput_0339 Δ Pput_4894)</td>
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<td>RPF004</td>
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<td>RPK001</td>
<td>KT2701 Δ mcpS</td>
<td>This study</td>
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<tr>
<td>XLF019</td>
<td>F1 Δ aer2</td>
<td>Luu et al., 2013</td>
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<td>XLF023</td>
<td>F1 Δ mcfS</td>
<td>This study</td>
</tr>
<tr>
<td>XLF026</td>
<td>F1 Δ mcfQ</td>
<td>This study</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pAW19</td>
<td>Cloning vector, sacB, Ap\textsuperscript{r}, Km\textsuperscript{r}</td>
<td>White &amp; Metcalf (2004)</td>
</tr>
<tr>
<td>pGCF101</td>
<td>mcrR (locus tag Pput_0339) from strain F1 cloned into HindIII-SalI sites of pRK415Km, constitutively expressed from lac promoter of plasmid, Km\textsuperscript{r}</td>
<td>This study</td>
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<td>pGCF123</td>
<td>mcfS (locus tag Pput_4520) from strain F1 cloned into BamHI-SalI sites of pRK415Km, constitutively expressed from lac promoter of plasmid, Km\textsuperscript{r}</td>
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<td>pGCF223</td>
<td>mcfS (locus tag Pput_4520) from strain F1 cloned into BamHI-SalI sites of pSRK-Km, inducibly expressed from lac promoter of plasmid in response to IPTG, Km\textsuperscript{r}</td>
<td>This study</td>
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<td>pGCK126</td>
<td>mcpQ (locus tag Pput_5020) from strain KT2701 cloned into HindIII-SalI sites of pRK415Km, constitutively expressed from lac promoter of plasmid, Km\textsuperscript{r}</td>
<td>This study</td>
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<td>pGCK223</td>
<td>mcpS from strain KT2701 in pSRK-Km, inducibly expressed from lac promoter of plasmid in response to IPTG, Km\textsuperscript{r}</td>
<td>This study</td>
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<td>pRK415mcpS</td>
<td>mcpS from strain KT2440 in pRK415, constitutively expressed from lac promoter of plasmid, Tc\textsuperscript{r}</td>
<td>Lacal et al., (2010a)</td>
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<td>pSRK-Km</td>
<td>Broad host range vector, Km\textsuperscript{r}</td>
<td>Khan et al., 2008</td>
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<td>pXLF001</td>
<td>mcrR (locus tag Pput_0339) upstream and downstream 1 kb PCR fragments fused and cloned into SpeI-SalI sites of pAW19, Ap\textsuperscript{r}, Km\textsuperscript{r}</td>
<td>This study</td>
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<td>pRK415Km</td>
<td>Broad host range vector, Km\textsuperscript{r}</td>
<td>Luu et al., 2013</td>
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<td>mcfS (locus tag Pput_4520) upstream and downstream 1 kb PCR fragments fused and cloned into SpeI-SalI sites of pAW19, Ap\textsuperscript{r}, Km\textsuperscript{r}</td>
<td>This study</td>
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<tr>
<td>pXLF026</td>
<td>mcfQ (locus tag Pput_4894) upstream and downstream 1 kb PCR fragments fused and cloned into SpeI-SalI sites of pAW19, Ap\textsuperscript{r}, Km\textsuperscript{r}</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Ap\textsuperscript{r}, ampicillin resistance; Km\textsuperscript{r}, kanamycin resistance; Sm\textsuperscript{r}, streptomycin resistance; Tc\textsuperscript{r}, tetracycline resistance.
Quantitative soft agar swim plate assays were used to screen for defective chemotactic responses to succinate, fumarate, malate, and citrate (1 mM). For these assays, *P. putida* strains were grown overnight in 5 ml LB medium at 30 °C with shaking. The overnight cultures were harvested by centrifugation, and the pellets were washed with 5 ml MSB and resuspended in MSB to an OD660 of 0.38–0.44. Then, 2 μl of the suspensions was used to inoculate 15 mm Petri plates containing soft agar. Plates were incubated at 30 °C for 22–26 h. When appropriate, 0.5 mM IPTG, 50 μg ml⁻¹ kanamycin or 20 μg ml⁻¹ tetracycline was included in the medium. Colony diameter images were taken using backlighting (Parkinson, 2007). For each individual experiment, the measured diameters of all mutant and complemented strains were normalized to 100 % of the wild-type colony diameter (or in some cases to the Δmcr2 mutant). Data are represented as the mean ± so of at least three independent experiments with three technical replicates each. All statistical analyses were conducted using JMP Pro Version 10.0 or GraphPad Software.

