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Authors

Zhang, Xiangsheng

Hughes, Jonathan G

Subuyuj, Gabriel A

et al.

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PHYSIOLOGY

Chemotaxis of *Pseudomonas putida* F1 to Alcohols Is Mediated by the Carboxylic Acid Receptor McfP

Xiangsheng Zhang,[a,b] Jonathan G. Hughes,[a] Gabriel A. Subuyuj,[a] Jayna L. Ditty,[c] Rebecca E. Parales[a]

[a]Department of Microbiology and Molecular Genetics, College of Biological Sciences, University of California Davis, Davis, California, USA
[b]Jiangsu Provincial Key Laboratory of Coastal Wetland Bioresources and Environmental Protection, Yancheng Teachers University, Yancheng, China
[c]Department of Biology, College of Arts and Sciences, University of St. Thomas, St. Paul, Minnesota, USA

ABSTRACT Although alcohols are toxic to many microorganisms, they are good carbon and energy sources for some bacteria, including many pseudomonads. However, most studies that have examined chemosensory responses to alcohols have reported that alcohols are sensed as repellents, which is consistent with their toxic properties. In this study, we examined the chemotaxis of *Pseudomonas putida* strain F1 to *n*-alcohols with chain lengths of 1 to 12 carbons. *P. putida* F1 was attracted to all *n*-alcohols that served as growth substrates (C_2 to C_{12}) for the strain, and the responses were induced when cells were grown in the presence of alcohols. By assaying mutant strains lacking single or multiple methyl-accepting chemotaxis proteins, the receptor mediating the response to C_2 to C_{12} alcohols was identified as McfP, the ortholog of the *P. putida* strain KT2440 receptor for C_2 and C_3 carboxylic acids. Besides being a requirement for the response to *n*-alcohols, McfP was required for the response of *P. putida* F1 to pyruvate, L-lactate, acetate, and propionate, which are detected by the KT2440 receptor, and the medium- and long-chain carboxylic acids hexanoic acid and dodecanoic acid. β-Galactosidase assays of *P. putida* F1 carrying an *mcfP-lacZ* transcriptional fusion showed that the *mcfP* gene is not induced in response to alcohols. Together, our results are consistent with the idea that the carboxylic acids generated from the oxidation of alcohols are the actual attractants sensed by McfP in *P. putida* F1, rather than the alcohols themselves.

IMPORTANCE Alcohols, released as fermentation products and produced as intermediates in the catabolism of many organic compounds, including hydrocarbons and fatty acids, are common components of the microbial food web in soil and sediments. Although they serve as good carbon and energy sources for many soil bacteria, alcohols have primarily been reported to be repellents rather than attractants for motile bacteria. Little is known about how alcohols are sensed by microbes in the environment. We report here that catabolizable *n*-alcohols with linear chains of up to 12 carbons serve as attractants for the soil bacterium *Pseudomonas putida*, and rather than being detected directly, alcohols appear to be catabolized to acetate, which is then sensed by a specific cell-surface chemoreceptor protein.

KEYWORDS *Pseudomonas*, alcohol, butanol, catabolism, chemoreceptor, chemotaxis, ethanol, methyl-accepting chemotaxis protein, propanol

Pseudomonads are ubiquitous members of soil and water microbial communities and are known for their ability to catabolize a wide range of organic substrates (1). Most *Pseudomonas putida* and *Pseudomonas aeruginosa* isolates screened in the classic taxonomic study by Stanier et al. were found to be capable of utilizing short-chain *n*-alcohols, including ethanol, propanol, and butanol, as sources of carbon and energy (2). The metabolism of ethanol has been examined in detail in *Pseudomonas aeruginosa*, and the enzymes required have been identified and characterized (3–5). Alcohols

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Address correspondence to Rebecca E. Parales, reparales@ucdavis.edu.

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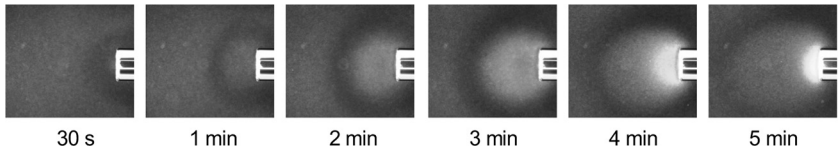

FIG 1 Time course of the response of *P. putida* strain F1 to ethanol in a qualitative capillary assay. Wild-type *P. putida* F1 was pregrown in MSB containing 10 mM pyruvate plus 2.5 mM ethanol. The response to 10 mM ethanol diffusing from a 1-μl capillary was photographed over 5 min; the accumulation of cells at the mouth of the capillary appears as a white cloud in darkfield microscopy. No response was seen to chemotaxis buffer (negative control; not shown).

utilized by *Pseudomonas* spp. also include longer-chain *n*-alcohols, as well as secondary alcohols and polyols (6, 7), and genes encoding the oxidation of long-chain alcohols/aldehydes were recently identified in *P. aeruginosa* (8).

In addition to their catabolic diversity, members of the genus *Pseudomonas* are known to use chemotaxis to sense concentration gradients of a large number of organic chemicals (9, 10). A conserved set of cytoplasmic chemotaxis proteins (CheA, CheY, CheW, CheR, and CheB), together with $\geq$20 membrane-spanning methyl-accepting chemotaxis proteins (MCPs), mediate responses to a wide range of organic and inorganic attractants in pseudomonads (9, 10). For example, known attractants for *Pseudomonas putida* strains include various amino acids, organic acids (11, 12), purines and pyrimidines (13, 14), polyamines (15), aromatic acids (16–18), and aromatic hydrocarbons (19–21). However, to our knowledge, chemotaxis to alcohols by *Pseudomonas* spp. has not been examined, and with the exception of the demonstration of chemoattraction to phenol by *Escherichia coli* (22), alcohols have generally been shown to be repellents for motile bacteria. For example, various alcohols, including straight-chain alcohols with up to four carbons, are repellents for *E. coli* (23, 24), and Young and Mitchell showed that ethanol elicited a negative chemotaxis response by a marine pseudomonad (25). Similarly, ethanol and butanol were repellents for *Borrelia burgdorferi* (26), and repellent responses of *Ralstonia pseudosolanacearum* strain Ps29 to a variety of alcohols were demonstrated (27).

In this study, chemotaxis assays were used to examine the responses of *P. putida* strain F1 to *n*-alcohols with 1 to 12 carbons, and positive responses were seen with all *n*-alcohols that served as growth substrates. The receptor for alcohol chemotaxis was identified by testing chemotaxis to ethanol by mutant strains lacking single or multiple MCP genes. Our results suggest that, rather than the alcohols themselves, carboxylic acids generated from the oxidation of alcohols are the actual attractants sensed by *P. putida* F1.

