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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.

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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 & Ordal, 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

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Abbreviations: CheA, sensory histidine kinase; CheY, response regulator; MCP, methyl-accepting chemotaxis protein.

Three supplementary figures and two tables are available with the online version of this paper.

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 environs. 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 McpS 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 McpS 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(*λpir*) 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 (BD Biosciences). 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_1257, 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, 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 *mcfR*, *mcfS* and *mcfQ* (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 *Spe*I-*Sac*I-digested pAW19 and sequenced. The resulting plasmids (Table 1) were introduced into *E. coli* DH5α(*λpir*) and mated into *P. putida* F1 by conjugation in the presence of *E. coli* HB101(pRK2013) (Simon *et al.*, 1983). Kanamycin-resistant F1 exoconjugants 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 KT2440Δ*mcpS* 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 *mcfR*, *mcfS* and *mcfQ* 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 and pGCF126. 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_5020 were designated *mcpR* and *mcpQ*, respectively). Genes *mcpS* and *mcfS* were also amplified using 4520BamHI-for and 4520SalI-rev (Table S2) and cloned into pSRK-Km (Khan *et al.*, 2008), generating plasmids pGCK223 and pGCF223, respectively. Plasmids in DH5α were introduced 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 [OD_{660}] 0.3−0.45) by centrifugation at 4500 r.p.m. for 5 min and washed once with chemotaxis buffer (CB;

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics*	Source or reference
E. coli		
DH5α	Cloning host	Life Technologies
DH5α(*λpir*)	Cloning host	William W. Metcalf
HB101	Host for plasmid pRK2013 for plasmid mobilization	Sambrook *et al.* (1989)
P. putida		
F1	Wild-type	Finette *et al.* (1984); Gibson *et al.* (1970)
GC001	F1 Δ*aer2* Δ*mcfS* (Δ*Pput_3628* Δ*Pput_4520*)	This study
GC017	F1 Δ*aer2* Δ*mcfQ* (Δ*Pput_3628* Δ*Pput_4894*)	This study
GC021	F1 Δ*aer2* Δ*mcfR* (Δ*Pput_3628* Δ*Pput_0339*)	This study
GC023	F1 Δ*mcfS* Δ*mcfQ* (Δ*Pput_4520* Δ*Pput_4894*)	This study
GC103	F1 Δ*aer2* Δ*mcfS* Δ*mcfQ* (Δ*Pput_3628* Δ*Pput_4520* Δ*Pput_4894*)	This study
KT2701	Sm^r derivative of KT2440	Franklin *et al.* (1981); Sarand *et al.* (2008)
RPF003	F1 Δ*aer2* Δ*mcfR* Δ*mcfQ* (Δ*Pput_3628* Δ*Pput_0339* Δ*Pput_4894*)	This study
RPF004	F1 Δ*mcfR* Δ*mcfS* Δ*mcfQ* (Δ*Pput_0339* Δ*Pput_4520* Δ*Pput_4894*)	This study
RPK001	KT2701 Δ*mcpS*	This study
XLF019	F1 Δ*aer2*	Luu *et al.*, 2013
XLF023	F1 Δ*mcfS*	This study
XLF026	F1 Δ*mcfQ*	This study
Plasmids		
pAW19	Cloning vector, *sacB*, Ap^r, Km^r	White & Metcalf (2004)
pGCF101	*mcfR* (locus tag Pput_0339) from strain F1 cloned into *Hin*dIII-*Xba*I sites of pRK415Km, constitutively expressed from *lac* promoter of plasmid, Km^r	This study
pGCF123	*mcfS* (locus tag Pput_4520) from strain F1 cloned into *Bam*HI-*Sal*I sites of pRK415Km, constitutively expressed from *lac* promoter of plasmid, Km^r	This study
pGCF126	*mcfQ* (locus tag Pput_4894) from strain F1 cloned into *Hin*dIII-*Eco*RI sites of pRK415Km, constitutively expressed from *lac* promoter of plasmid, Km^r	This study
pGCF223	*mcfS* (locus tag Pput_4520) from strain F1 cloned into *Bam*HI-*Sal*I sites of pSRK-Km, inducibly expressed from *lac* promoter of plasmid in response to IPTG, Km^r	This study
pGCK101	*mcpR* (locus tag Pput_0317) from strain KT2701 cloned into *Hin*dIII-*Sac*I sites of pRK415Km, constitutively expressed from *lac* promoter of plasmid, Km^r	This study
pGCK126	*mcpQ* (locus tag Pput_5020) from strain KT2701 cloned into *Hin*dIII-*Sac*I sites of pRK415Km, constitutively expressed from *lac* promoter of plasmid, Km^r	This study
pGCK223	*mcpS* from strain KT2701 in pSRK-Km, inducibly expressed from *lac* promoter of plasmid in response to IPTG, Km^r	This study
pRK415*mcpS*	*mcpS* from strain KT2440 in pRK415, constitutively expressed from *lac* promoter of plasmid, Tc^r	Lacal *et al.*, (2010a)
pSRK-Km	Broad host range vector, Km^r	Khan *et al.*, 2008
pXLF001	*mcfR* (locus tag Pput_0339) upstream and downstream 1 kb PCR fragments fused and cloned into *SpeI-SacI* sites of pAW19, Ap^r, Km^r	This study
pRK415Km	Broad host range vector, Km^r	Luu *et al.*, 2013
pXLF023	*mcfS* (locus tag Pput_4520) upstream and downstream 1 kb PCR fragments fused and cloned into *Spe*I-*Sac*I sites of pAW19, Ap^r, Km^r	This study
pXLF026	*mcfQ* (locus tag Pput_4894) upstream and downstream 1 kb PCR fragments fused and cloned into *Spe*I-*Sac*I sites of pAW19, Ap^r, Km^r	This study

*Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance.

50 mM potassium phosphate buffer [pH 7.0], 10 μM disodium EDTA, 0.05 % glycerol) (Parales *et al.*, 2000). Washed cells were suspended in CB to an OD_{660} 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 μl) 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 ×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.).

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 OD_{660} 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 Δ*aer2* mutant). Data are represented as the mean ± SD 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' DNase 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 *Taq* 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_0317INFFor-*Hin*dIII and pp_0317M87LRev (Table S2) were used to generate a 300 bp fragment, and primers pp_0317M87LFor and pp_0317INFRev-*Sac*I were used to generate a 1400 bp product using *Pfu* polymerase under standard conditions. PCR products and plasmid pRK415Km were digested with *Sac*I and *Hin*dIII 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 Tn*5* 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 *mcfS* (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*::Tn*5* (Lacal *et al.*, 2010a), this strain, RPK001, was unable to detect succinate, malate, fumarate or citrate as measured by qualitative capillary assays (Fig. 2).

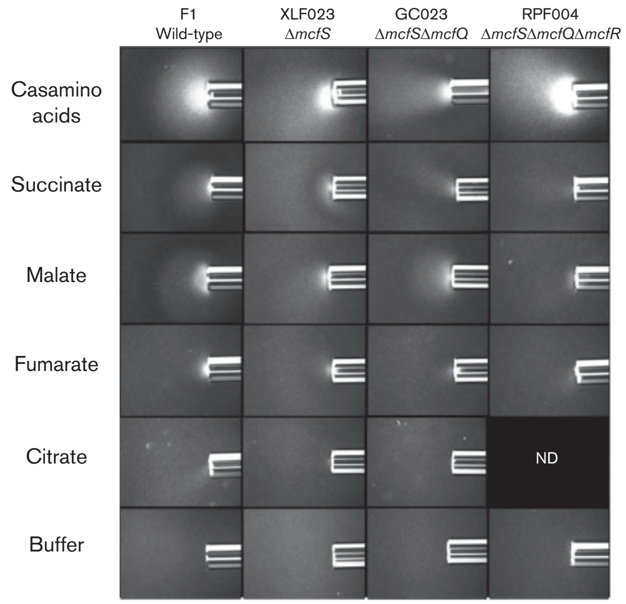

Fig. 1. Qualitative capillary assays comparing responses of wild-type *P. putida* F1 and the single (XLF023, Δ*mcfS*), double (GC023, Δ*mcfS* Δ*mcfQ*), and triple (RPF004, Δ*mcfS* Δ*mcfQ* Δ*mcfR*) mutants to succinate, malate, fumarate and citrate. Succinate, malate and fumarate were provided at 10 mM; citrate was provided at 50 mM. Also shown are positive and negative control responses to 2 % Casamino acids and chemotaxis buffer, respectively. Assays were repeated at least three times and representative photographs are shown. All photographs were taken after 7 min. ND, Not determined.