**Reverse transcriptase (RT)-PCR.** *P. putida* KT2701 grown in MSB containing 10 mM succinate was harvested in exponential phase and treated with RNAprotect cell reagent (Qiagen) according to the manufacturer’s instructions and stored at −80 °C. RNA was isolated from frozen cells using the Qiagen RNeasy mini kit. DNA was eliminated using the Qiagen ‘on column’ DNease procedure followed by precipitation with lithium chloride. RT-PCR was performed using the OneStep RT-PCR kit (Qiagen), and the primers are shown in Table S2. Absence of DNA in the RNA samples was verified by PCR with the same primers and *Tag* polymerase. PCR and RT-PCR products were visualized on agarose gels.

**Site-directed mutagenesis.** A mutant form of *mcpR* (KT2440 locus tag PP_0317) encoding a variant with a M87L amino acid substitution was generated as follows. Primers pp_0317NFFor-HindIII and pp_0317M87LRev (Table S2) were used to generate a 300 bp fragment, and primers pp_0317M87LFor and pp_0317M87LRev-SacI were used to generate a 1400 bp product using *Pfu* polymerase under standard conditions. PCR products and plasmid pRK415Km were digested with *SacI* and *HindIII* and gel purified using the Fermentas GeneJet Extraction kit (Thermo Fisher Scientific). The purified products were joined using the Infusion HD Cloning kit (Clontech) and used to transform competent DH5α. Inserts were verified by restriction digestion and sequencing.

**RESULTS**

**Deletion of mcfS (locus tag Pput_4520) alone did not result in a loss of response to organic acids**

A Tn5 insertion in *mcpS* (locus tag PP_4658) in *P. putida* KT2440 was previously shown to eliminate the chemotactic responses to the TCA cycle intermediates succinate, malate, fumarate, citrate, isocitrate and oxaloacetate, as well as butyrate (Lacal et al., 2010a). We found that wild-type *P. putida* F1 was also attracted to these compounds, and the relative strengths of the responses (malate, succinate, oxaloacetate and fumarate; strong attractants; citrate, isocitrate and butyrate: weak attractants) were the same in both strains (Fig. 1 and data not shown). Therefore, we were surprised to find that deletion of the *mcpS* orthologue (locus tag Pput_4520) in *P. putida* F1 (mutant strain XLF023) did not show an obvious loss of the response to any of the tested organic acids in qualitative capillary assays (Fig. 1 and data not shown). We designated the *mcpS* orthologue in *P. putida* F1 as *mcs* (methyl-accepting chemotaxis protein from strain F1) to indicate its host origin. These results suggested that different or additional receptors function in the detection of TCA cycle intermediates in *P. putida* F1. For this study, we decided to focus on the strong attractants succinate, malate and fumarate and the weak attractant citrate. To confirm the role of *mcpS* in the KT2440 background, we deleted the *mcpS* gene from a streptomycin-resistant derivative of *P. putida* KT2440 (strain KT2701) using the same PCR primers (Table S2) that were used to generate the *mcfS* deletion in *P. putida* F1 (strain XLF023). Like KT2440*mcpS::Tn5* (Lacal et al., 2010a), this strain, RPK001, was unable to detect succinate, malate, fumarate or citrate as measured by qualitative capillary assays (Fig. 2).