RESULTS

***P. putida* F1 is attracted to metabolizable short-chain *n*-alcohols, and the chemotactic response is inducible.** *P. putida* strain F1 efficiently utilized ethanol, 1-propanol, and 1-butanol in minimal medium (MSB [2]), with doubling times of approximately 1.5 h in aerobic batch culture, but was unable to grow on methanol or 2-propanol (data not shown). A strong and rapid chemotactic response to ethanol was observed in qualitative capillary assays using strain F1 cells grown in the presence of ethanol (Fig. 1). Chemical-in-plug assays were carried out to test the response of *P. putida* F1 to a variety of alcohols and whether the responses required induction. Cells were pregrown with 20 mM pyruvate (uninduced) or 10 mM pyruvate and 2.5 mM methanol, ethanol, 1-propanol, 2-propanol, or 1-butanol (induced). All cultures showed strong positive responses to 10 mM succinate (positive control), as shown by the accumulation of a ring of cells around the attractant-containing agar plug (Fig. 2A, white arrows). In contrast, no response was observed to the negative control (chemotaxis buffer, not shown). Only cultures pregrown in the presence of alcohols responded to alcohols as attractants (Fig. 2A), indicating that the response to alcohols is inducible. Some of the responses, particularly those of methanol- and 2-propanol-

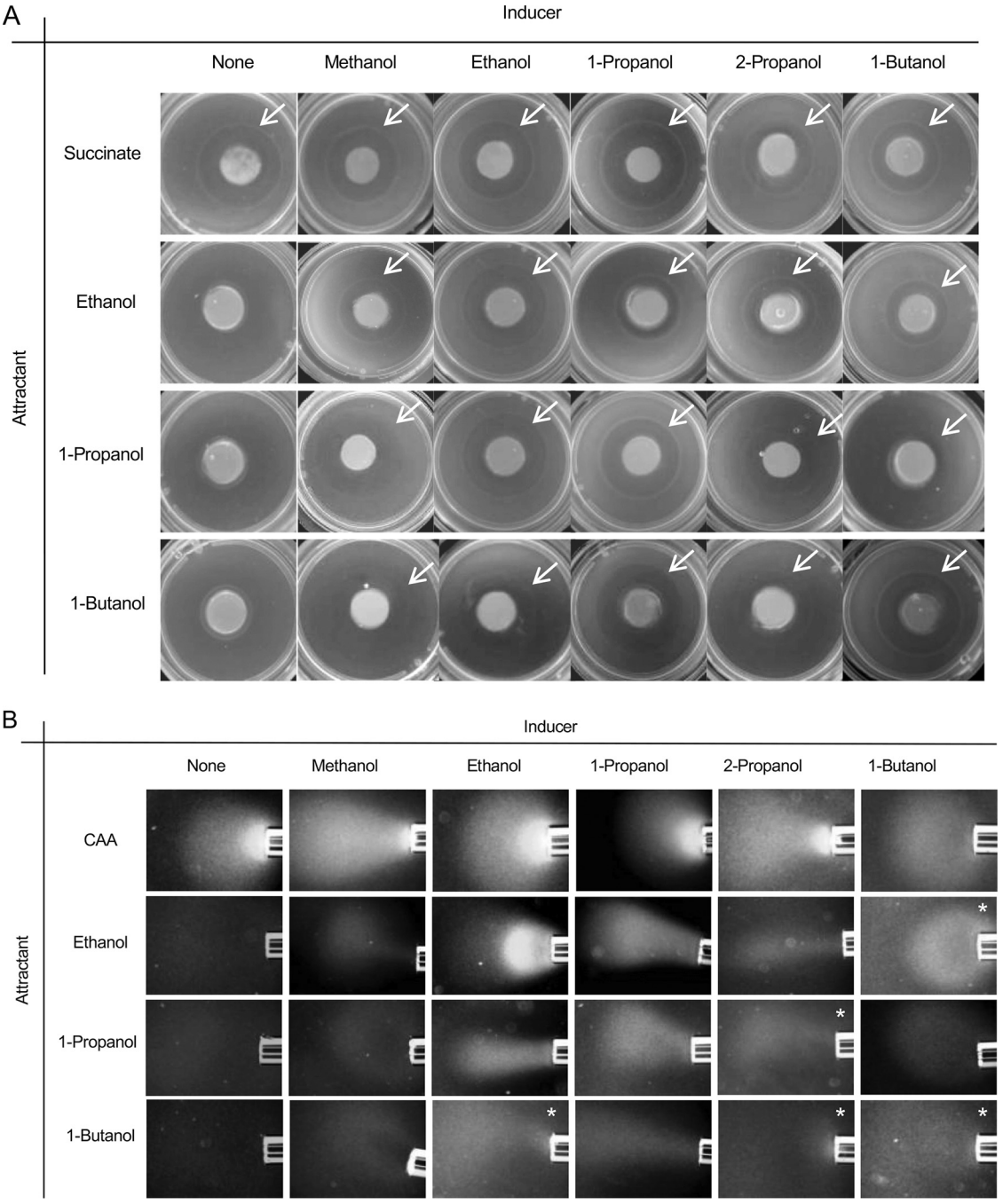

FIG 2 Induction of the *P. putida* F1 chemotaxis response to alcohols in chemical-in-plug assays and qualitative capillary assays. Wild-type *P. putida* F1 was pregrown in MSB containing 20 mM pyruvate (None) or 10 mM pyruvate plus 2.5 mM alcohol (methanol, ethanol, 1-propanol, 2-propanol, or 1-butanol as indicated). (A) Chemical-in-plug assays. Cells are suspended in soft agar, which surrounds the white central agar plug containing the attractant. Responses to 10 mM succinate (positive control) and 5 mM ethanol, 1-propanol, and 1-butanol after incubation for 1 h at room temperature are shown. Rings of cells accumulating around the plugs at the optimal concentration of attractant are indicated by white arrows. (B) Qualitative capillary assays. Responses to 2% Casamino Acids (CAA; positive control) and 10 mM ethanol, 1-propanol, and 1-butanol (or in some cases 5 mM, as indicated by white asterisks) are shown. Photographs were taken after 5 min. No responses were seen to chemotaxis buffer (negative control), or to 10 mM methanol or 10 mM 2-propanol (not shown).

induced cells and those to 1-butanol, were quite weak and not consistently observed. Therefore, to confirm the results of the plug assays, qualitative capillary assays were carried out. All cultures showed strong responses to 2% Casamino Acids (CAA; positive control), as shown by the accumulation of cells at the capillary tips (Fig. 2B). Again, only

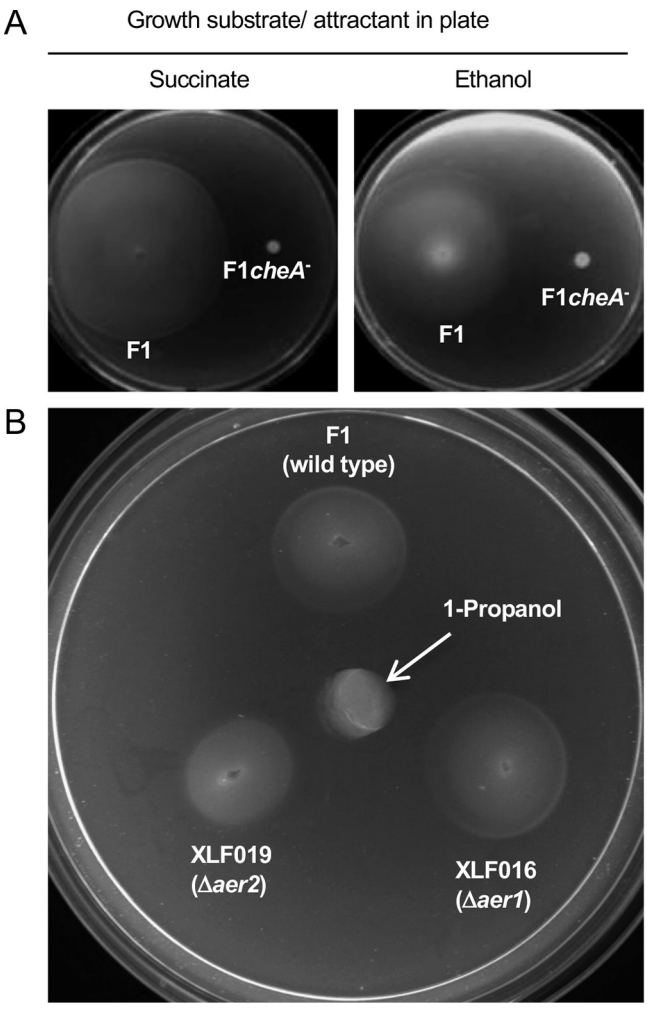

FIG 3 Role of CheA and energy taxis in the response to alcohols. (A) Soft agar swim plate assays to examine the role of CheA in the chemotactic response to ethanol. Wild-type *P. putida* F1 and mutant F1 *cheA*::Km were inoculated into MSB soft agar plates containing 5 mM succinate (positive control; photograph taken after 16 h of incubation at 30°C) and 5 mM ethanol (photograph taken after 24 h of incubation at 30°C). (B) Representative responses of *P. putida* F1, XLF016 (Δ*aer1*), and XLF019 (Δ*aer2*) to propanol in the gradient plate assay. Strains were inoculated at equivalent distances from the central plug containing 5 mM propanol. The photograph was taken after 24 h.

