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Authors

Hughes, Jonathan G
Zhang, Xiangsheng
Parales, Juanito V
et al.

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Pseudomonas putida F1 uses energy taxis to sense hydroxycinnamic acids

Jonathan G. Hughes,[1] Xiangsheng Zhang,[1,2] Juanito V. Parales,[1] Jayna L. Ditty[3] and Rebecca E. Parales[1,*]

Abstract

Soil bacteria such as pseudomonads are widely studied due to their diverse metabolic capabilities, particularly the ability to degrade both naturally occurring and xenobiotic aromatic compounds. Chemotaxis, the directed movement of cells in response to chemical gradients, is common in motile soil bacteria and the wide range of chemicals detected often mirrors the metabolic diversity observed. *Pseudomonas putida* F1 is a soil isolate capable of chemotaxis toward, and degradation of, numerous aromatic compounds. We showed that *P. putida* F1 is capable of degrading members of a class of naturally occurring aromatic compounds known as hydroxycinnamic acids, which are components of lignin and are ubiquitous in the soil environment. We also demonstrated the ability of *P. putida* F1 to sense three hydroxycinnamic acids: *p*-coumaric, caffeic and ferulic acids. The chemotaxis response to hydroxycinnamic acids was induced during growth in the presence of hydroxycinnamic acids and was negatively regulated by HcaR, the repressor of the hydroxycinnamic acid catabolic genes. Chemotaxis to the three hydroxycinnamic acids was dependent on catabolism, as a mutant lacking the gene encoding feruloyl-CoA synthetase (Fcs), which catalyzes the first step in hydroxycinnamic acid degradation, was unable to respond chemotactically toward *p*-coumaric, caffeic, or ferulic acids. We tested whether an energy taxis mutant could detect hydroxycinnamic acids and determined that hydroxycinnamic acid sensing is mediated by the energy taxis receptor Aer2.

INTRODUCTION

Soil bacteria have been widely studied due to their diverse catabolic capabilities. In contrast to enteric organisms, which typically utilize sugars and amino acids as carbon and energy sources, soil bacteria are generally capable of using a much wider range of compounds, including aromatic compounds, which are ubiquitous in the soil environment. Motile soil bacteria are capable of using chemotaxis, directed movement in response to chemical gradients, to seek out sources of carbon and energy [1]. Paralleling their catabolic diversity, soil bacteria such as pseudomonads are able to detect a great number of different chemoattractants [2, 3]. This broad sensing ability is reflected by an average of 33 chemoreceptor-encoding genes in pseudomonad genomes [4], in contrast to the 5 receptors found in *E. coli* [5]. *Pseudomonas* species are particularly well adapted to growth on aromatic compounds and are also attracted to many of the aromatic compounds that they degrade [1–3].

Recent studies have examined the coordination of catabolism and chemotaxis toward a variety of aromatic compounds in *P. putida* F1, including aromatic acids and aromatic hydrocarbons [6–8]. Although they are widespread in nature, hydroxycinnamic acids constitute a class of aromatic acids that have rarely been investigated as possible bacterial chemoattractants. These derivatives of the aromatic amino acids phenylalanine and tyrosine have antimicrobial, antioxidant and antitumour activities [9]. Hydroxycinnamic acids are liberated during the biodegradation of lignin, and thus are ubiquitous in the environment and constitute major components of the plant–soil carbon cycle [10, 11]. Although many reports of bacterial growth on hydroxycinnamic acids have been published [12–19], to the best of our knowledge there has only been one report of chemotaxis to hydroxycinnamic acids [20]. The response to hydroxycinnamic acids by *Bradyrhizobium japonicum* was not characterized in detail, and the receptor involved was not identified. In this study, we demonstrated that *P. putida* F1 is capable of growth on and taxis toward the hydroxycinnamic acids, *p*-coumaric, caffeic and ferulic acids, and examined the connection between the chemotaxis and catabolism of these three compounds.

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Author affiliations: [1]Department of Microbiology and Molecular Genetics, College of Biological Sciences, University of California Davis, Davis, California, USA; [2]Jiangsu Provincial Key Laboratory of Coastal Wetland Bioresources and Environmental Protection, Yancheng Teachers University, Yancheng, PR China; [3]Department of Biology, College of Arts and Sciences, University of St Thomas, St Paul, MN, USA.

***Correspondence:** Rebecca E. Parales, reparales@ucdavis.edu

Keywords: chemotaxis; energy taxis; aromatic compounds; hydroxycinnamic acids; *Pseudomonas putida*; chemoreceptor.

Abbreviations: CB, chemotaxis buffer; Gm, gentamicin; Km, kanamycin; LB, lysogeny broth; MSB, minimal salts broth; Tc, tetracycline.

METHODS

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α λ*pir* and *E. coli* HST08, which are used for the cloning and propagation of plasmids, and *E. coli* HB101, which is used as a helper strain in triparental matings, were grown in lysogeny broth (LB) or on LB plates [21] at 37 °C, with the addition of 100 μg ml^{-1} kanamycin or 12 μg ml^{-1} gentamicin when appropriate. *P. putida* F1 and its derivatives were grown at 30° C in minimal medium (MSB [22]) or on MSB plates with the addition of 10 mM succinate, 20 mM pyruvate, or a combination of 10 mM pyruvate and 2.5 mM *p*-coumaric, ferulic or caffeic acid, as indicated. Kanamycin and gentamicin were added at 50 and 15 μg ml^{-1}, respectively, for plasmid selection and maintenance in *P. putida* strains.

DNA manipulations

The manipulation of DNA fragments and plasmids was carried out by standard methods [21]. *E. coli* strains were transformed with plasmids following standard procedures [21]. Genomic DNA was isolated using a 5′ ArchivePure DNA kit (5 Prime, Gaithersburg, MD, USA), and plasmid DNA was isolated using commercial kits from Fermentas (Glen Burnie, MD, USA). Restriction endonucleases were purchased from New England BioLabs (Beverly, MA, USA). DNA fragments were purified by gel extraction using a Fermentas Gene Jet gel extraction kit. All PCR reactions were carried out using *Pfu* high-fidelity DNA polymerase [*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^{-1} 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^{-1} of PCR product). The sequences of all the 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.

Plasmids were mobilized into *P. putida* F1 by conjugation using *E. coli* HB101 (pRK2013) as a helper strain in triparental matings, as described previously [23]. Exconjugants were selected on MSB plates containing 10 mM succinate and the appropriate antibiotic. Deletion mutants were created by allowing double-crossover events after matings and antibiotic selection as follows: cells were grown in MSB medium containing 10 mM succinate and 20 % sucrose, without antibiotics, and single colonies were screened for the loss of antibiotic resistance. Gene deletions were verified by PCR using appropriate primers (Table 2).