**McfS and McpS are functionally equivalent**

In order to determine whether McfS is functional and if so, which compounds it recognizes as attractants, cloned copies of *mcfS* from *P. putida* F1 and *mcs* from KT2440 were expressed in the Δ*mcpS* mutant RPK001. In this strain, both genes were able to complement the chemotaxis defect (Fig. 2). These results demonstrate that McfS is functional and it recognizes all four organic acids. Thus...
McfS and McpS appeared to be functionally equivalent, and further experiments were required to understand the phenotype of the *P. putida ΔmcfS* mutant XLF023.

**McfS is one of multiple receptors that detects TCA cycle intermediates in *P. putida* F1**

Subtle defects in chemotaxis responses are difficult to detect in qualitative capillary assays; therefore, we needed a simple quantitative method to compare responses of wild-type and mutant strains. Aerotaxis (or energy taxis) has been shown to mask chemotaxis phenotypes of *Pseudomonas aeruginosa* mutants in soft agar plates (Alvarez-Ortega & Harwood, 2007), so in order to identify partial chemotaxis defects using quantitative capillary assays, we constructed double mutants lacking Aer2, the energy taxis receptor in *P. putida* F1 (Luu et al., 2013), and each canonical MCP gene. To determine whether deletion of *mcfS* resulted in any reduction in the response to organic acids, we compared the responses of an aer2 deletion mutant (strain XLF019), an *mcfS* deletion mutant (strain XLF023) and a double deletion mutant lacking both *mcfS* and aer2 (strain GC001) to succinate, malate, citrate and fumarate in soft agar plates. Compared to wild-type, the mutant lacking *mcfS* (strain XLF023) had a slight but significant defect in the response to malate, but responded normally to succinate and fumarate (Fig. 3a). For reasons we do not understand, the *ΔmcfS* mutant formed a significantly larger colony diameter in response to citrate compared to the wild-type. Compared to the *Δaer2* mutant, the strain lacking both *mcfS* and aer2 had significantly reduced responses to both malate and succinate (Fig. 3a), indicating that the product of *mcfS* contributes to the response to some attractants, in this case, succinate. Complementation with *mcfS* on a plasmid (strain GC001 [pGCF223]) restored the responses (Fig. 3b).

**Identification of McfQ (locus tag Pput_4894) as a receptor for citrate and fumarate in *P. putida* F1**

Bioinformatic analyses indicated that 18 of the 27 putative MCPs in *P. putida* F1 have the canonical MCP domain structure (Falke et al., 1997), with two hydrophobic transmembrane regions flanking a periplasmic sensing domain, a HAMP (histidine kinases, adenyl cyclases, methyl-accepting chemotaxis proteins and phosphatases) domain and a cytoplasmic signalling domain (Table S1). Known receptors for organic acids [McpS, Tcp, PA2652 (Alvarez-Ortega & Harwood, 2007; Yamamoto & Imae, 1993)] have canonical domain structures, so we expected that additional receptors for these compounds would be of similar structure. To screen for additional receptors that detect organic acids in *P. putida* F1, each of the remaining 17 genes encoding MCPs with canonical structure was individually deleted, and double mutants lacking each MCP gene and the aer2 gene were also constructed. The resulting strains were screened on soft agar swim plates for defects in the responses to succinate, malate, fumarate and citrate. From this screen, a strain designated GC017 lacking aer2 and the gene at locus tag Pput_4894 (which was designated *mcfQ*) was found to have significantly reduced responses to citrate and fumarate relative to the aer2 deletion strain (Fig. 3c). A defective response to only citrate was detected in the single mutant XLF026 lacking *mcfQ*, again showing that the presence of Aer2 can mask a mutant phenotype. Complementation restored the responses to both citrate and fumarate (Fig. 3d). No other strains with obvious chemotaxis defects were detected in this screen.