cultures that had been pregrown in the presence of alcohols showed responses to alcohols (Fig. 2B). It should be noted that methanol and 2-propanol, which are not metabolized by *P. putida* F1, were capable of inducing weak chemotactic responses to metabolizable alcohols (Fig. 2). However, *P. putida* F1 did not respond to methanol or 2-propanol (10 mM) under any of the conditions tested (data not shown).

CheA is required for signal transduction in response to alcohols. To determine whether alcohols are sensed through the conventional two-component chemotaxis signal transduction pathway, the response of a *cheA* mutant was tested. CheA is a histidine kinase that phosphorylates CheY, a response regulator, which in its phosphorylated state interacts with the flagellar motor to control the direction of flagellar rotation in response to stimuli (28). As shown by the results in Fig. 3A, a *P. putida* F1 *cheA* mutant (13) did not respond to either succinate (positive control) or ethanol in soft agar swim plate assays. In contrast, the wild-type strain formed a large chemotactic ring on both substrates, indicating that the chemotaxis response to ethanol is mediated through the standard two-component chemotaxis signal transduction pathway.

Attraction to alcohols is not mediated by energy taxis receptors. To test whether the response to alcohols is mediated by energy taxis, the responses of

wild-type strain F1, strain XLF019 (Δ*aer2*), and strain XLF016 (Δ*aer1*) were compared in gradient plate assays. Aer1 is a homolog of the energy taxis receptor Aer2 in *P. putida* F1 (17), and although Aer1 has not been demonstrated to play a role in energy taxis in *P. putida* F1, Aer1 has been reported to mediate energy taxis in *P. putida* strain PRS2000 (29). Similarly to the wild type, the Δ*aer1* and Δ*aer2* mutants responded to ethanol, 1-propanol, and 1-butanol in gradient plate assays. A representative example of the response to 1-propanol is shown in Fig. 3B, and the response index (RI) and standard deviation were calculated for three replicate plates. Values were 0.55 ± 0.02 (mean ± standard deviation), 0.53 ± 0.02, and 0.57 ± 0.01 for strains F1, XLF016, and XLF019, respectively; RI values greater than 0.52 are considered to indicate a positive response (22). Similar responses were seen with 10 mM ethanol and 5 mM 1-butanol (RI values for ethanol were 0.53 ± 0.02, 0.54 ± 0.02, and 0.55 ± 0.01 for strains F1, XLF016, and XLF019, respectively, and RI values for 1-butanol were 0.55 ± 0.01, 0.54 ± 0.01, and 0.55 ± 0.01 for strains F1, XLF016, and XLF019, respectively). No responses were seen to chemotaxis buffer (negative control; all RI values were ≤0.52). Positive responses of strains F1, XLF016, and XLF019 to all three alcohols were also seen with chemical-in-plug assays (data not shown). These results indicate that the response to alcohols is not mediated by an energy taxis receptor in *P. putida* F1.

The receptor for chemotaxis to alcohols is McfP (Pput_2828). To identify the chemoreceptor for alcohols, mutant strains with various receptor gene deletions were screened for the ability to respond to ethanol. We have been working toward generating a "gutted" strain lacking all 27 of the putative MCP and MCP-like genes in *P. putida* F1 and currently have a mutant lacking 11 of the 21 genes encoding canonical MCPs (those with two transmembrane domains flanking a periplasmic ligand binding domain and a cytoplasmic signaling domain). Strain RPF018 (F1Δ11) lacks genes encoding the tricarboxylic acid (TCA) cycle receptors McfS, McfR, and McfQ (12), the amino acid receptors McfA (Pput_3489) (30) and McfG (Pput_4352) (31), the aromatic acid receptor PcaY (16), and the nicotinic acid/cytosine receptor McpC (13, 18). In addition, four genes encoding receptors of unknown function were also deleted in this strain (locus tags *Pput_0342*, *Pput_3459*, *Pput_4234*, and *Pput_4764*). As expected, this strain responded only weakly to Casamino Acids, since two of the known amino acid receptors are absent (Fig. 4A). However, the F1Δ11 mutant did respond to propionate (Fig. 4A), demonstrating that the strain is motile and retains the ability to carry out chemotaxis. The ethanol-induced mutant (F1Δ11) retained the ability to respond to ethanol (Fig. 4A), which allowed us to eliminate 11 MCPs as the primary chemoreceptor for ethanol.

Next, we individually tested the responses of the remaining mutants with single MCP gene deletions and found that all mutants except strain XLF014 (54), which lacks McfP (locus tag *Pput_2828*), responded to ethanol (Fig. 4B). The MCP encoded by McfP is the ortholog of the *P. putida* strain KT2440 receptor McpP, which has been shown to mediate chemotaxis to C_2 and C_3 carboxylic acids, including acetate, pyruvate, L-lactate, and propionate (32). We recently confirmed that McfP is one of several chemoreceptors that mediate chemotaxis to propionate in *P. putida* F1 (54). Here, we verified the role of McfP in detecting C_2 and C_3 carboxylic acids in *P. putida* F1 using qualitative capillary assays (Fig. 5A). Similar responses were seen regardless of whether cells were pregrown in the presence of pyruvate, acetate, or succinate, suggesting that the response is not inducible. In addition, we compared the responses of strain XLF014(pRK415Km) and the complemented strain XLF014(pGCF114) to 10 mM acetate, pyruvate, L-lactate, and propionate (Fig. 5B). As expected, McfP was required for chemotaxis to all four short-chain carboxylic acids.