Generation of *fcs* and *hcaR* mutants and complementation plasmids

To construct the *fcs* (locus tag Pput_2402) deletion mutant, 1 kb stretches of DNA directly upstream and downstream of *fcs* were PCR-amplified using the primers pEX_2402_del_-For, Pput_2402_del_For, Pput_2402_del_Rev and

Table 1. Strains and plasmids used in this study

Strain, plasmid, or primer name	Relevant characteristics*	References
E. coli		
DH5α λ*pir*	Cloning host	[45]
HB101	Host for mobilization plasmid pRK2013	[21]
HST08	Cloning host	TaKaRa Bio Inc. Otsu, Shiga, Japan
P. putida		
F1	Wild-type	[46, 47]
F1 *cheA* :: Km	F1 *cheA* :: mini-Tn5; Km^r	[48]
JGH001	F1 Δ*fcs* (Pput_2402); hydroxycinnamic acids catabolic mutant	This study
JGH002	F1 Δ*hcaR* (Pput_2399); hydroxycinnamic acids catabolic pathway regulatory mutant	This study
XLF019	F1 Δ*aer2*; energy taxis mutant	[7]
XLF010	F1 Δ*pcaY*; aromatic acid chemoreceptor mutant	[8]
Plasmids		
pEX18Gm	*sacB* containing cloning vector; Gm^r	[49]
pJGH018	pRK415Km carrying *aer2*; Km^r	This study
pJGH019	pRK415Km carrying *fcs*; Km^r	This study
pJGH020	pRK415Km carrying *hcaR*; Km^r	This study
pJGH119	*fcs* deletion construct in pEX18Gm; Gm^r	This study
pJGH120	*hcaR* deletion construct in pEX18Gm; Gm^r	This study
pRK2013	ColE1 *ori*, RP4 mobilization function; Km^r	[50]
pRK415Km	Broad host range cloning vector; Km^r	[7]
pXLF219	pRK415 carrying the *aer2* gene from *P. putida* F1; Tc^r	[7]

*Km^R, kanamycin resistance; Gm^R, gentamicin resistance; Tc^R, tetracycline resistance.

Table 2. Oligonucleotide primers used in this study

Primer name	Sequence (5′–3′)*
Pput_2399_HindIII_For	CGCCAAGCTTACCCATGATCGAGGAGCATGAACC
Pput_2399_SacI_Rev	ATTCGAGCTCCTCGCCTCTGGGTTGATTTGCC
pEX_2399_del_For	CGACGGCCAGTGCCAAGCTTCACGCAGCTCGGCCAGCGGCACACTG
Pput_2399_del_For	GGAACCTCACTAGCCGAATCGCGGACTGACTCAGTCAACCCAGCGTGG
Pput_2399_del_Rev	CCACGCTGGGTTGACTGAGTCAGTCCGCGATTCGGCTAGTGAGGTTCC
Pput_2402_BamHI_For	CGCCGGATCCCCATCTAGCCGCGGCAGGCCC
Pput_2402_SacI_Rev	ATTCGAGCTCGCCGCACTCAAGGCCGCACCTTGG
pEX_2402_del_For	CTGCAGGTCGACTCTAGAGGATCCGCTGGTGCTCGACGATGCCGACCTGG
Pput_2402_del_For	CCAGGCCACTGGCAAGATGACCGCCGCCACGCATTGCACGCCAAGGTGCGGC
Pput_2402_del_Rev	GCCGCACCTTGGCGTGCAATGCGTGGCGGCGGTCATCTTGCCAGTGGCCTGG
pEX_2402_del_Rev	GCTATGACCATGATTACGAATTCCCAGTGCGGCGGCATCGGTAGGCCTC

*Restriction sites are underlined.

pEX_2402_del_Rev (Table 2), gel-purified and directionally cloned into the BamHI and EcoRI sites of pEX18Gm using an In-Fusion HD cloning kit (Clonetech, Mountain View, CA, USA). Exconjugants were isolated and verified as described above. To complement the mutant, the wild-type *fcs* gene was amplified by PCR using the primers Pput_2402_BamHI_For and Pput_2402_SacI_Rev (Table 2), and directionally inserted into the BamHI and SacI sites of pRK415Km [7] to generate the plasmid pJGH019. To construct the *hcaR* (locus tag Pput_2399) deletion mutant, 1 kb stretches of DNA directly upstream and downstream of *hcaR* were PCR-amplified using the primers pEX_2399_-del_For, Pput_2399_del_For, Pput_2399_del_Rev and pEX_2399_del_Rev (Table 2), gel-purified and directionally cloned into the HindIII and EcoRI sites of pEX18Gm. To complement the mutant, the wild-type *hcaR* gene was amplified by PCR using the primers Pput_2399_HindIII_-For and Pput_2399_SacI_Rev (Table 2), and directionally inserted into the HindIII and SacI sites of pRK415Km to generate the plasmid pJGH020.

Generation of the *aer2* complementation plasmid

To construct a plasmid to complement the *aer2* in-frame deletion mutant XLF019 [7], wild-type *aer2* from *P. putida* F1 was excised from pXLF219 [7] using the restriction enzymes BamHI and HindIII and ligated into dephosphorylated BamHI/HindIII-digested pRK415Km. The resulting plasmid, pJGH018, was used to transform *E. coli* DH5α and was introduced into *P. putida* XLF019 by triparental mating with *E. coli* HB101 (pRK2013) as previously described [21].

Soft agar swim plate assays

Chemotaxis was measured in MSB soft agar plates. Plates contained 0.3 % Noble agar and 1 mM caffeic acid, 2 mM *p*-coumaric acid, or 2 mM *p*-ferulic acid. When necessary, kanamycin was supplied at 50 µg ml^{-1}. Cultures were grown overnight in MSB with 5 mM *p*-coumaric acid (and 50 µg ml^{-1} kanamycin where necessary). Cultures were washed and resuspended in MSB to an OD_{660} of approximately 0.4. The plates were inoculated by pipetting 2 µl of cell suspension into the soft agar and incubated at 30° C for approximately 24 h. Positive chemotaxis responses, illustrated as isotropic growth outward from the point inoculation, were backlit [24] for diameter measurement and photography using a Canon EOS T2i camera.

Chemical-in-plug assays

The chemotaxis responses were also monitored via chemical-in-plug assays, which were performed according to the method of Storch *et al.* [25, 26]. *P. putida* strains were harvested in the mid-exponential phase (OD_{660} 0.4–0.5) and pellets were washed and resuspended in 2× chemotaxis buffer [CB; 50 mM potassium phosphate buffer (pH 7.0), 0.05 % v/v glycerol, 10 µM EDTA]. Cell suspensions were mixed with an equal volume of cooled molten 0.5 % Noble agar and dispensed into Petri dishes. Before the soft agar cell suspension was allowed to solidify, a 2 % agar plug containing CB only or an attractant dissolved in CB was inserted in the centre of the Petri dish. The assay plates were incubated at room temperature (approx. 25 °C) for 1 h. Positive chemotaxis resulted in the formation of a distinct ring of cells around the plug, which was imaged with backlighting [24].