Although results in Fig. 2 demonstrated that McfS recognizes fumarate, the response to fumarate by the *Δaer2 ΔmcfS* double mutant (strain XLF023) was not significantly different from that of the *aer2* single mutant XLF019 (Fig. 3a). However, the response of a *Δaer2 ΔmcfS ΔmcfQ* triple mutant (strain GC103) to fumarate was significantly weaker than the *Δaer2 ΔmcfS* double mutant (strain GC001) or the *Δaer2 ΔmcfQ* double mutant (strain GC017) (Fig. S1), confirming that McfS contributes to the detection of fumarate in *P. putida* F1.
Fig. 3. Participation of McfS and McfQ in the chemotactic responses to organic acids in *P. putida* F1 demonstrated in quantitative swim plate assays. (a) Comparison of the responses of strains F1 (wild type), XLF019 (Δaer2), XLF023 (ΔmcfS) and GC001 (Δaer2ΔmcfS). (b) Complementation of the ΔmcfS defect; comparison of strains XLF019(pSRK-Km) [Δaer2 vector control strain], GC001(pSRK-Km) [Δaer2ΔmcfS vector control strain] and GC001(pGCF223) [Δaer2ΔmcfS strain carrying mcfS under the control of an IPTG-inducible promoter on pSRK-Km]. In addition to 1 mM attractants, MSB plates in (b) contained 50 μg ml⁻¹ kanamycin and 0.5 mM IPTG. (c) Comparison of the responses of wild-type F1, XLF019 (Δaer2), XLF026 (ΔmcfQ) and GC017 (Δaer2ΔmcfQ). (d) Complementation of the mcfQ defect showing strains XLF019 (pRK415Km) [Δaer2 vector control strain], GC017(pRK415Km) [Δaer2ΔmcfQ vector control strain] and GC017(pGCF126) [Δaer2ΔmcfQ strain carrying mcfQ on pRK415Km]. In addition to 1 mM attractants, MSB plates in (d) contained 50 μg ml⁻¹ kanamycin. For reasons that are not fully understood but are most likely related to gene expression levels, introduction of pRK415Km carrying mcfS was unable to complement the mcfS deletion mutant, so the alternative vector pSRK-Km was used for complementation of both mcfS and mcpS. Y-axes are slightly different in each graph, so for clarity, 100% is indicated by the dotted line. Error bars represent the SD values for at least three independent assays conducted in triplicate. Within the data for each organic acid tested, means with the same letter are not significantly different. In (a) and (c), *P*, <0.05; one-way ANOVA interaction, Tukey’s multiple comparison test and (b) and (d), *P*<0.05; Student’s t-test. Ninety-five percent confidence intervals (indicated by asterisks) are used to describe significant differences from the normalized wild-type controls. Growth studies demonstrated that all strains had similar growth rates in MSB medium with individual organic acids (succinate, malate, fumarate or citrate; data not shown), indicating that the defects in the swim plate assay were solely due to chemotaxis defects.
Identification of McfR (locus tag Pput_0339) as a receptor for succinate, malate and fumarate in *P. putida* F1

