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Flagella-Driven Chemotaxis Towards Exudate Components Is an Important Trait for Tomato Root Colonization by *Pseudomonas fluorescens*

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Motility is a major trait for competitive tomato root-tip colonization by *Pseudomonas fluorescens*. To test the hypothesis that this role of motility is based on chemotaxis toward exudate components, *cheA* mutants that were defective in flagella-driven chemotaxis but retained motility were constructed in four *P. fluorescens* strains. After inoculation of seedlings with a 1:1 mixture of wild-type and nonmotile mutants all mutants had a strongly reduced competitive root colonizing ability after 7 days of plant growth, both in a gnotobiotic sand system as well as in nonsterile potting soil. The differences were significant on all root parts and increased from root base to root tip. Significant differences at the root tip could already be detected after 2 to 3 days. These experiments show that chemotaxis is an important competitive colonization trait. The best competitive root-tip colonizer, strain WCS365, was tested for chemotaxis toward tomato root exudate and its major identified components. A chemotactic response was detected toward root exudate, some organic acids, and some amino acids from this exudate but not toward its sugars. Comparison of the minimal concentrations required for a chemotactic response with concentrations estimated for exudates suggested that malic acid and citric acid are among major chemo-attractants for *P. fluorescens* WCS365 cells in the tomato rhizosphere.

It is evident that colonization of at least part of the root system is required for the beneficial effects of inoculant bacteria for applications such as biocontrol of soil-borne diseases (Bull et al. 1991; Chin-A-Woeng et al. 2000; Lugtenberg et al. 1991; Schippers et al. 1987; Suslow and Schroth 1981; Weller and Thomashow 1994), biofertilization, and phytostimulation (Okon et al. 1998) and phytoremediation (Kuiper et al. 2001). To obtain a better understanding of the mechanism behind competitive tomato root-tip colonization, traits were identified that are required for efficient competitive root colonization, a topic which has recently been reviewed by Lugtenberg and associates (2001). One of these traits is motility. Although Howie and associates (1987) and Scher and associates (1988) reported that nonmotile mutants of fluorescent pseudomonads on wheat and of *Pseudomonas* and *Serratia* species on soybean are not impaired in root colonization, nonmotile mutants of *P. fluorescens* WCS374 (de Weger et al. 1987), *P. fluorescens* WCS365 (Dekkers et al. 1998b; Simons et al. 1996), and *P. putida* WCS358 (Simons et al. 1996) show a clear defect in competitive root-tip colonization of potato, wheat, radish, and tomato. Although the differences between the results of Howie and associates (1987) and Scher and associates (1988) with the results found in our group can be due to different assays, plants, and experimental conditions, it should be emphasized that, in our tests, nonmotile mutants belong to the most defective class of competitive root-tip colonization mutants (Lugtenberg et al. 2001). Since it seems reasonable to hypothesize that the role of motility in root colonization is to reach the nutrients exuded by the roots (Lugtenberg et al. 1999; Simons et al. 1997; A. H. M. Wijfjes, *unpublished data*), we decided to investigate whether the best competitive tomato root-tip colonizer we have tested so far, strain *P. fluorescens* WCS365 (Dekkers et al. 1998a; Geels and Schippers 1983) shows chemotactic behavior toward tomato root exudate and, if so, to its individual exudate components. To this end, we constructed mutants in the *cheA* gene, which controls flagella-driven chemotaxis. Furthermore, *P. fluorescens* WCS365 appeared to show chemotaxis toward root exudate as well as toward some individual exudate components. We show that chemotaxis plays a major role in competitive tomato root-tip colonization.

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RESULTS

Characterization of *cheA* mutants of various *P. fluorescens* strains.

cheA mutants were generated from the four different *P. fluorescens* wild-type strains OE28.3, SBW25, F113, and WCS365 and were designated as FAJ2061, FAJ0761, FAJ0758, and FAJ2060, respectively (Table 1). No effect of the *cheA* mutation on growth rate in King's medium B (KB) medium was observed, neither when strains were tested individually nor when tested in competition (data not shown). Microscopic image analysis of these *cheA* mutants revealed that they remain motile but have a much lower tumbling frequency compared with the wild-type cells. Movies showing the swimming behavior of the wild-type strains and the corresponding *cheA* mutants can be viewed on the Catholic University of Leuven Agricultural and Applied Biological Sciences Faculty PGPR and biodegradation web page.