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 *mcpS* 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

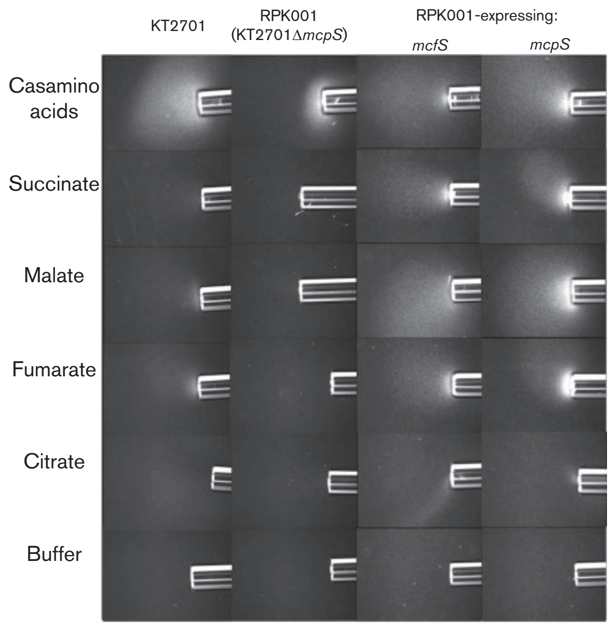

Fig. 2. Qualitative capillary assays demonstrating the function of McfS and McpS. Responses of *P. putida* KT2701 (wild type), RPK001 (KT2701Δ*mcpS*), and RPK001-expressing *mcfS* from *P. putida* F1 or *mcpS* from *P. putida* KT2701 are shown. Succinate, malate and fumarate were provided at 10 mM, and citrate was provided at 50 mM. Also shown are positive and negative control responses to 2 % Casamino acids and CB, respectively. Assays were repeated at least three times, and representative photographs are shown. All photographs were taken after 7 min.

McfS and McpS appeared to be functionally equivalent, and further experiments were required to understand the phenotype of the *P. putida* Δ*mcfS* 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 swim plate 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 Δ*mcfS* mutant formed a significantly larger colony diameter in response to citrate compared to the wild-type. Compared to the Δ*aer2* 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 malate and succinate chemotactic responses. Therefore, as expected based on similar findings in *P. aeruginosa* (Alvarez-Ortega & Harwood, 2007), the presence of a functional *aer* gene masked the defect in 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, adenylyl 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 Δ*aer2* Δ*mcfS* double mutant (strain XLF023) was not significantly different from that of the *aer2* single mutant XLF019 (Fig. 3a). However, the response of a Δ*aer2* Δ*mcfS* Δ*mcfQ* triple mutant (strain GC103) to fumarate was significantly weaker than either the Δ*aer2* Δ*mcfS* double mutant (strain GC001) or the Δ*aer2* Δ*mcfQ* double mutant (strain GC017) (Fig. S1), confirming that McfS contributes to the detection of fumarate in *P. putida* F1.