A double mutant lacking *mcfS* and *mcfQ* (strain GC023) retained some ability to respond to succinate, malate and fumarate in qualitative capillary assays (Fig. 1), indicating that at least one additional receptor in *P. putida* F1 contributes to the responses to these compounds. We were, however, unable to detect any residual response of this mutant to citrate even at a high concentration (50 mM). Because screening the aer2-MCP double mutants in swim plate assays did not identify any additional candidate MCP genes, we cloned each of the 16 other genes predicted to encode MCPs with the canonical structure and expressed them individually in the *P. putida* KT2440 mcpS deletion mutant RPK001, which is unable to respond to any of these compounds. These strains were screened for the ability to respond to organic acids in swim plate assays. The KT2440 strain expressing the gene at locus tag Pput_0339 (strain RPK001[pGCF101]) was found to respond to succinate, malate, citrate and fumarate (Fig. 4a); this gene was designated *mcfR*. When the *P. putida* F1 mutant lacking ΔmcfR and Δaer2 was tested (strain GC021), we did detect very slight but significant defects in the responses to succinate and malate compared to the Δaer2 mutant XLF019 (Fig. 4b). These subtle differences were not obvious in our initial mutant screens, which explains why we did not identify this mutant strain. Interestingly, expression of the *mcfR* gene from a multi-copy plasmid in the ΔmcfR Δaer2 double mutant (strain GC021[pGCF101]) resulted in statistically significant stronger responses to succinate, malate and fumarate (Fig. 4b), further confirming the role of this MCP in detecting these organic acids. In a previous study, an enhanced response to cytosine by *P. putida* F1 was also seen when the chemoreceptor for cytosine (McpC) was expressed from a multi-copy plasmid (Liu et al., 2009). The KT2440 strain lacking *mcpS* (strain RPK001) expressing *mcfR* was observed to respond to succinate, malate and fumarate (Fig. 5), which further confirms these results. A weak response to citrate was also detected (Fig. 5).

At least one additional receptor appears to function in the detection of organic acids in *P. putida* F1

Qualitative capillary assays demonstrated that a triple mutant lacking *mcfS*, *mcfQ* and *mcfR* (strain RPF004) had significantly reduced responses to succinate, malate and...
fumarate compared to wild-type (Fig. 1). Weak residual responses to these compounds were detected (Fig. 1), indicating that additional receptors function in the detection of these compounds in \textit{P. putida} F1. However, repeated screens in soft agar plates using RPK001 expressing each of the other 15 typical MCP genes did not identify additional receptors for organic acids.

**Function of the KT2440 MCP orthologues**

Bioinformatic analysis showed that \textit{P. putida} KT2440 has orthologues of \textit{mcfQ} and \textit{mcfR} (Table S1), so we were interested in determining whether the encoded proteins are functional. The products of \textit{PP_5020} and \textit{mcfQ} are 98 \% identical, those of \textit{PP_0317} and \textit{mcfR} are >99 \% identical, and each pair of orthologues is the same length (Table 2).

**Table 2.** Amino acid differences between MCPs in \textit{P. putida} F1 and KT2440

<table>
<thead>
<tr>
<th>Receptor orthologue pair (F1/KT2440)</th>
<th>Amino acid identity (%)</th>
<th>Total no. of residues</th>
<th>Total no. of different residues</th>
<th>Different residues in periplasmic binding domain*</th>
</tr>
</thead>
<tbody>
<tr>
<td>McfS/McpS</td>
<td>99.5</td>
<td>639/639</td>
<td>3</td>
<td>2 (A222E, T224A)</td>
</tr>
<tr>
<td>McfQ/McpQ</td>
<td>98.3</td>
<td>638/638</td>
<td>11</td>
<td>7 (H69Q, R95I, R106H, T118I, E149D, Y169H, Y169H, T272A)</td>
</tr>
<tr>
<td>McfR/McpR</td>
<td>99.4</td>
<td>541/541</td>
<td>3</td>
<td>1 (L87M)</td>
</tr>
</tbody>
</table>