Gradient swim plate assays

Gradient swim plate assays were performed as previously described [7, 27]. Agar plugs (2 % agar) containing attractants were placed on the surface of MSB plates containing 0.3 % soft agar, 1 mM glycerol and 50 µg ml^{-1} kanamycin, where necessary. The cultures were prepared as described for the swim plate assay and 2 µl of cell suspension was inoculated 2.0 cm from the centre of the 2 % agar plug. The plates were incubated at 30° C for 24 h and visualized with backlighting [24]. Positive chemotaxis was exhibited by asymmetric growth of the resulting colonies, skewed in the direction of the agar plug. Gradient plates were quantified using the method of Pham and Parkinson [27], i.e. measuring both the near and far radii of the colonies and generating a response index (RI) using the following equation:

$$RI = \frac{R_{near}}{R_{near}+R_{far}},$$

where R_{near} is the colony radius nearest the plug, and R_{far} is the radius farthest from the plug. An RI=0.50 denotes a neutral response, whereas an RI≥0.53 was deemed to be a positive chemotaxis response and an RI≤0.47 was categorized as a repellent response [27]. Significant differences were determined using Student's *t*-test.

RESULTS

Identification of genes for hydroxycinnamic acid catabolism in *P. putida* F1

The genes *fcs*, *ech* and *vdh*, encoding feruloyl-CoA synthetase, enoyl-CoA hydratase/aldolase and vanillin dehydrogenase were shown to be required for ferulic acid catabolism in *P. putida* strains KT2440 [28] and CSV86 [29]. The same three genes were predicted to be required for *p*-coumaric and caffeic acid catabolism in strain KT2440 [30] (Fig. 1). The complete genome of *P. putida* F1 has been sequenced (IMG JGI Taxon ID 640427132), and a BLAST search revealed homologues of the *P. putida* KT2440 *ech*, *fcs* and *vdh* genes (locus tags Pput_2400-Pput_2402) in a single cluster. The deduced amino acid sequences for the *P. putida* F1 *ech*, *fcs* and *vdh* were 100, 99 (two amino acid differences) and 99 % (three amino acid differences) identical to the *P. putida* KT2440 orthologues, respectively. A MarR-type repressor gene (locus tag Pput_2399) was located upstream of and divergently transcribed from the *ech–vdh–fcs* cluster in the genome of *P. putida* F1 (Fig. 1).

To confirm whether the first step in the catabolism of all three hydroxycinnamic acids is catalyzed by feruloyl-CoA synthetase, which has also been referred to as ferulate-CoA ligase [13], we deleted the *fcs* gene (locus tag Pput_2402) from the *P. putida* F1 chromosome, generating strain JGH001. This mutant was unable to grow on *p*-coumaric, caffeic, or ferulic acid as sole sources of carbon and energy. The ability of the Δ*fcs* mutant to grow on all three compounds was restored when the *fcs* gene was provided *in trans* on plasmid pJGH019 (data not shown). These findings indicate that *fcs* encodes a hydroxycinnamoyl-CoA ligase that is essential for the catabolism of hydroxycinnamic acids in *P. putida* F1.

The catabolism of hydroxycinnamic acids is regulated in a variety of organisms by MarR-type repressors, which have been variously designated FerR, CouR, or HcaR [31–34]. To test whether the divergently transcribed MarR-type regulator (designated here as HcaR; Fig. 1) functions to control expression of the *ech-vdh-fcs* cluster in *P. putida* F1, we generated a deletion derivative of *P. putida* F1 lacking the gene at locus tag Pput_2399 and found that the mutant strain (JGH002) grew on *p*-coumaric acid at a rate equivalent to that of the wild type, but with a reduced lag time compared to the wild type when transferred from uninduced conditions (growth on pyruvate) to medium containing *p*-coumaric acid (data not shown), which is consistent with this regulatory protein functioning as a repressor. In contrast, the complemented strain JGH002 (pJGH020) exhibited an increased lag time during growth on *p*-coumaric acid following growth on pyruvate, most likely due to the increased copy number of the *hcaR* gene, which would result in stronger repression of the hydroxycinnamic acid catabolic genes.

P. putida F1 is chemotactic to *p*-coumaric, caffeic and ferulic acids, and the responses are inducible and require a functional CheA

The chemotaxis responses of wild-type *P. putida* F1 to *p*-coumaric, caffeic and ferulic acids were initially tested using swim plate assays. Wild-type *P. putida* F1 grew on, and responded positively to, all three hydroxycinnamic acids, forming large spreading colonies in response to the gradient generated by catabolism of the respective substrate. In contrast, the generally nonchemotactic mutant strain F1 *cheA*:: Km was unable to move away from the point of inoculation (Fig. 2a), indicating that the classical chemotaxis signal transduction pathway is mediating the response. Chemical-in-plug assays were then used to test whether the responses of *P. putida* F1 to hydroxycinnamic acids are inducible. Prior to the assays, wild-type strain F1 was grown in minimal medium with pyruvate (uninduced) or pyruvate plus the test attractant (induced). The responses to *p*-coumaric, caffeic and ferulic acids were only observed for induced cultures (Fig. 2b). Uninduced cells that had been pre-grown in minimal medium containing pyruvate alone did not respond to any of the hydroxycinnamic acids in chemical-in-plug assays, but were attracted to the positive control succinate (Fig. 2b; data not shown). These results indicate that the chemotaxis response to hydroxycinnamic acids is inducible by growth in the presence of hydroxycinnamic acids.

The chemotaxis response of *P. putida* F1 to hydroxycinnamic acids is not mediated by the aromatic acid chemoreceptor PcaY

Previous work in our laboratory identified the methyl-accepting chemotaxis protein PcaY as the chemoreceptor for aromatic acids (benzoate, 4-hydroxybenzoate, protocatechuate and vanillate), as well as the hydroaromatic compounds quinate and shikimate, all of which are catabolized via the β-ketoadipate pathway [8]. Because *p*-coumaric, caffeic and ferulic acids are structurally similar to substituted benzoates, and because their metabolites feed into the same catabolic pathway, these compounds might also be sensed by PcaY. The responses of the *P. putida* F1 Δ*pcaY* mutant (XLF010) to hydroxycinnamic acids, however, was similar to that of the wild type, as judged by chemical-in-plug assays (Fig. 3), indicating that a different receptor is involved in their detection.