To confirm that McfP is required for chemotaxis to ethanol, 1-propanol, and 1-butanol, we examined the responses of strains F1(pRK415Km) and XLF014(pRK415Km) and the complemented strain XLF014(pGCF114) after growth in the presence (induced) and absence (uninduced) of ethanol. The mutant lacking *mcfP* did not respond to any of the alcohols under either condition, but the response was restored when *mcfP* was provided in *trans* (Fig. 6). The responses of the complemented strain were stronger,

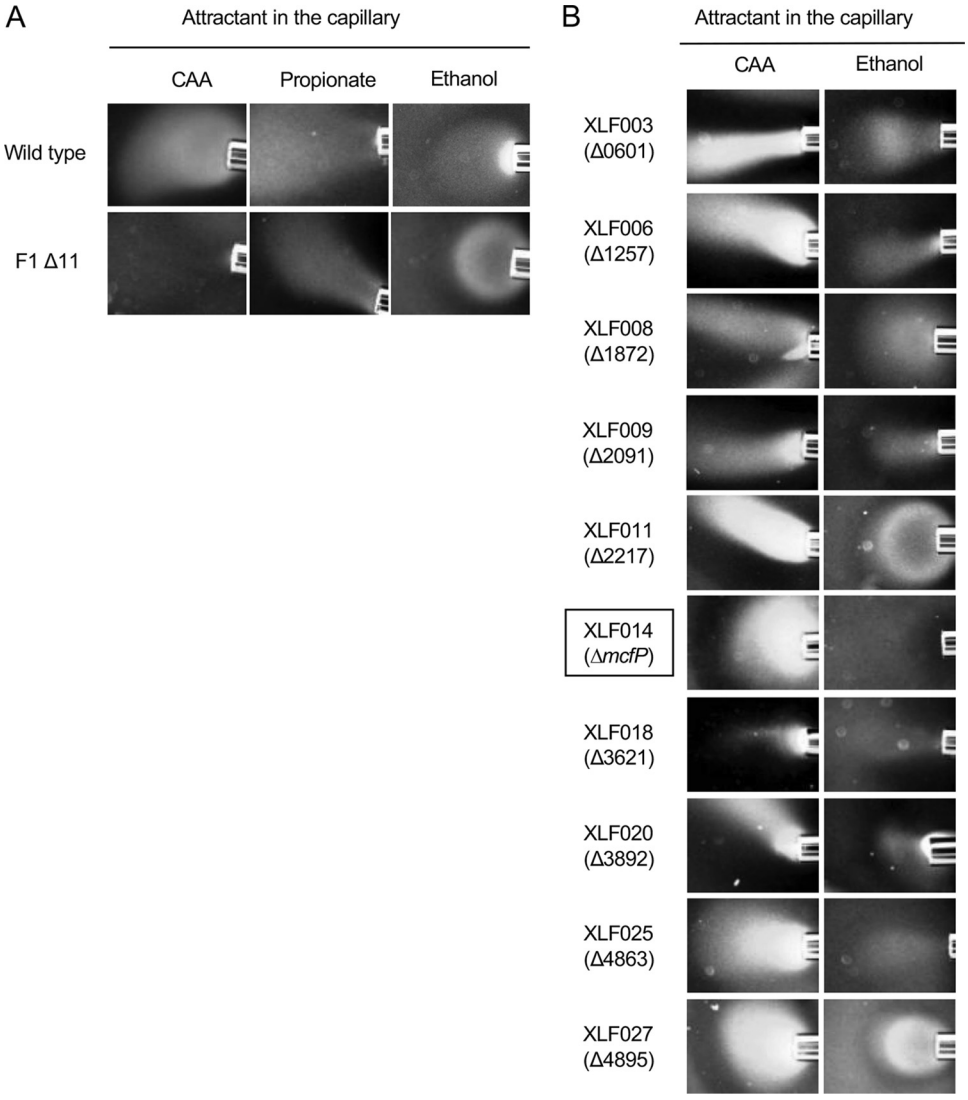

FIG 4 Screening *P. putida* F1 mutants for the ethanol chemoreceptor. (A) Response of a *P. putida* F1 mutant lacking 11 MCP genes (F1Δ11) to ethanol. Wild-type *P. putida* F1 and F1Δ11 were pregrown in MSB containing 10 mM pyruvate plus 2.5 mM ethanol (induced). Responses to 2% Casamino Acids (CAA), 10 mM propionate, and 10 mM ethanol are shown. Strain F1Δ11 lacks 11 MCP genes (Table 1), including two for amino acids (*Pput_3489* and *Pput_4352*), so only a very weak response to CAA was seen. However, strain F1Δ11 retains the propionate chemoreceptor gene (*Pput_2828*; *mcfP*), so propionate was used as the positive control. (B) Responses of *P. putida* F1 mutants lacking single MCP genes. The box indicates the mutant that was unable to respond to ethanol. Mutant strains were pregrown in MSB containing 10 mM pyruvate plus 2.5 mM ethanol (induced). Responses to 2% Casamino Acids (CAA; positive control) and 10 mM ethanol are shown. Photographs were taken after 5 min.

most likely because the *mcfP* gene was carried on a multicopy plasmid, and induction was unnecessary since the *mcfP* gene was expressed from the constitutive plasmid promoter. Similar results were obtained when cultures were induced with 1-propanol or 1-butanol (data not shown).

***P. putida* F1 responds to *n*-alcohols with up to 12 carbons and the responses require McfP.** To examine the range of *n*-alcohols sensed by *P. putida* F1, we tested growth and chemotaxis with *n*-alcohols with up to 12 carbons. *P. putida* F1 was capable of using pentanol, hexanol, heptanol, octanol, decanol, and dodecanol as sole carbon and energy sources (data not shown). Qualitative capillary assays with *P. putida* F1, the *mcfP* mutant, and the complemented strain showed that, unlike the *mcfP* mutant, the induced wild type and the complemented mutant were attracted to each alcohol (Fig. 7). These findings demonstrate that McfP also mediates chemotaxis to long-chain *n*-alcohols.

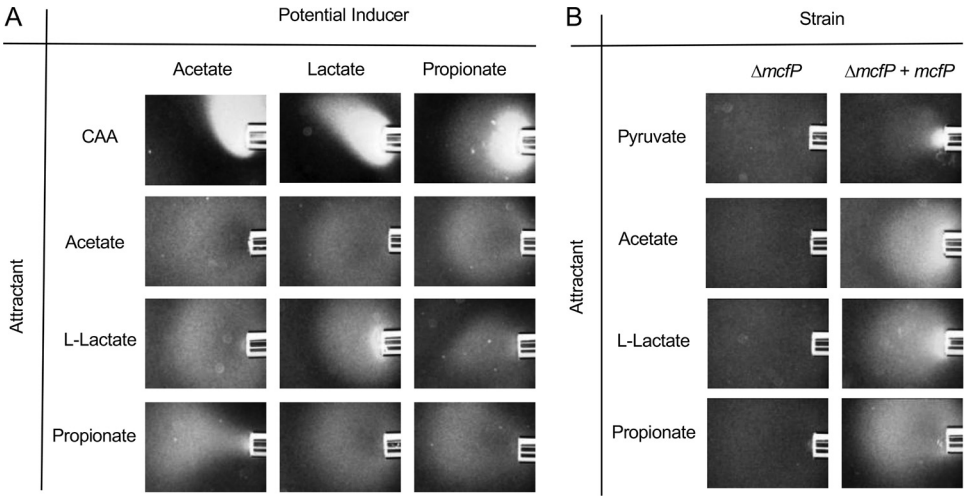

FIG 5 Chemotactic responses of wild-type *P. putida* F1 and the *mcfP* mutant to C_2 and C_3 carboxylic acids. (A) Responses of *P. putida* F1 to C_2 and C_3 carboxylic acids in qualitative capillary assays after growth in MSB containing 5 mM succinate plus 2.5 mM indicated carboxylic acid as a potential inducer of the chemotaxis response. (B) Responses of uninduced *mcfP* mutant and the complemented mutant to C_2 and C_3 carboxylic acids in qualitative capillary assays. Mutant strain XLF014(pRK415Km) lacking the receptor encoded by *mcfP* (Δ*mcfP*) and the complemented strain XLF014(pGCF114) (Δ*mcfP* + *mcfP*) were pregrown in MSB containing 20 mM pyruvate and 50 μg/ml kanamycin. Responses to 2% Casamino Acids (CAA; positive control) and 10 mM acetate, L-lactate, pyruvate, and propionate are shown as indicated. Photographs were taken after 5 min. No responses were seen to chemotaxis buffer (negative control; not shown).