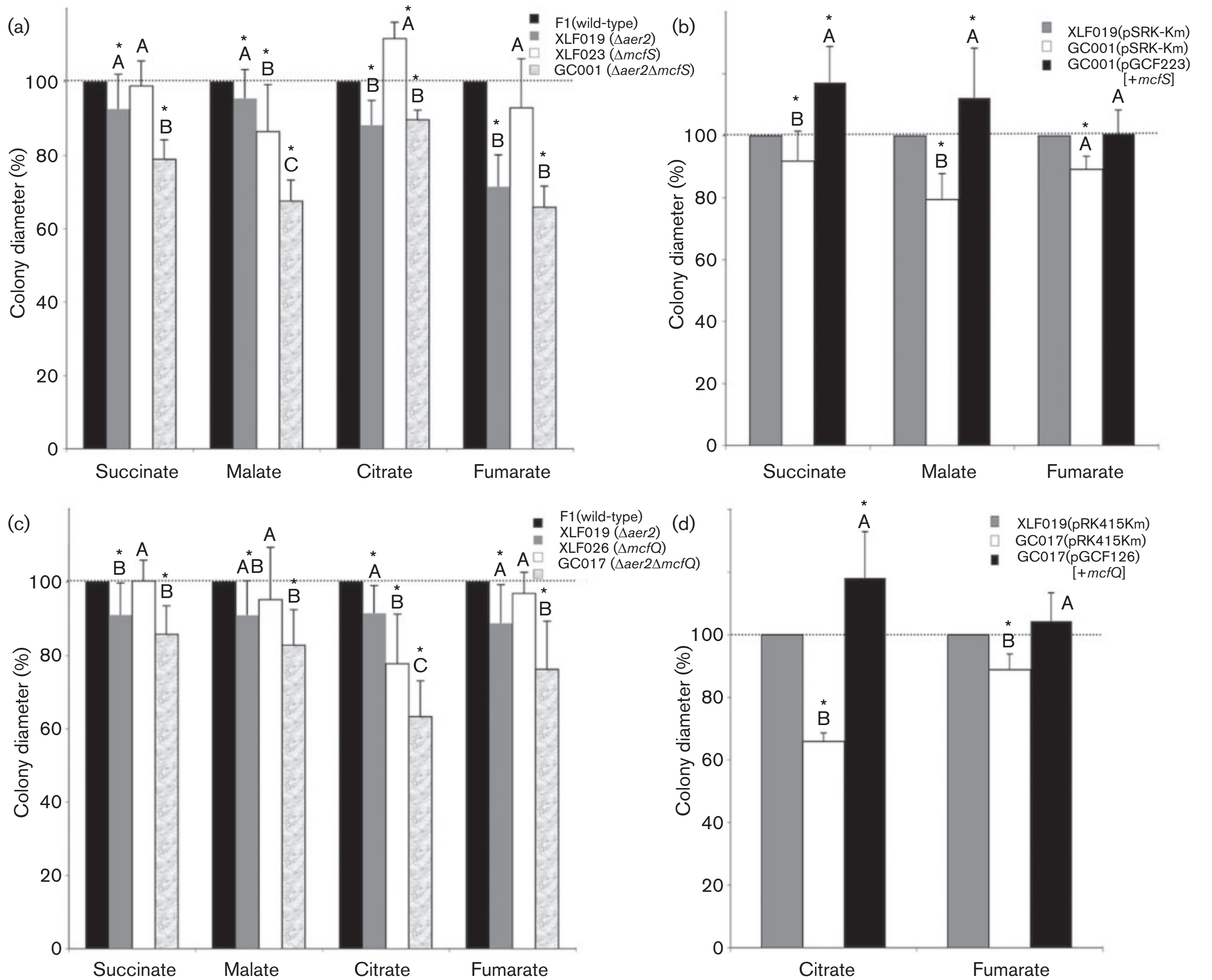

Fig. 3. Participation of McfS and McfQ in the chemotactic responses to organic acids in *P. putida* F1 demonstrated in quantitative swim plate assays. (a) Comparison of the responses of strains F1 (wild type), XLF019 (Δ*aer2*), XLF023 (Δ*mcfS*) and GC001 (Δ*aer2*Δ*mcfS*). (b) Complementation of the Δ*mcfS* defect; comparison of strains XLF019(pSRK-Km) [Δ*aer2* vector control strain], GC001(pSRK-Km) [Δ*aer2*Δ*mcfS* vector control strain] and GC001(pGCF223) [Δ*aer2*Δ*mcfS* 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 (Δ*aer2*), XLF026 (Δ*mcfQ*) and GC017 (Δ*aer2*Δ*mcfQ*). (d) Complementation of the *mcfQ* defect showing strains XLF019 (pRK415Km) [Δ*aer2* vector control strain], GC017(pRK415Km) [Δ*aer2*Δ*mcfQ* vector control strain] and GC017(pGCF126) [Δ*aer2*Δ*mcfQ* 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 Δ*mcfR* and Δ*aer2* was tested (strain GC021), we did detect very slight but significant defects in the responses to succinate and malate compared to the Δ*aer2* 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 Δ*mcfR* Δ*aer2* 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