The chemotaxis response to hydroxycinnamic acids is metabolism-dependent and coordinately controlled with hydroxycinnamic acid catabolism

To test whether metabolism is required for the chemotaxis response to hydroxycinnamic acids, the responses of the Δ*fcs* mutant JGH001, which is unable to catabolize hydroxycinnamic acids, were analysed using chemical-in-plug

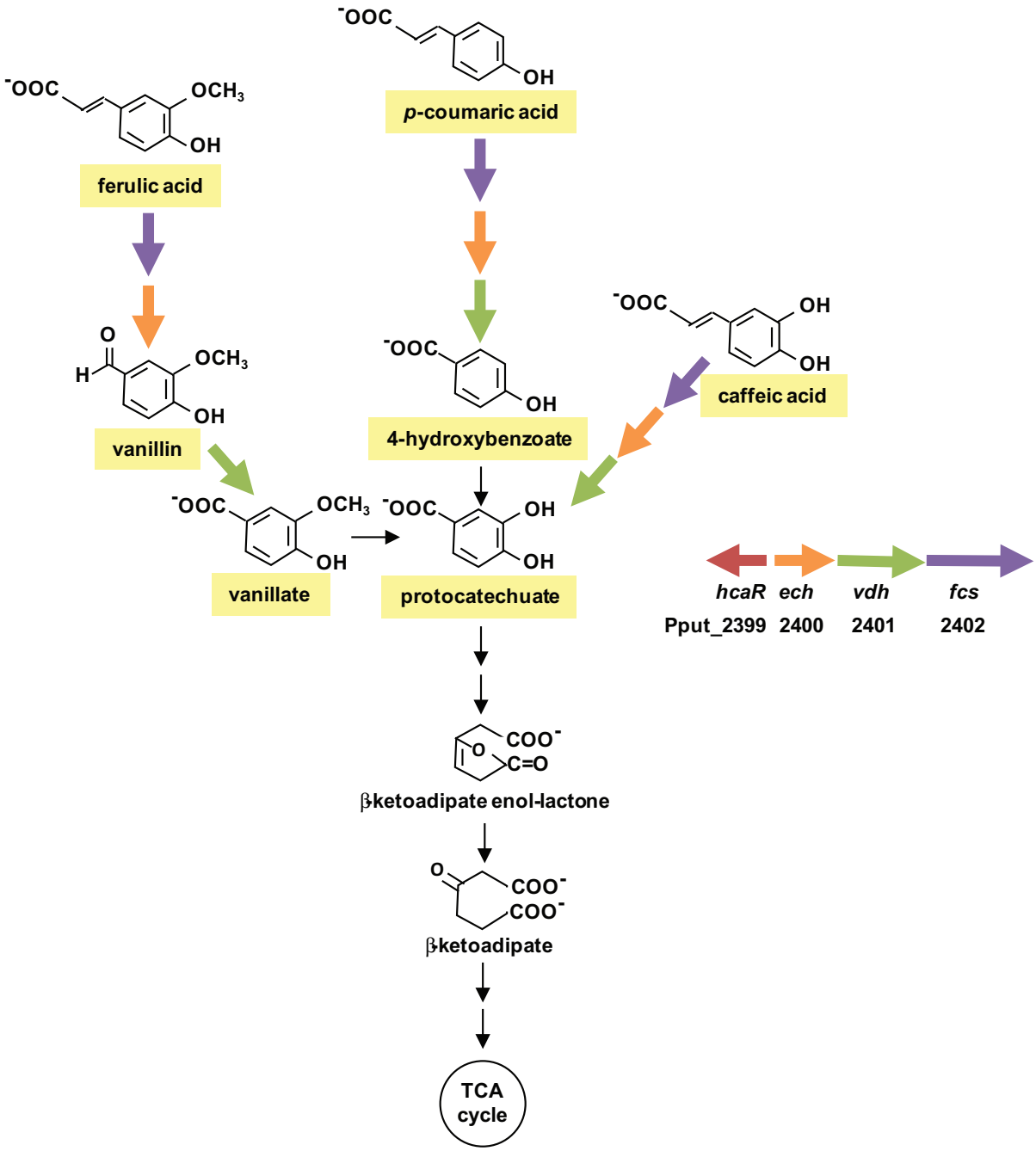

Fig. 1. Catabolic pathway and gene organization for hydroxycinnamic acid degradation in *P. putida* F1. *fcs* encodes feruloyl-CoA synthetase, *ech* encodes an enoyl-CoA hydratase/aldolase and *vdh* encodes vanillin dehydrogenase. The three genes are located in a cluster with a *marR*-type repressor gene (*hcaR*) located upstream and divergently transcribed. The gene names and corresponding locus tag numbers are indicated, and the correspondingly coloured arrows in the pathway indicate the reactions catalyzed by each enzyme. The intermediates in hydroxycinnamic acid degradation are further degraded, as indicated, via the β-ketoadipate pathway. The intermediates vanillin, vanillate, 4-hydroxybenzoate and protocatechuate are sensed by the methyl-accepting chemotaxis protein PcaY [8]. The expression of *pcaY* is coordinately controlled with catabolic genes for the conversion of protocatechuate to TCA cycle intermediates by the transcriptional activator PcaR when sufficient β-ketoadipate accumulates [8].

assays. In contrast to the wild type, when the Δ*fcs* mutant JGH001 was grown on pyruvate in the presence of *p*-coumaric acid, it failed to accumulate in a ring surrounding the plug containing any of the hydroxycinnamic acids in chemical-in-plug assays, although the response to the positive control succinate was indistinguishable from that of the