Expression of *mcfP* is not induced in the presence of alcohols in *P. putida* F1. Although our results clearly show that alcohol chemotaxis requires McfP, they do not demonstrate whether alcohols are directly detected by McfP. Based on our findings that responses to L-lactate, propionate, and acetate do not require induction, whereas chemotaxis to *n*-alcohols requires induction in the presence of alcohols, we hypothesized that induction was necessary to allow the expression of genes for alcohol oxidation rather than for the chemotactic response. To rule out the possibility that the

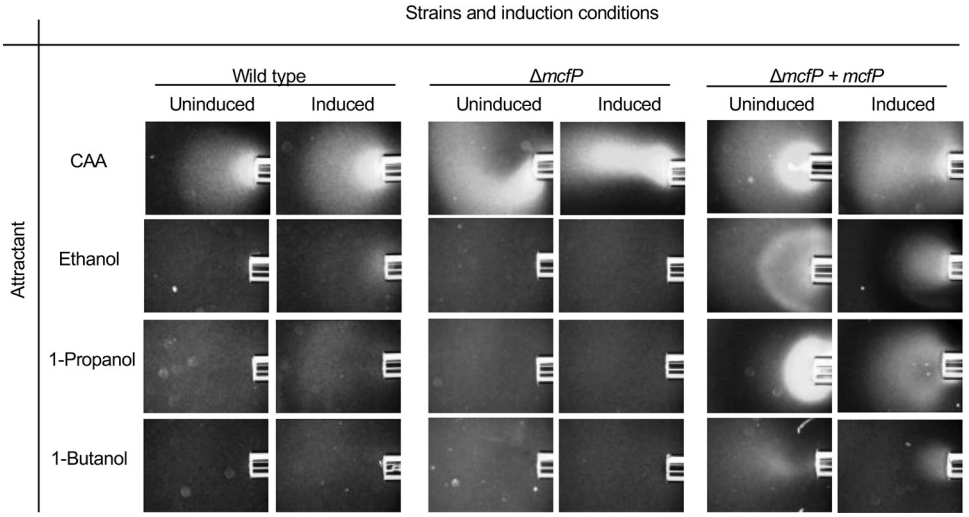

FIG 6 Responses of uninduced and ethanol-induced wild-type *P. putida* F1, the mutant strain lacking *mcfP*, and the complemented mutant to alcohols in qualitative capillary assays. F1(pRK415Km) (wild type), mutant XLF014(pRK415Km), (Δ*mcfP*), and the complemented strain XLF014(pGCF114) (Δ*mcfP* + *mcfP*) were pregrown in MSB containing 20 mM pyruvate (Uninduced) or 10 mM pyruvate plus 2.5 mM ethanol (Induced). Kanamycin (50 μg/ml) was included in all cultures. Responses to 2% Casamino Acids (CAA; positive control) and 10 mM ethanol, 1-propanol, and 1-butanol are shown. Photographs were taken after 5 min. No responses were seen to chemotaxis buffer (negative control; not shown).

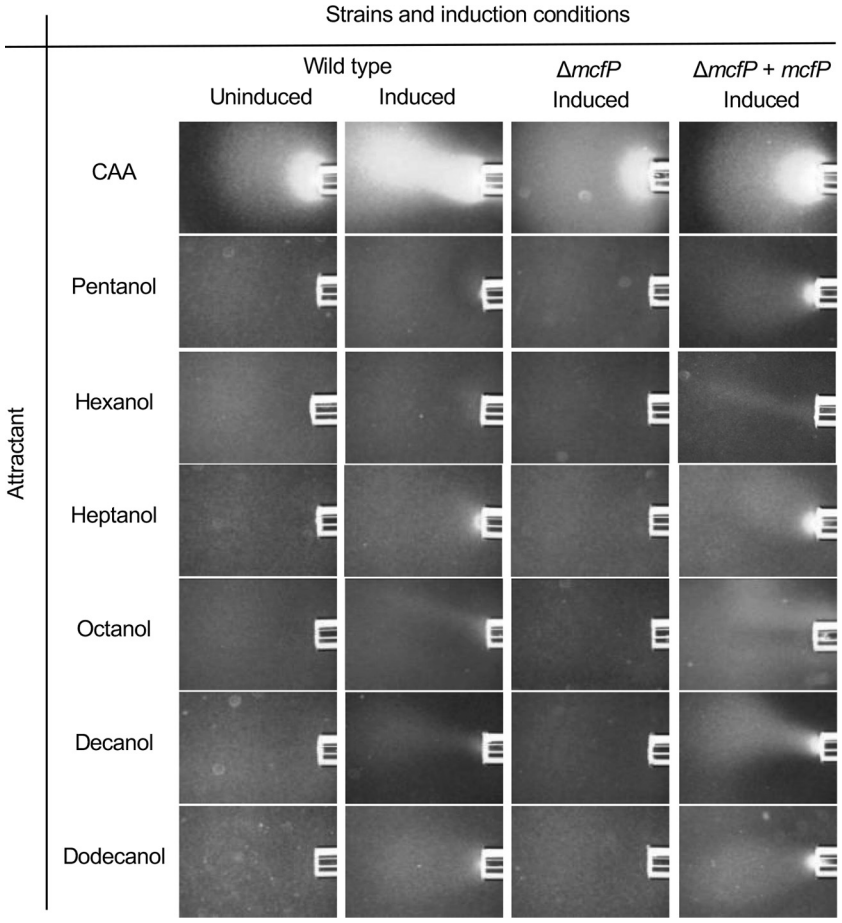

FIG 7 Responses of uninduced and induced *P. putida* wild type, Δ*mcfP* mutant, and the complemented mutant to longer-chain (C_5, C_6, C_7, C_8, C_{10}, and C_{12}) *n*-alcohols in qualitative capillary assays. F1(pRK415Km) (wild type), mutant XLF014(pRK415Km), lacking the receptor encoded by *mcfP* (Δ*mcfP*), and the complemented strain XLF014(pGCF114) (Δ*mcfP* + *mcfP*) were pregrown in MSB containing 20 mM pyruvate (Uninduced) or 10 mM pyruvate plus 2.5 mM alcohol (Induced; in each case, the same alcohol being tested as the attractant was used as the inducer). Kanamycin (50 μg/ml) was included in all cultures. Responses to 2% Casamino Acids (CAA; positive control) and 5 mM alcohol are shown. All of the induced cultures responded to CAA; only those induced by 1-pentanol are shown. Photographs were taken after 5 min. No responses were seen to chemotaxis buffer (negative control; not shown).

mcfP gene is induced in response to the presence of alcohols, we constructed an *mcfP-lacZ* transcriptional fusion in the plasmid pHRP309. *P. putida* F1 carrying the cloned *mcfP-lacZ* fusion was grown in MSB containing 20 mM pyruvate (uninduced) or 10 mM pyruvate plus 2.5 or 10 mM ethanol (induced). A significant change in the level of β-galactosidase activity in Miller units (uninduced, 191 $\pm$ 40) in response to 2.5 or 10 mM ethanol (111 $\pm$ 54 and 228 $\pm$ 22, respectively) was not detected, indicating that the expression of *mcfP* is not altered in the presence of ethanol.

If the response to alcohols is actually mediated via McfP binding to carboxylic acids produced during alcohol catabolism, *P. putida* F1 should grow on and be attracted to medium- and long-chain fatty acids. We showed that *P. putida* F1 could grow in MSB containing hexanoic acid (C_6) and dodecanoic acid (lauric acid; C_{12}) (data not shown), and chemotaxis to these compounds was tested. Induced cultures showed clear responses, and McfP was required for the response to both compounds (Fig. 8).