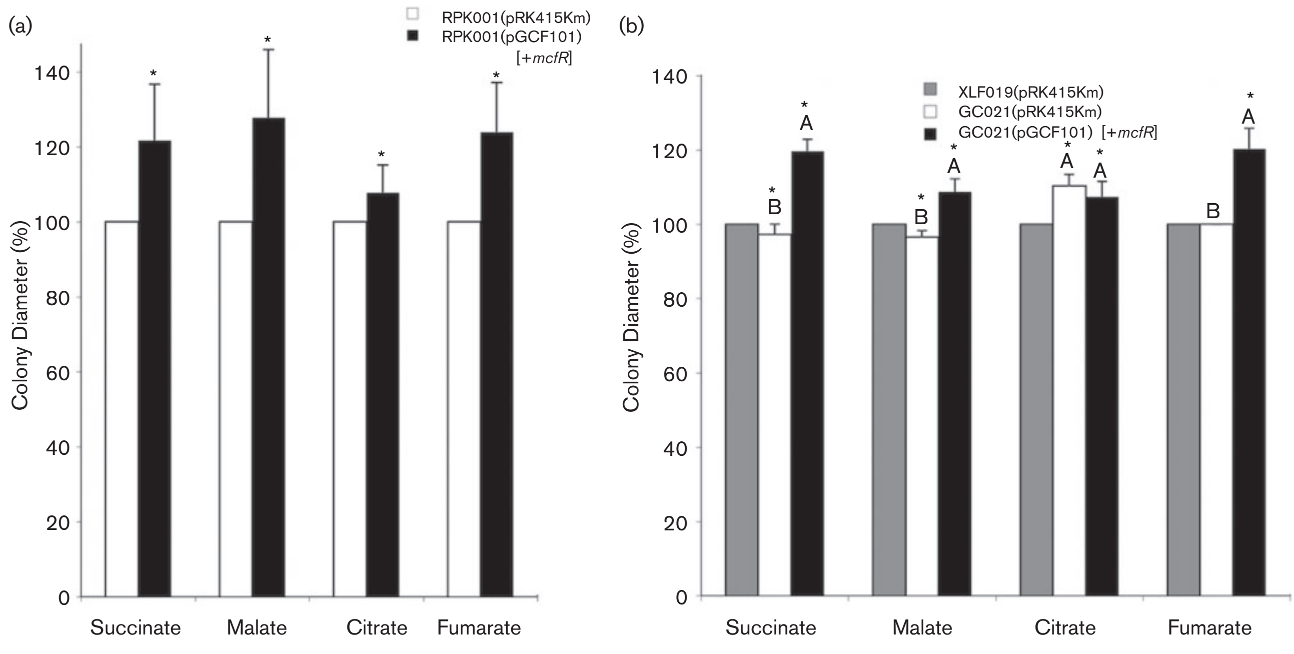

Fig. 4. Participation of McfR in the chemotactic responses to organic acids demonstrated in swim plate assays. (a) Function of McfR from *P. putida* F1 was demonstrated in the KT2440 derivative RPK001 (Sm^r, Δ*mcpS*) by comparing responses of RPK001 carrying the vector pRK415Km or pGCF101, which has *mcfR* cloned in pRK415Km. (b) Responses of the Δ*aer2* mutant XLF019 and the Δ*aer2*Δ*mcfR* double mutant GC021 carrying the vector pRK415Km were compared to GC021(pCGF101), which has *mcfR* cloned on pRK415Km. Attractants were provided at 1 mM, and plates contained 50 μg ml^{-1} kanamycin. In (a), $P<0.05$, one sample *t*-test. In (b), means with the same letter are not significantly different. $P<0.05$, one-way ANOVA interaction, Tukey's multiple comparison test. Ninety-five percent confidence intervals (indicated by asterisks) are used to indicate significant differences from the normalized wild-type controls.