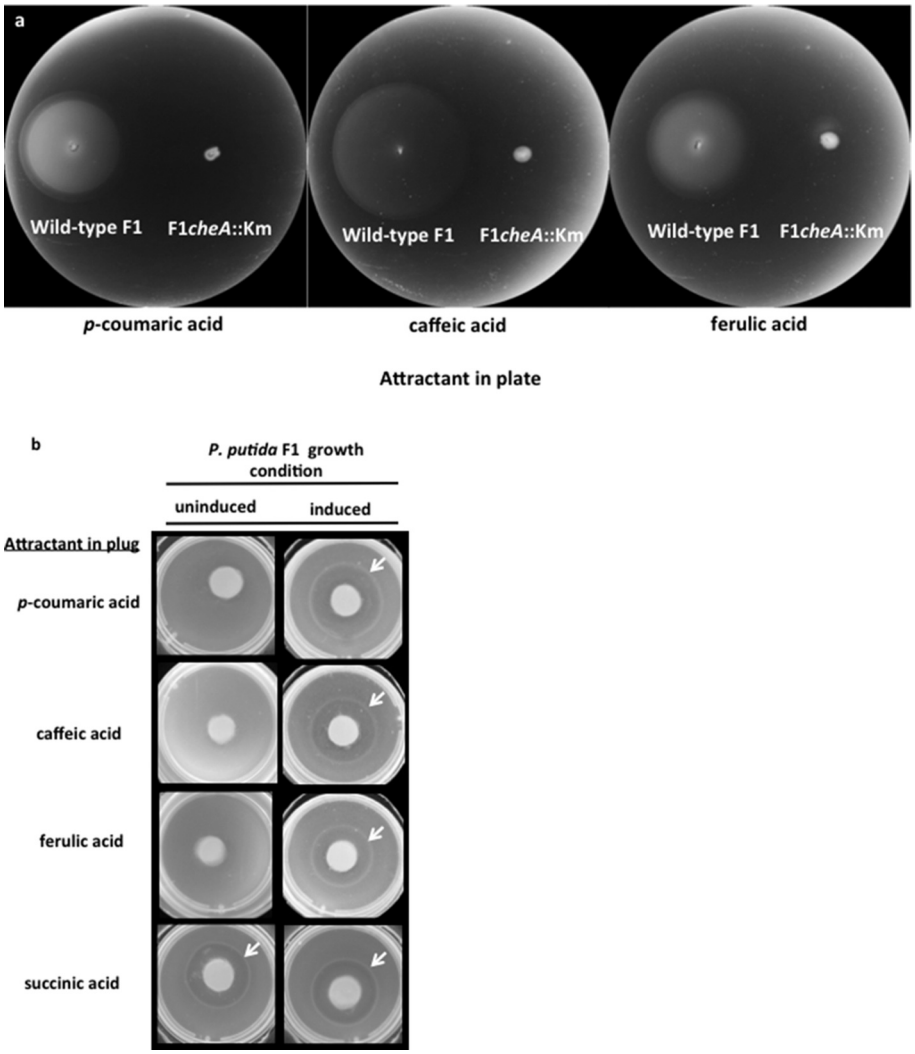

Fig. 2. Chemotaxis of *P. putida* F1 and its derivatives to hydroxycinnamic acids. (a) Responses of wild-type *P. putida* F1 and the generally nonchemotactic mutant F1 *cheA :: km* to *p*-coumaric, caffeic and ferulic acids in soft agar swim plates. The plates contained minimal medium with 0.3 % Noble agar and 2 mM *p*-coumaric acid, 1 mM caffeic acid, or 2 mM ferulic acid, concentrations that resulted in optimal responses. Photographs were taken after the plates had been incubated for ~24 h at 30 °C. (b) Response of *P. putida* F1 to *p*-coumaric, caffeic and ferulic acids in chemical-in-plug assays. The cells were pregrown in minimal medium containing 10 mM pyruvate (uninduced) or 10 mM pyruvate +2.5 mM of the respective attractant (induced) for each set of assays. The plugs contained 10 mM *p*-coumaric, caffeic, or ferulic acid. Photographs were taken after 1 h at room temperature. The responses of uninduced and *p*-coumaric acid-induced cultures of *P. putida* F1 to 10 mM succinate are shown for comparison; similar results were seen with caffeic acid-induced and ferulic acid-induced cultures (not shown). Positive responses are indicated by a white ring of cells collecting around the attractant plug (white arrows). No chemotactic rings were observed in response to control plugs lacking added attractant (not shown).

wild-type (Fig. 4a). The responses were restored in a complemented strain expressing *fcs* from a plasmid (Fig. 4a).

To confirm these results and definitively demonstrate that hydroxycinnamic acids are being sensed as attractants by wild-type cells, gradient plate assays were carried out. The colony growth of wild-type strain F1 was skewed toward the hydroxycinnamic acid-containing plugs, indicating positive chemotaxis responses (Fig. 4b and Table 3). In contrast, the Δ*fcs* mutant JGH001 did not respond positively to *p*-coumaric, caffeic, or ferulic acids. In fact, the colonies of JGH001 grew away from the plugs containing caffeic and ferulic acids (Fig. 4b and Table 3), indicating a weak repellent response (RI of JGH001 for ferulic and caffeic acid <0.47). Expression of the *fcs* gene *in trans* from a plasmid complemented the mutant phenotype, allowing JGH001(pJGH019) to positively respond to all three hydroxycinnamic acids (Fig. 4b and Table 3).

To examine whether the repressor of the catabolic genes also represses chemotaxis to hydroxycinnamic acids, we carried out chemical-in-plug assays with the uninduced wild

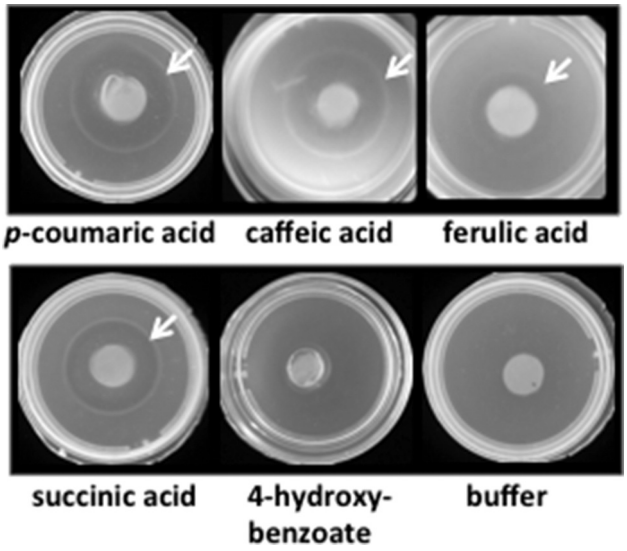

Fig. 3. Response of the F1Δ*pcaY* mutant XLF010 to *p*-coumaric, caffeic and ferulic acids in chemical-in-plug assays. Cultures were pregrown in minimal medium containing 10 mM pyruvate + 2.5 mM *p*-coumaric acid for assays with *p*-coumaric and caffeic acids, and with 10 mM pyruvate + 2.5 mM ferulic acid for assays with ferulic acid. The plugs contained 10 mM *p*-coumaric, caffeic, or ferulic acid. The responses to 10 mM succinate (positive control) and buffer only or 10 mM 4-hydroxybenzoate (negative controls) are also shown. Photographs were taken after 1 h at room temperature. Positive responses are indicated by a white ring of cells collecting around the attractant plug (white arrows).

type and the regulatory mutant. Unlike the wild-type *P. putida* F1, strain JGH002 (Δ*hcaR*) exhibited chemotaxis toward *p*-coumaric, caffeic and ferulic acids following growth on pyruvate alone (Fig. 5), indicating that HcaR represses the chemotaxis response to hydroxycinnamic acids. The Δ*hcaR* mutant phenotype was complemented by expression of the wild-type *hcaR* gene from a plasmid (pJGH020). JGH002 (pJGH020) did not respond to hydroxycinnamic acids in chemical-in-plug assays when cells were pre-grown with pyruvate, but responded positively after growth in the presence of *p*-coumaric acid (Fig. 5). These findings are consistent with a metabolism-dependent response in which hydroxycinnamic acids are not detected directly. Rather, the catabolism of hydroxycinnamic acids appears to be necessary to generate one or more intermediates that serve as the attractant, or the response may be mediated via energy taxis.