*The first and second amino acid residues listed surrounding the numerical amino acid position are present in the \textit{P. putida} F1 and KT2440 proteins, respectively.
PP_5020 and PP_0317 from KT2440 were designated mcpQ and mcpR, respectively. To determine whether mcpS, mcpQ and mcpR are expressed in the P. putida KT2440 background, RT-PCR was carried out using primers specific for the three genes (Table S2). All three genes were expressed as judged by the detection of single RT-PCR fragments of the expected sizes (Fig. S2). In order to determine whether the gene products are functional, each gene was cloned and expressed in the ΔmcpS mutant RPK001. Responses of RPK001 carrying cloned copies of the F1 and KT2440 orthologues of each receptor to succinate, malate, fumarate and citrate were compared in qualitative capillary assays (Fig. 5). The responses of the strains carrying the F1 and KT2440 orthologues to the positive control, Casamino acids, were similar (Fig. 5). Compared to the strain RPK001, which was unable to detect any of the organic acids (Fig. 2), all of the strains were able to detect some of the attractants (Fig. 5), indicating that the MqpC and McpR receptors from KT2440 are functional. In the qualitative capillary assay, a response to only citrate was detected in the ΔmcpS mutant carrying cloned mcfQ or mcpR (Fig. 5). Interestingly, the strain expressing mcfR (strain F1 orthologue) responded to succinate, malate and fumarate and very weakly to citrate, while responses only to malate and fumarate were detected by the strain expressing mcpR. Overall, the responses of the strain carrying mcfR to organic acids were consistently stronger than those of the strain carrying mcpR (KT2440 orthologue) (Fig. 5). The role of residue 87 in the function of McpR

Only a single amino acid difference at position 87 was identified in the ligand-binding domains of McfR and McpR (Table 2). Because the functions of McpR and McfR in the KT2440ΔmcpS background appeared to be different (Fig. 5), we generated a site-directed mutation that resulted in a single amino acid substitution (M87L) in the periplasmic binding domain of McpR, which renders this region of the KT2440 protein identical to the F1 protein. Responses of the ΔmcpS mutant RPK001 carrying the mutant form of McpR to malate and fumarate were consistently stronger than those of the same strain carrying the wild-type McpR, and a clear response to succinate was detected (Fig. 5). Overall, the responses of the mutant carrying McpR-M87L were similar to those of the strain expressing mcfR (Fig. 5).

DISCUSSION

In contrast to P. putida KT2440, which appears to utilize a single receptor (McpS) to respond to succinate, malate, fumarate and citrate (Lacal et al., 2010a), the results presented here demonstrate that in P. putida F1, McfS (the McpS orthologue) and McfR both contribute to the responses to succinate, malate, fumarate and citrate, while McfQ is primarily responsible for the detection of citrate and also contributes to the response to fumarate. Deletion of all three genes did not completely eliminate the responses to succinate, malate or fumarate, indicating that at least one other receptor also participates. It is quite possible that more than one additional MCP with the canonical structure contributes to the weak residual responses, and individual deletion or overexpression of any single gene did not result in a detectable phenotype using the available assays. Alternatively, it is possible that one or more of the noncanonical MCP-like proteins mediates the residual responses to succinate, malate and fumarate; we did not clone and express these nine putative receptor genes in the KT2440 ΔmcpS background; nor did we test the responses of mutants of P. putida F1 lacking them. Of the nine MCP-like proteins encoded in the P. putida F1 genome, three are predicted to have a single transmembrane domain, six have no identifiable transmembrane regions and are predicted to be soluble, and six of the nine MCP-like proteins (including the energy taxis receptor Aer2) contain one or two PAS domains (Table S1).

In the F1 background, mcfR was responsible for significant responses to succinate, malate and fumarate (Fig. 4b), and the product of mcfS compensated for the absence of mcfR; this made it difficult to detect a mutant phenotype for the ΔmcfR mutant. In contrast, in the KT2440 background, mcpR (the mcpR orthologue) seemed to play a less important role in detecting organic acids. When expressed from a multi-copy plasmid in a strain lacking mcpS, mcpR mediated only weak responses to malate and fumarate (Fig. 5). Furthermore, the demonstration that the amino acid at position 87 (the only amino acid residue that differs between the ligand-binding domains of McpR and McfR) plays an important role in the ability of the protein to respond to succinate, malate and fumarate may explain why the absence of mcpS alone in KT2440 results in an essentially null phenotype.

McpQ and McfQ seemed to function similarly in the KT2440 ΔmcpS background; in qualitative capillary assays, only citrate was detected (Fig. 5). However, in these experiments the genes were expressed under the control of a constitutive lac promoter on the multi-copy plasmid pRK415Km, which may have resulted in an enhanced response to this very weak attractant. The mcpQ gene is expressed in KT2701 (Fig. S2), but the level of expression was not quantified. The absence of a detectable response to citrate in the single mutant lacking only mcpS could be due to low expression of mcpQ in the KT2440 background.