DISCUSSION

To date, most studies have reported that alcohols are sensed as repellents by bacteria and few receptors for alcohols have been identified (23–27, 33). In *E. coli*, Tar mediates chemoattraction to phenol, whereas Tsr, Trg, and Tap mediate repellent

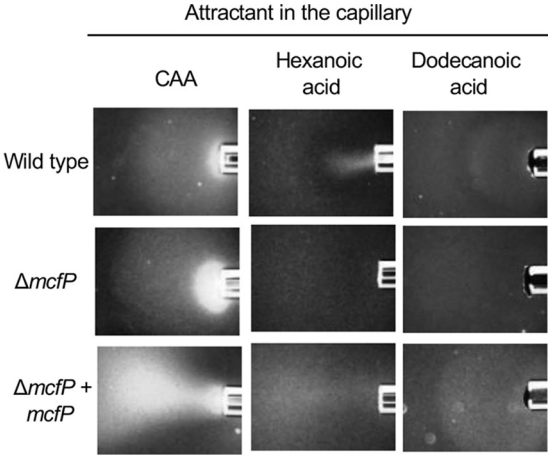

FIG 8 Responses of *P. putida* F1 wild type, the Δ*mcfP* mutant, and the complemented mutant to hexanoic (C_6) and dodecanoic (C_{12}) acids in qualitative capillary assays. F1(pRK415Km) (wild type), mutant XLF014(pRK415Km), lacking the receptor encoded by *mcfP* (Δ*mcfP*), and the complemented strain XLF014(pGCF114) (Δ*mcfP* + *mcfP*) were pregrown in MSB containing 10 mM pyruvate plus 2.5 mM hexanoic acid or MSB containing 5 mM dodecanoic acid. Kanamycin (50 μg/ml) was included in all cultures. Responses to 2% Casamino Acids (CAA; positive control) and 10 mM carboxylic acids are shown. All cultures responded to CAA; only those grown in the presence of 1-dodecanoic acid are shown. Photographs were taken after 5 min. No responses were seen to chemotaxis buffer (negative control; not shown).

responses to the same chemical (33, 34). Detailed analyses involving site-directed and random mutagenesis, as well as hybrid Tar-Tsr proteins, have indicated that phenol is likely sensed by diffusing into the cytoplasmic membrane and perturbing the transmembrane domains of MCPs rather than via direct binding to the periplasmic ligand binding domain (22). A similar mechanism may be involved in the repellent response of *Ralstonia pseudosolanacearum* Ps29 to various alcohols, including methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, 1,3-propanediol, and prenol, which was shown to be mediated by multiple MCPs (27).

In contrast, we report here that a single receptor in *P. putida* F1 mediates chemoattraction to *n*-alcohols of up to 12 carbons in length. McpP, the McfP ortholog in *P. putida* KT2440, is responsible for detection of C_2 and C_3 carboxylic acids, and direct binding of McpP to acetate, propionate, pyruvate, and L-lactate was demonstrated by isothermal titration calorimetry (32). Although linear alcohols and carboxylic acids are structurally similar and could potentially bind to the same receptor, several lines of evidence suggest that during catabolism, alcohols are oxidized to carboxylic acids, which then serve as the actual ligands for McfP. Our finding that only alcohols that serve as carbon and energy sources for *P. putida* F1 were capable of eliciting a chemotactic response is consistent with this idea and suggests that metabolism is required for the response. Energy taxis is a metabolism-dependent type of response (35), but we showed that the energy taxis receptors Aer1 and Aer2 were not required for the response. Another form of metabolism-dependent chemotaxis involves the detection of catabolic intermediates. Detection of catabolic intermediates rather than initial substrates has been observed previously in other soil bacteria. For example, chemotaxis toward aromatic compounds by *Comamonas testosteroni* strain CNB-1 is metabolism dependent; the actual ligands that bind the relevant MCP are TCA cycle intermediates produced during catabolism (36, 37). Similarly, a major portion of the response of *Acidovorax* sp. strain JS42 toward 2-nitrotoluene results from the detection of nitrite, which is produced during the oxidation of 2-nitrotoluene by 2-nitrotoluene 2,3-dioxygenase (38). In both cases, pathway mutants blocked at steps that prevent the formation of the relevant intermediates were incapable of responding to the initial aromatic substrates, although they were still capable of responding to the relevant intermediates directly (36, 38). Consistent with these findings, induction of the

2-nitrotoluene catabolic genes was shown to be required for the chemotactic response to 2-nitrotoluene (38).

The simplest explanation for the inducible response to alcohols in *P. putida* F1 would be that the gene encoding the chemoreceptor is induced in the presence of alcohols. There is a precedent for inducible MCP genes in *P. putida* F1; *pcaY*, which encodes the chemoreceptor for aromatic acids, is induced by β-ketoadipate, an intermediate in aromatic acid catabolism (16). However, we showed that the *mcfP* gene is not induced in the presence of alcohols, and we hypothesize that pregrowth in the presence of alcohols is necessary to induce genes for alcohol catabolism, which would allow the oxidation of alcohols to carboxylic acids. *P. putida* KT2440 grown in rich medium was capable of sensing C_2 and C_3 carboxylic acids (32), suggesting that *mcpP* is also constitutively expressed.

Genes and enzymes necessary for growth on alcohols have not been identified in *P. putida*, but alcohol catabolism has been studied in *P. aeruginosa*. *P. aeruginosa* catabolizes ethanol aerobically via a two-step oxidative pathway, yielding acetate. Ethanol is converted to acetaldehyde by the periplasmic quinoprotein ethanol dehydrogenase (ExaA), shuttling electrons to cytochrome c_{550} oxidase (ExaB). Acetaldehyde is then oxidized by the NAD^+-dependent aldehyde dehydrogenase (ExaC), yielding acetate and NADH. Similarly, the products of *laoABC* (encoding a flavin-containing oxidoreductase, a small protein of unknown function, and an aldehyde dehydrogenase, respectively) are involved in catabolism of long-chain alcohols like *n*-dodecanol in *P. aeruginosa* (8). Growth on *n*-dodecanol was completely eliminated only when both *exaA* and *laoA* were deleted, indicating that there is overlapping specificity of the two alcohol oxidation systems (8). The expression of both *exaABC* and *laoABC* in *P. aeruginosa* is regulated; transcription of *exaA* and *exaC* is the under the control of the two-component regulatory system encoded by *exaDE* (5), and the *laoABC* genes are under the control of the TetR family repressor LaoR (8). ExaD is a HAMP domain-containing integral membrane histidine kinase, and ExaE is a LuxR-type transcriptional activator. Analysis of the genome indicates that *P. putida* F1 has homologs of *exaABCDE* (*Pput_3083* and *Pput_3088* to *Pput_3092*) and *laoABCR* (*Pput_4993* to *Pput_4996*), as well as additional putative alcohol and aldehyde dehydrogenases. Some or all of the encoded proteins may be involved in alcohol catabolism, and it is likely that such genes are expressed in response to the presence of alcohols. Therefore, it is likely that induction of alcohol oxidation genes is required for the chemotactic response to alcohols, as the cells are actually sensing the carboxylic acids that are produced by the action of alcohol/aldehyde dehydrogenases. Future studies with *P. putida* mutants in which alcohol catabolism is blocked could provide further evidence for this hypothesis.