Chemotaxis to hydroxycinnamic acids is mediated by the energy taxis receptor Aer2

The gene *aer2* encodes the primary receptor for energy taxis in *P. putida* F1 [7]. To test whether the response to hydroxycinnamic acids is a form of energy taxis, we tested the response of an *aer2* deletion mutant XLF019 using swim plate and gradient plate assays. Strain XLF019 formed colonies that were significantly smaller than those of wild-type

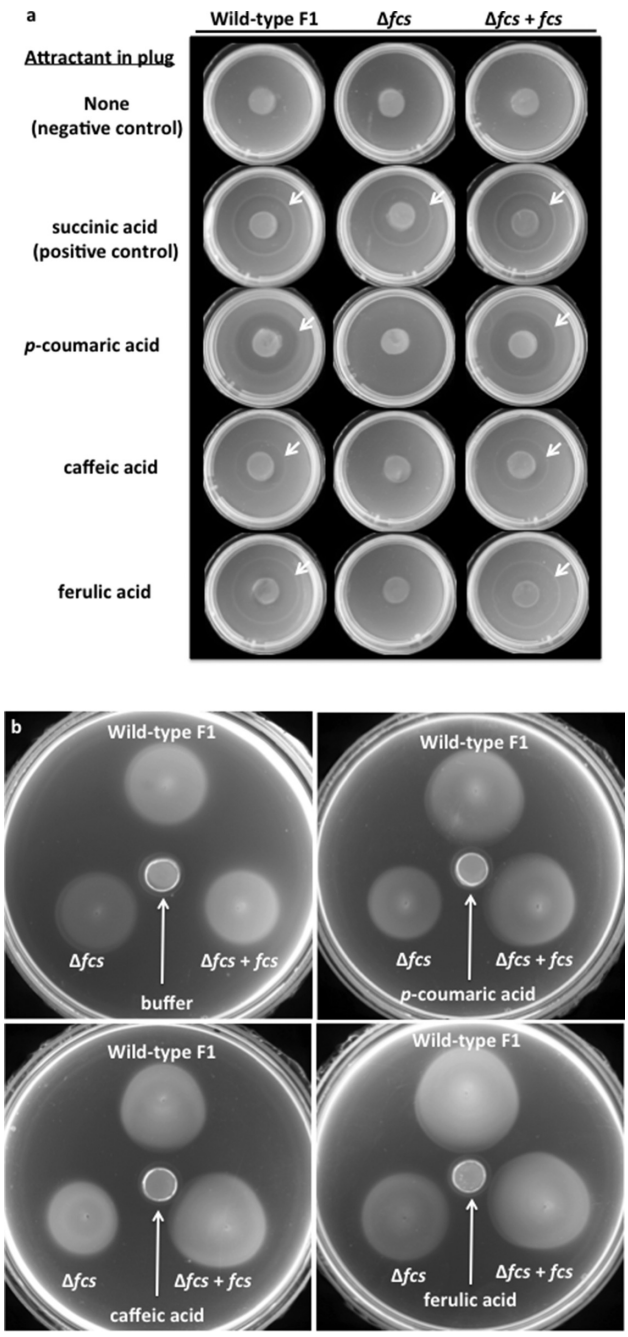

Fig. 4. Responses of wild-type *P. putida* F1 (pRK415Km), the Δ*fcs* mutant JGH001(pRK415Km) and the complemented Δ*fcs* mutant JGH001(pJGH019) (Δ*fcs+fcs*) to *p*-coumaric, caffeic and ferulic acids. (a) Chemical-in-plug assays. Strains were pregrown in minimal medium containing pyruvate plus 2.5 mM *p*-coumaric acid and 50 µg ml^{-1} kanamycin. The plugs contained 10 mM *p*-coumaric, caffeic, or ferulic acid. Positive responses are indicated by a white ring of cells collecting around the attractant plug (white arrows). Photographs were taken after 1 h at room temperature. (b) Gradient plate assays. The plates contained 1 mM glycerol and the plugs contained buffer only or 10 mM caffeic acid, 20 mM *p*-coumaric or 20 mM ferulic acid. Photographs were taken after plates had been incubated for ~24 h at 30 °C.

Table 3. Response indices (RI) for gradient plate assays

Wild-type F1(pRK415Km), the Δ*fcs* mutant JGH001(pRK415Km) and its complement (carrying pJGH019), and the energy taxis mutant XLF019 (pRK415Km) and its complement (carrying pJGH018) in gradient plate assays. Hydroxycinnamic acids were supplied at 20 mM. The results are the averages of at least five assays from two independent experiments.

Attractant	F1 (pRK415Km) wild type	JGH001 (pRK415Km) Δ*fcs*	JGH001 (pJGH019) Δ*fcs*+*fcs*	XLF019 (pRK415Km) Δ*aer2*	JFH002 (pJGH018) Δ*aer2*+*aer2*
None	0.49±0.03	0.49±0.01	0.48±0.03	0.48±0.01	0.48±0.01
p-coumaric acid	0.58±0.01	0.49±0.02*	0.58±0.03†	0.51±0.01*	0.57±0.03†
Caffeic acid	0.58±0.03	0.39±0.0*	0.57±0.03†	0.49±0.01*	0.55±0.03†
Ferulic acid	0.56±0.02	0.48±0.02*	0.56±0.05†	0.51±0.01*	0.56±0.03†

*Significantly different from wild-type F1 (P<0.05).

†Significantly different from the mutant strain (P<0.05).

strain F1 in swim plates containing *p*-coumaric, caffeic, or ferulic acid, whereas the complemented strain XLF019 (pJGH018) formed colonies with sizes similar to those of the wild-type (Fig. 6a, b). Furthermore, XLF019 formed uniformly circular colonies in gradient plate assays with plugs containing *p*-coumaric, ferulic, or caffeic acids, indicating there was no directed movement toward the plugs. However both wild-type F1 and the complemented Δ*aer2* mutant XLF019(pJGH018) formed oblong colonies skewed in the direction of plugs containing *p*-coumaric, caffeic and ferulic acids (Fig. 6c and Table 3).