Pairwise sequence comparisons revealed that McfS and McfQ are 55 % identical overall, and their ligand-binding domains are 27 % identical. In fact, McfS and McfQ are more closely related to each other than to any of the other 25 MCP-like proteins encoded in the P. putida F1 genome, which may explain their functional equivalence in the detection of organic acids. In contrast, McfS and McfQ are each approximately 34 % identical to McfR, and their respective ligand-binding domains share ≤ 15 % sequence identity; multiple sequence alignments of the ligand-binding
domains show very little sequence conservation (Fig. S3). In addition, while the ligand-binding domains of McfS and McfQ are approximately 250 amino acids in length, that of McfR is 100 amino acids shorter, indicating that it is more similar to E. coli MCPs. Furthermore, the ligand-binding domain of McfR is predicted to form a four-helix bundle module, similar to the four receptors in E. coli (Ulrich & Zhulin, 2005). Pineda-Molina et al. (Pineda-Molina et al., 2012) showed that McpS forms a six-helix bundle that could represent a novel signal detection motif, essentially forming two adjacent four-helix bundles, each with its own ligand-binding site. The long ligand-binding domains of McfS and McfQ are both predicted to form six helices based on analysis using NPS@ (Combet et al., 2000). The six helices predicted for both McfS and McfQ have approximately the same size and distribution (two shorter helices followed by one longer helix, repeated) as those in McpS (Lacal et al., 2010a), indicating that both MCPs are likely to form similar six-helix bundles.

The ligand-binding domains of the other two functionally characterized receptors for TCA cycle intermediates, PA2652 from P. aeruginosa, which detects malate (Alvarez-Ortega & Harwood, 2007), and Tcp from Salmonella enterica serovar Typhimurium, which detects citrate (Yamamoto & Imae, 1993), do not share any significant sequence similarity with the P. putida receptors. In fact, only two leucine residues are conserved between PA2652 and the three P. putida F1 receptors McfS, McfR and McfQ (Fig. S3).

Functional redundancy of MCPs has been reported previously, particularly in Pseudomonas species. For example, P. aeruginosa and P. fluorescens each have three receptors with overlapping specificity for amino acids (Kato et al., 1999; Oka et al., 2012; Taguchi et al., 1997). It is not known at this time whether detection of organic acids is the only function of McfR, McfS and McfQ; it is quite possible that they are responsible for sensing other compounds as well. It is also not clear why bacteria would have multiple receptors to detect the same set of compounds — it may be an insurance policy in case some receptors are lost or mutated, or it could be a consequence of the need to detect many compounds with similar structures. Perhaps these particular receptors have different affinities for certain attractants, and as a result can sense different concentrations of specific chemicals. One example of this is illustrated by the two receptors in P. aeruginosa for sensing high and low concentrations of phosphate (Wu et al., 2000).

An important finding illustrated here is that strain-to-strain differences may be critical in determining the functional importance or specific role of orthologous chemoreceptors. One cannot assume that the role of a particular receptor is identical in different, albeit closely related, strains of the same species. Another example is the identification of two different primary energy taxis receptors in P. putida strains PRS2000, which uses Aer1 (Nichols & Harwood, 2000), and KT2440 and F1, which use Aer2 (Liu, 2009; Sarand et al., 2008), even though all three strains carry nearly identical copies of both aer1 and aer2. In the KT2440 background, aer2 was more highly transcribed than aer1 in cells grown in minimal medium, and the Aer2 protein was more abundant than Aer1 (Sarand et al., 2008). It is likely that the observed differences in chemotactic responses to organic acids in the F1 and KT2440 strain backgrounds are due to differences in gene expression levels and/or MCP abundances.

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encoded receptor required for chemotaxis of

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