Consistent with the idea that McfP detects carboxylic acids generated during alcohol catabolism is the demonstration that *P. putida* F1 grows on and is chemotactic to hexanoic acid and dodecanoic acid, which are expected to be produced as intermediates during catabolism of hexanol and dodecanol. A standard β-oxidation pathway is present in *Pseudomonas putida* (39, 40) and is likely the main route responsible for conversion of fatty acids of various chain lengths to acetyl-coenzyme A (CoA) (and some propionyl-CoA in the case of fatty acids with an odd number of carbons). Medium- and long-chain carboxylic acids would therefore be converted to acetate (and some pyruvate in the case of fatty acids with an odd number of carbons); both acetate and pyruvate were shown to bind directly to the purified ligand binding domain (LBD) of McpP from *P. putida* KT2440 (32). The McpP LBD did not bind to butyrate, succinate, fumarate, malate, citrate, or malonate in isothermal titration calorimetry assays (32), suggesting that it is not responsible for directly detecting carboxylic acids with more than three carbons. In addition, the LBD of McpP from strain KT2440 did not bind to 1-propanol (32), which is consistent with our proposed metabolism-dependent mechanism of alcohol taxis in *P. putida* F1, in which acetate produced during the catabolism of alcohols is the primary metabolite detected by McfP.

TABLE 1 Bacterial strains used in this study

Strain	Relevant characteristic(s)[a]	Source or reference
E. coli strains		
DH5α	Cloning host	Life Technologies, Gaithersburg, MD
DH5α λ*pir*	Cloning host	William W. Metcalf
HB101	Host for mobilization plasmid pRK2013	41
P. putida strains		
F1	Wild type	51, 52
F1 *cheA*	F1 *cheA*::miniTn*5*; Km^r	13
RPF018 (F1Δ11)	F1 Δ*Pput_3489* Δ*Pput_4352* Δ*mcpC* Δ*mcfR* Δ*mcfQ* Δ*mcfS* Δ*Pput_0342* Δ*pcaY* Δ*Pput_4234* Δ*Pput_4764* Δ*Pput_3459*	54
XLF003	F1 Δ*Pput_0601*	This study
XLF006	F1 Δ*Pput_1257* (Δ*mcfU*)	54
XLF008	F1 Δ*Pput_1872*	This study
XLF009	F1 Δ*Pput_2091*	This study
XLF011	F1 Δ*Pput_2217*	This study
XLF014	F1 Δ*Pput_2828* (Δ*mcfP*)	54
XLF016	F1 Δ*aer1*	17
XLF018	F1 Δ*Pput_3621*	This study
XLF019	F1 Δ*aer2*	17
XLF020	F1 Δ*Pput_3892*	This study
XLF025	F1 Δ*Pput_4863*	This study
XLF027	F1 Δ*Pput_4895*	This study

[a]Km^r, kanamycin resistance.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2. *E. coli* strains were grown in lysogeny broth (LB) or on LB plates (41) at 37°C in the presence of 100 μg/ml kanamycin or 12 μg/ml gentamicin when appropriate. *P. putida* F1 strains were grown at 30°C in MSB medium (2) containing 10 mM succinate, 20 mM pyruvate, or a combination of 10 mM pyruvate and 2.5 mM ethanol, 1-propanol, 1-butanol, or another potential inducer as indicated in the text or figure legend. Kanamycin was added to final concentrations of 50 μg/ml for plasmid selection and maintenance in *P. putida*.

DNA manipulations. Genomic DNA was isolated using a 5′ ArchivePure DNA kit (5 Prime, Gaithersburg, MD), and plasmid DNA was isolated using commercial kits from Fermentas (Glen Burnie, MD). Manipulation of DNA fragments and plasmids and transformation of *E. coli* strains were carried out by standard methods (41). Restriction endonucleases were purchased from New England BioLabs (Beverly,

TABLE 2 Plasmids used in this study

Plasmid	Relevant characteristics[a]	Source or reference
pAW19	Suicide vector; *sacB*, Km^r, Ap^r	43
pGCF114	*Pput_2828* (*mcfP*) cloned in pRK415Km, Km^r	54
pHRP309	Broad-host-range *lacZ* transcriptional fusion vector, Gm^r	42
pHRP310	pK19 with Ω Sm^r/Sp^r cassette, Km^r	42
pJPF100	pHRP310 carrying the *mcfP* promoter	This study
pJPF101	pHRP309 carrying the *mcfP* promoter fused to *lacZ*	This study
pRK2013	ColE1 *ori*, RP4 mobilization function, Km^r	53
pXLF003	Gene *Pput_0601* upstream and downstream 1-kb PCR fragments fused and cloned into SpeI-SacI sites of pAW19, Ap^r, Km^r	This study
pXLF008	Gene *Pput_1872* upstream and downstream 1-kb PCR fragments fused and cloned into SpeI site of pAW19, Ap^r, Km^r	This study
pXLF009	Gene *Pput_2091* upstream and downstream 1-kb PCR fragments fused and cloned into SpeI-SacI sites of pAW19, Ap^r, Km^r	This study
pXLF011	Gene *Pput_2217* upstream and downstream 1-kb PCR fragments fused and cloned into SpeI-SacI sites of pAW19, Ap^r, Km^r	This study
pXLF018	*Gene Pput_3621* upstream and downstream 1-kb PCR fragments fused and cloned into SpeI site of pAW19, Ap^r, Km^r	This study
pXLF020	Gene *Pput_3892* upstream and downstream 1-kb PCR fragments fused and cloned into SpeI-SacI sites of pAW19, Ap^r, Km^r	This study
pXLF025	Gene *Pput_4863* upstream and downstream 1-kb PCR fragments fused and cloned into SpeI-SacI sites of pAW19, Ap^r, Km^r	This study
pXLF027	Gene *Pput_4895* upstream and downstream 1-kb PCR fragments fused and cloned into SpeI-SacI sites of pAW19, Ap^r, Km^r	This study

[a]Km^r, kanamycin resistance; Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Sm^r, streptomycin resistance; Sp^r, spectinomycin resistance.

TABLE 3 Primers used in this study[a]