DISCUSSION

Previous studies have identified other aromatic compounds that are sensed via energy taxis. In *P. putida*, for example, the energy taxis receptor Aer2 is also responsible for the metabolism-dependent detection of phenylacetic acid [7] and (methyl)phenols [35]). In contrast, *Comamonas testosteroni* CNB-1 uses a different type of metabolism-dependent chemotaxis mechanism to indirectly sense a wide array of aromatic acids via methyl-accepting chemotaxis proteins that bind to intermediates of the TCA cycle generated during the catabolism of aromatic acids [36, 37]. In some cases, multiple taxis mechanisms are used simultaneously. *Acidovorax* sp. strain JS42 senses 2-nitrotoluene directly via a metabolism-independent mechanism, and also uses metabolism-dependent taxis to sense the energy it obtains during growth on 2-nitrotoluene via the Aer receptor. In addition, *Acidovorax* sp. strain JS42 has multiple receptors that sense the nitrite that is released during 2-nitrotolune degradation [38]. In contrast, single methyl-accepting chemotaxis proteins are responsible for metabolism-independent detection of naphthalene in *P. putida* G7 [39] and 2-nitrobenzoic acid in *Pseudomonas fluorescens* KU-7 [40].

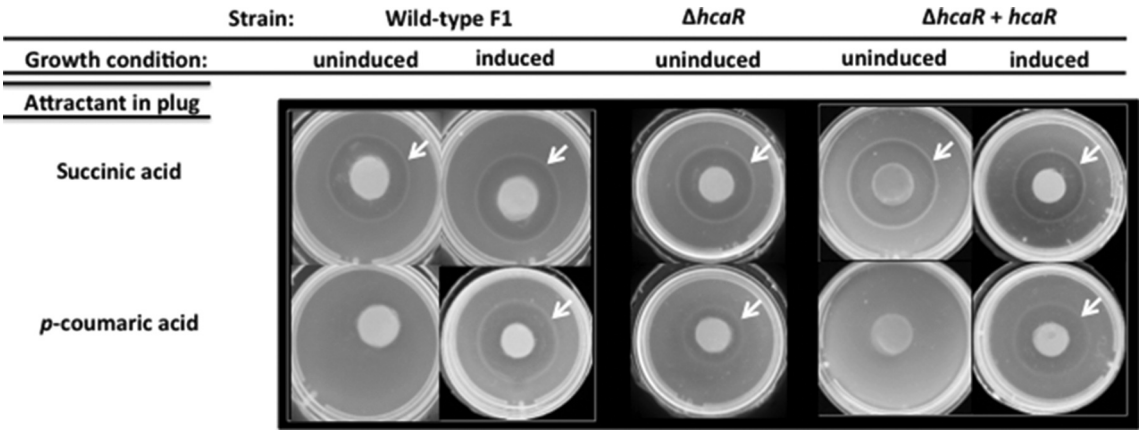

Fig. 5. Responses of wild-type *P. putida* F1 (pRK415Km), the F1 Δ*hcaR* mutant JGH002(pRK415Km) and the complemented mutant JGH002 (pJGH020) (Δ*hcaR*+*hcaR*) to 10 mM *p*-coumaric acid in chemical-in-plug assays. Cells were pregrown in minimal medium containing 50 μg ml^{-1} kanamycin and 20 mM pyruvate (uninduced) or 10 mM pyruvate plus 2.5 mM *p*-coumaric acid (induced). The responses to the positive control attractant, 10 mM succinate, are also shown. Positive responses are indicated by a white ring of cells collecting around the attractant plug (white arrows). Photographs were taken after 1 h at room temperature. Similar results were obtained with caffeic acid and ferulic acid (data not shown).