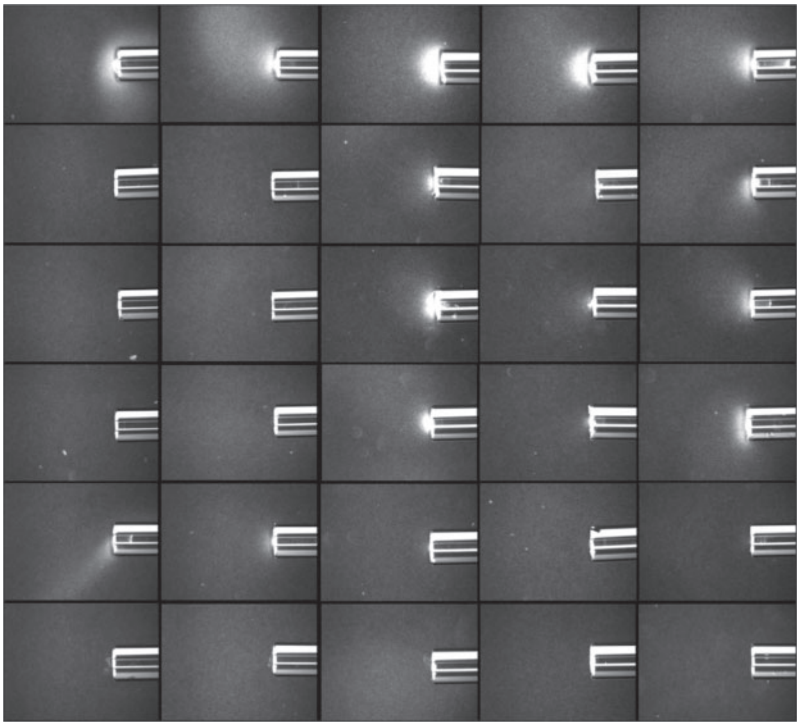

Fig. 5. Qualitative capillary assays comparing responses of the KT2440 derivative RPK001 (Sm^r, Δ*mcpS*) expressing the *P. putida* F1 *mcfQ* and *mcfR*, the KT2440 orthologues *mcpQ* and *mcpR*, and the mutant form of *mcpR* that encodes a protein with a single amino acid substitution in the periplasmic ligand-binding domain (M87L). Succinate, malate and fumarate were provided at 10 mM; citrate was provided at 50 mM. Also shown are positive and negative control responses to 2 % Casamino acids and chemotaxis buffer, respectively; see Fig. 2 to compare the responses of RPK001. Assays were repeated at least three times and representative photographs are shown. All photographs were taken after 7 min.

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

Table 2. Amino acid differences between MCPs in *P. putida* F1 and KT2440

Receptor orthologue pair (F1/KT2440)	Amino acid identity (%)	Total no. of residues	Total no. of different residues	Different residues in periplasmic binding domain*
McfS/McpS	99.5	639/639	3	2 (A222E, T224A)
McfQ/McpQ	98.3	638/638	11	7 (H69Q, R95L, R106H, T118I, E149D, Y169H, T272A)
McfR/McpR	99.4	541/541	3	1 (L87M)

*The first and second amino acid residues listed surrounding the numerical amino acid position are present in the *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 Δ*mcpS* 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 McpQ and McpR receptors from KT2440 are functional. In the qualitative capillary assay, a response to only citrate was detected in the Δ*mcpS* mutant carrying cloned *mcfQ* or *mcpQ* (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 KT2440Δ*mcpS* 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 Δ*mcpS* 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 Δ*mcpS* 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 Δ*mcfR* mutant. In contrast, in the KT2440 background, *mcpR* (the *mcfR* 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 Δ*mcpS* 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 $\leqslant$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; Oku *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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