Primer name	Sequence (5′–3′)
2828ProF	GGGGGTACCTTACAGCGACTGTTGGGCGC
2828ProR	GGGGGGAATTCATCCATCAGCTCCCGCATTG
0601 SpeI up-for	GGAGCTACTAGTGGTTGGCGACGTTGTTCAGG
0601 up-rev (p)	p-CGAGCATTCAGGGTCTGAGTCAGC
0601 dn-for (p)	p-CAGCAATAGGGTGTTGAAATTGGTCAGC
0601 SacI dn-rev	GTATCTGAGCTCTGATCATCCTCGACCTGTACATGCC
1872 SpeI up-for	AGGTCTACTAGTGATGATGTGTTCCGGGTCCAGTTCAC
1872 up-rev (p)	p-CGACATTATCTTCACCATACGCGACATCG
1872 dn-for (p)	p-GTAACGCACCTGTCCGGTCATCG
1872 SpeI dn-rev	GTGACGACTAGTTGCCTTGTACTGGGTGGACATTCC
2091 SpeI up-for	CCTGACACTAGTACCGCTTCATCCATATCCTCAACCGC
2091 up-rev (p)	p-ATGGTGGCGCAGTTCAAGGTTTGATC
2091 dn-for (p)	p-GGAATGCTCCTTGACGGTGGCG
2091 SacI dn-rev	GACTCTGAGCTCTTCGCCAACTCGCAATACCGTGG
2217 SpeI up-for	GGATGTACTAGTTCAGGCGATCGATGGGCAGG
2217 up-rev (p)	p-ATGGACCAGTTCCGCGTCTGAAGC
2217 dn-for (p)	p-GATGGAAAGTTGTCGCAAAGGCATGGG
2217 SacI dn-rev	GACTCAGAGCTCCAGCAAGGTCAGCGGGTTGTAC
3621 SpeI up-for	GCATGAACTAGTCCAATGTCGGCCACGGAAATCTGC
3621 up-rev (p)	p-TTGTCAGGGCAGTTGGGCGAAGTC
3621 dn-for (p)	p-TCGGAACCAGAGGGAGTAGGCTGAG
3621 SpeI dn-rev	GGAGTTACTAGTGAGGTGATCGGCGAACTGCAACG
3892 SpeI up-for	GGCGCGCCACTAGTCACACTAAGTTCGGCAAAGGG
3892 up-rev	CCGGCACAGGCAACGAGACGCCGATCCCCTTTCAGC
3892 dn-for	GCTGAAAGGGGATCGGCGTCTCGTTGCCTGTGCCGG
3892 SacI dn-rev	GTCATGGAGCTCTACATTACCTGTGCCTTGCTGG
4863 SpeI up-for	AGCTACACTAGTCGGACTCCAGGTAATCGC
4863 up-rev (p)	p-CCGCGTTAAGGAATAGTGACCATG
4863 dn-for (p)	p-GTTCGCAGGTCCTATAACGCCAC
4863 SacI dn-rev	CTGATCGAGCTCTCAACAACACGCACCCTGAC
4895 SpeI up-for	AGATGAACTAGTCCTCAACGCAGCAATCGAAGCC
4895 up-rev (p)	p-ATATTTCGTCCTGTGCCGTCCTC
4895 dn-for (p)	p-TTCAGCCTCTGATTTCGCCTCGC
4895 SacI dn-rev	CCTGTAGAGCTCATCGCCATCACCGAACTGACCAAG

[a]Underlined sequences indicate restriction sites. (p) or p, the primer was phosphorylated at the 5′ end.

MA). DNA fragments were purified by gel extraction using a Fermentas GeneJET gel extraction kit. All PCRs were carried out using *Pfu* polymerase in *Pfu* reaction buffer [200 mM Tris-Cl (pH 8.8), 100 mM $(NH_4)_2PO_4$, 100 mM KCl, 1% Triton X-100, 1 mg/ml bovine serum albumin, 20 mM Mg_2SO_4] under standard conditions (95°C denaturation, 55°C annealing, 72°C elongation, with an elongation time of 1 min/kb of PCR product). Sequences of cloned PCR products were verified by fluorescent automated DNA sequencing at the University of California Davis DNA Sequencing Facility with an Applied Biosystems 3730 automated sequencer.

Construction of the *mcfP-lacZ* fusion plasmid. To examine the regulation of *mcfP*, the promoter region upstream from the *mcfP* gene was amplified using primers 2828ProF and 2828ProR (Table 3) with *P. putida* F1 genomic DNA as the template. The resulting PCR fragment was digested and cloned into the KpnI and EcoRI restriction sites of the cohort vector pHRP310 (42), generating plasmid pJPF100. The plasmid containing the correct construct was verified by DNA sequence analysis and digested with XbaI and EcoRI, and the resulting fragment was cloned upstream from the promoterless *lacZ* gene in pHRP309 (42) to generate plasmid pJPF101.

Construction of the single MCP mutants and the F1Δ11 mutant RPF018. *P. putida* F1 MCP gene deletion mutants were constructed using the suicide vector pAW19 (43). The 1-kb regions upstream and downstream from each MCP gene were amplified by PCR using the primers listed in Table 3. The resulting PCR fragments were fused by either overlap extension PCR (44) or blunt-end ligation. Each product was further amplified by 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) and then inserted into the SpeI (or the SpeI and SacI) site(s) of pAW19. The resulting plasmids were introduced into *E. coli* DH5α λ*pir* and mated into *P. putida* F1 by conjugation using *E. coli* HB101(pRK2013) as a helper strain in triparental matings, as described previously (45). Kanamycin-resistant F1 exconjugants were selected and grown in MSB minimal medium containing 10 mM succinate. To select for deletion mutants that arose from double crossover events, cells were plated on MSB agar containing 10 mM succinate and 20% sucrose. Individual colonies were then screened for kanamycin sensitivity, and deletions were verified by PCR using appropriate primers.

β-Galactosidase enzyme assays. β-Galactosidase assays were carried out as previously described (46). Cells were grown to a final optical density at 660 nm (OD_{660}) of 0.45 to 0.55 in MSB containing 20 mM pyruvate (uninduced) or 20 mM pyruvate plus 2.5 or 10 mM ethanol (induced).

Qualitative capillary assays. Qualitative capillary assays were carried out as previously described (47). Cells were grown in MSB medium with the appropriate carbon source(s) as indicated and harvested

during mid-exponential phase (OD_{660} of 0.3 to 0.45). Cell pellets were washed and resuspended in chemotaxis buffer (CB; 50 mM potassium phosphate buffer [pH 7.0], 0.05% glycerol, 10 μM EDTA) to a final OD_{660} of approximately 0.1. Microcapillaries (1 μl) were filled with attractants dissolved in CB or CB alone (negative control) with 2% low-melting-temperature agarose (NuSieve GTG; Lonza, Rockwell, ME) and introduced into the cell suspensions. Chemotactic responses were viewed at room temperature for up to 15 min under ×40 total magnification on a Nikon Eclipse TE2000-S microscope (Melville, NY). Photographs were taken using an Evolution Micropublisher 3.3 RTV camera and Evolution MP/QImaging software (Media Cybernetics, Inc., Rockville, MD).

Chemical-in-plug assays. Chemotaxis responses were monitored using chemical-in-plug assays (48, 49). *P. putida* strains were grown under the indicated conditions (induced or uninduced) and harvested in mid-exponential phase (OD_{660} of 0.4 to 0.5). Cell pellets were resuspended in 2× CB, and suspensions were mixed with an equal volume of cooled molten 0.5% Noble agar and dispensed into 35-mm petri dishes. A 2% Noble agar plug containing the test attractant dissolved in CB (or CB only; negative control) was inserted in the center of the petri dish. Assay plates were incubated at room temperature (approximately 25°C) for 1 h. Chemotactic responses were observed as a distinct ring of cells around the plug, and photographs were taken with backlighting (50).

Soft agar swim plate assays. Plates contained MSB with 0.3% Noble agar and 5 mM ethanol. Cultures were grown overnight in MSB containing 10 mM succinate and were harvested, washed, and resuspended in MSB to an OD_{660} of approximately 0.4. Plates were inoculated by pipetting 2 μl of cell suspension into the soft agar and were incubated at 30°C for approximately 24 h. Photographs were taken with backlighting (50).

Gradient swim plate assays. Gradient plate assays were based on the Pham and Parkinson protocol (22). MSB soft agar plates contained 0.3% agar and 1 mM glycerol. Agar plugs contained 10 mM ethanol, 5 mM 1-propanol, or 5 mM 1-butanol, and after harvesting and resuspending in MSB as described above, cell suspensions (2 μl each) were inoculated 2 cm from the plugs. Photographs were taken with backlighting (50) after incubation at 30°C for ~24 h. The response index (RI) was calculated using the equation $RI = D_1/(D_1 + D_2)$, where D_1 is the distance measured from the site of inoculation to the colony edge closest to the plug and D_2 is the distance from the site of inoculation to the colony edge furthest from the plug. As reported previously (22), RI values greater than 0.52 were concluded to indicate an attractant response.

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