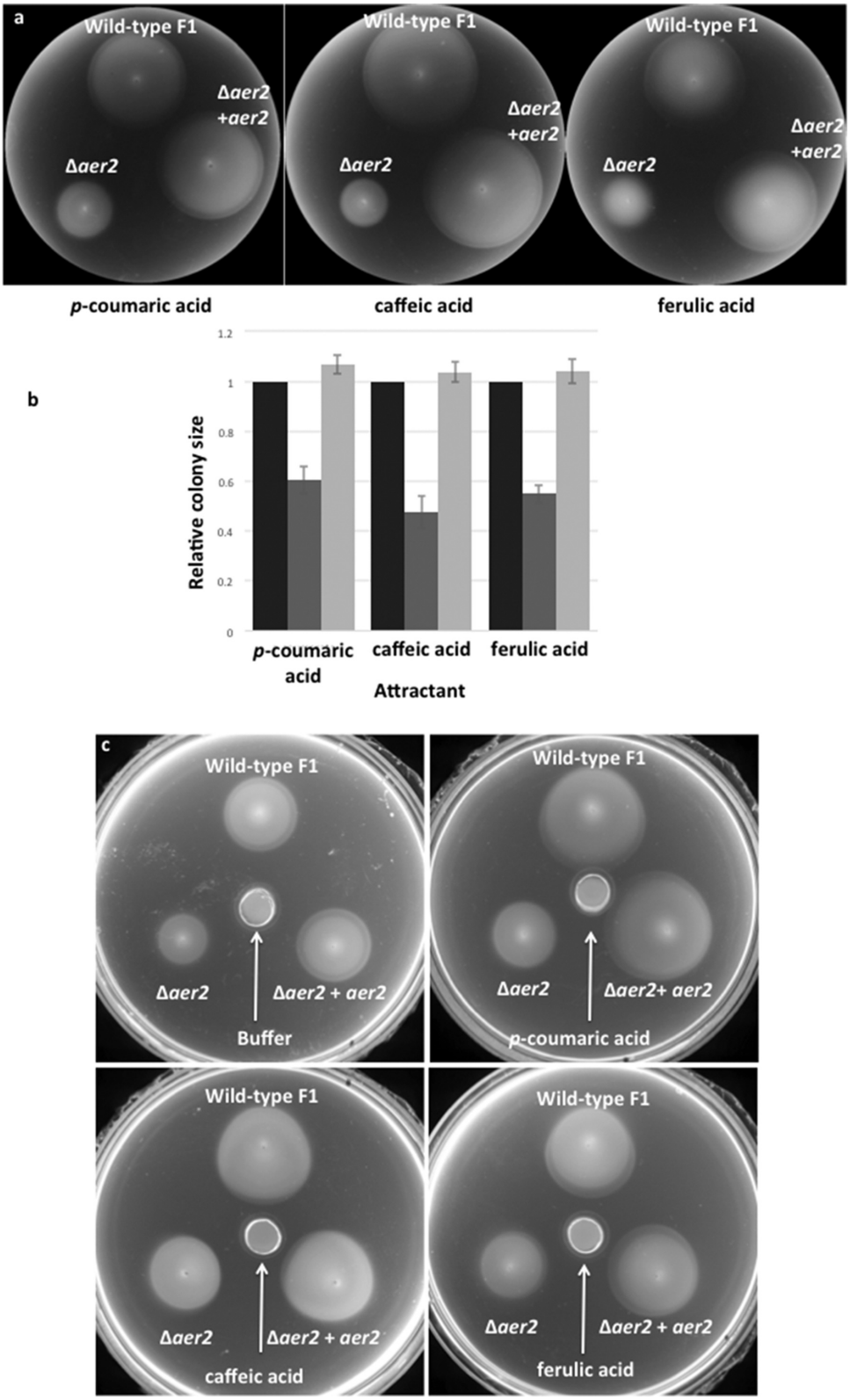

Fig. 6. Responses of wild-type *P. putida* F1(pRK415Km), the Δ*aer2* mutant strain XLF019(pRK415Km) and the complemented Δ*aer2* mutant strain XLF019(pJGH018) (Δ*aer2*+*aer2*) to *p*-coumaric, caffeic, or ferulic acid. (a) Swim plate assays. Swim plates contained minimal medium with 0.3 % Noble agar, 50 µg µl^{-1} kanamycin and 1 mM caffeic acid, 2 mM *p*-coumaric acid, or 2 mM ferulic acid. Photographs were taken after plates had been incubated for ~24 h at 30°. (b) Quantification of swim plate assay results. Colony diameters

were normalized to the wild-type F1(pRK415Km) response (black bars). Δ*aer2* mutant strain XLF019(pRK415Km), dark grey bars; complemented Δ*aer2* mutant strain XLF019(pJGH018), light grey bars. The columns represent the averages of two independent experiments with technical replicates ($n \geq 5$); the error bars represent standard deviations. (c) The responses of wild-type *P. putida* F1 (pRK415Km), the Δ*aer2* mutant XLF019(pRK415Km) and the complemented mutant XLF019(pJGH020) (Δ*aer2+aer2*) to *p*-coumaric, caffeic, or ferulic acid in gradient plate assays. The plates contained 1 mM glycerol and the plugs contained 10 mM caffeic acid, or 20 mM *p*-coumaric acid or ferulic acid. Photographs were taken after plates had been incubated for ~24 h at 30 °C.

As mentioned earlier, in *P. putida* F1 the inducible methyl-accepting chemotaxis protein PcaY is responsible for metabolism-independent chemotaxis to vanillate, protocatechuate, benzoate and a variety of both metabolizable and nonmetabolizable substituted benzoates [8, 41]. It is difficult to understand why the organism has evolved different mechanisms to sense structurally related chemicals that are catabolized through common pathways. It is also difficult to understand how the sensing mechanisms remain separate and are not cumulative. It seems, for example, to be counterintuitive that a good carbon and energy source like vanillate, which is an intermediate in ferulic acid catabolism (Fig. 1), is specifically sensed via PcaY and not by energy taxis, but we have shown that a PcaY mutant (which has an intact *aer2* gene) is unable to respond to vanillate [8]. Conversely, as shown here, ferulic acid is sensed by energy taxis and PcaY is not required for the response (Fig. 3). The chemotaxis response of the *pcaY* mutant to ferulic acid seemed somewhat weaker than that of the wild-type, so it seemed possible that PcaY might contribute to ferulic acid taxis. However, the absence of any detectable chemotaxis response to ferulic, *p*-coumaric and caffeic acids by the *aer2* mutant (which carries a functional copy of *pcaY*; Fig. 6c) seems to contradict this possibility.

Complex regulatory controls are present in *P. putida*, which has mechanisms for both induction and repression of aromatic acid catabolism and transport genes that result in preferential utilization of benzoate over 4-hydroxybenzoate when both substrates are present [42]. Therefore, one possibility is that there are additional layers of regulatory control in place that affect which receptor genes are expressed and/or which receptor proteins are synthesized when cells are growing with different substrates. *pcaY* is a member of the *pca* regulon, which includes genes for the catabolism of protocatechuate. Expression is induced by the transcriptional activator PcaR in the presence of the catabolic intermediate β-ketoadipate (Fig. 1 [8]). Because the inducer β-ketoadipate is also an intermediate in the degradation of hydroxycinnamic acids, we predicted that *pcaY* would be expressed in the presence of these chemicals. As expected, we found similar levels of *pcaY* expression when cells were grown with *p*-coumaric acid or 4-hydroxybenzoate (data not shown), indicating that PcaY is likely to be present in hydroxycinnamic acid-grown cultures. Therefore, cells growing on hydroxycinnamic acids should have the ability to use PcaY to sense aromatic intermediates generated during hydroxycinnamic acid catabolism; this, however, does not appear to be the case.

A similar lack of response occurs with downstream metabolites: although *P. putida* F1 has three methyl-accepting chemotaxis proteins (McfS, McfR and McfQ) that sense TCA cycle intermediates [43], cells growing on aromatic compounds that are degraded via TCA cycle intermediates do not sense these intermediates as attractants, as a *pcaY* mutant (with intact *mcfS*, *mcfR* and *mcfQ* genes) shows no response to vanillate [8]. In *C. testosteroni* CNB-1, the detection of TCA cycle intermediates that accumulate and leak out into the periplasm seems to be the primary mechanism for sensing aromatic acids rather than using an MCP that directly binds the aromatic acids themselves [36, 37]. A possible reason for the lack of a response to leaked TCA cycle intermediates in *P. putida* via the McfS, McfR and McfQ receptors could be that metabolism is so fast that insufficient amounts of TCA cycle intermediates accumulate during aromatic acid catabolism.

The deduced amino acid sequence of Aer2 from *P. putida* F1 is 31 % identical to Aer from *E. coli*, and its PAS domain, which is predicted to bind FAD, is 55 % identical to that in Aer [7]. In *E. coli*, energy taxis responses are mediated by Aer and Tsr, with the FAD cofactor of Aer sensing changes in redox status and Tsr sensing PMF [44]. Tsr is responsible for energy taxis toward the most efficient growth substrates for *E. coli* (mainly sugars), whereas Aer senses oxidizable substrates regardless of whether they serve as good growth substrates. In general, energy taxis responses mediated by both Tsr and Aer required higher threshold concentrations than receptors that bind attractant chemicals directly [44]. In the case of *E. coli*, chemoreceptor mutants expressing only Aer or Tsr were capable of sensing chemicals normally detected by the specialized receptors Tar or Trg when the attractants were provided at a significantly higher concentration [44]. This may be the case with hydroxycinnamic acids sensed by Aer2 vs substituted benzoates sensed directly by PcaY, although we have not determined the minimum concentrations detected.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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