Swarming assays conducted on a semisolid minimal basic medium (BM)-succinic acid plates revealed that wild-type WCS365, its *cheA* mutant FAJ2060, and its nonmotile mutant PCL1524 had ring diameters of 26 ± 2, 8 ± 1, and 2 ± 1 mm,

respectively (Fig. 1). Since PCL1524 in contrast to FAJ2060 is unable to swim, the ring formed by the latter strain is the result of smooth swimming. Similar differences between wild-type and *cheA* mutants were obtained for the other three strains. Assays on semisolid KB plates gave results similar to those in Figure 1.

Effect of *cheA* mutations on tomato root colonization in sand and soil systems.

The colonizing behavior of chemotatic mutants and their wild-type strains was initially analyzed using the gnotobiotic sand system described by Simons and associates (1996). The single homologous *cheA* mutants, FAJ2061 and FAJ2060, of OE28.3 and WCS365, respectively, appeared to be 100% stable in the rhizosphere, as judged after testing for the presence of antibiotic-resistant isolates released from the roots. Colonization assays in sand with wild-type strains or *cheA* mutants inoculated alone on a seedling show that *cheA* mutants colonize the root tip to the same extent as wild-type strains (data not shown). When tested in the gnotobiotic system in competition with their wild type after inoculation of seedlings with a 1:1 mixture, all *cheA* mutants show a 10- to 1000-fold reduced ability to colonize the root tip of tomato (Table 2). Isolation of attached bacteria immediately after inoculation of the seedlings confirmed that, for all strains, the ratio of wild type and mutants on the seedling was indeed 1:1, as in the inoculation mixture.

Colonization assays in nonsterile potting soil (Table 3) showed that CFU values in soil of both mutant and wild-type strains are about 100-fold less when compared with the CFU values obtained in sand (Table 2). After applying the *cheA* mutants or the wild-type strains alone on the seedling, an at least 100-fold impaired root-tip colonization phenotype was observed in potting soil for the *cheA* mutants when compared with the wild-type strains (data not shown). Competitive root-tip colonization assays in soil on tomato show an approximately 10-fold impaired colonization ability for the *cheA* mutants (Table 3).

A time-course experiment of competitive root-tip colonization in the gnotobiotic sand system analyzed for four days showed that the *cheA* mutants are already significantly outcompeted after 2 to 3 days (Table 4).

In order to gain insight into the distribution of bacteria along the root, competitive colonization assays were performed with WCS365 and its *cheA* mutant FAJ2060 on tomato in sand as well as in soil. The roots were divided into four parts: 1 cm just underneath the crown, the middle part of the root divided into two parts with an equal length of approximately 3 to 4 cm, and the root tip (1 cm). After 7 days of plant growth in sand (Table 5) or nonsterile soil (data not shown), the *cheA* mutant appeared to be present in significantly decreased numbers on the upper part of the root (Table 5), whereas it could hardly be detected on the root tip. The latter results confirm the results of our previous competition experiments (Tables 2 and 3).

Chemotaxis toward tomato root exudate and individual exudate components.

Since *P. fluorescens* strain WCS365 is the best competitive tomato root-tip colonizer of the four studied strains, this strain, its *cheA* mutant FAJ2060, and its nonmotile mutant PCL1524 were used to study the putative role of chemotaxis toward tomato root exudate in the drop assay of Fahrner and associates (1994) as described by Grimm and Harwood (1997). Concentrated (50-fold) tomato root exudate as well as individual exudate components were tested (Fig. 2 and Table 6). In contrast to the control with chemotaxis buffer (Fig. 2A), concentrated root exudate, as well as several individual organic acids including succinic acid (Fig. 2B) and malic acid (Fig. 2C), initiated a clear response of the WCS365 cells (Table 6). Neither the other tested organic acids nor exudate sugars induced a response (Table 6). The dominant amino acids present in the tomato root exudate, L-aspartic acid, L-glutamic acid, L-isoleucine, L-leucine, and L-lysine (Simons et al. 1997) all induced a response from WCS365 cells when tested in 10-µl drops at concentrations of 100 mM.

Various concentrations of organic acids and amino acids were tested in 10-µl drops to determine the threshold concentration that was able to induce chemotaxis. For citric acid, malic acid, fumaric acid, and pyroglutamic acid, concentrations as low as 10 to 20 mM initiated a response. For succinic acid, a 40 mM concentration initiated a significant response of the cells (Fig. 2). Four out of five major amino acids, L-aspartic acid, L-glutamic acid, L-isoleucine, and L-leucine (Table 6), initiated a response at concentrations of about 10 mM or lower. For L-lysine, a 20 mM concentration induced chemotaxis of the WCS365 cells. The responses toward these amino acids were faster (shorter incubation time) and stronger (lower concentrations detectable) than toward organic acids tested at 10 mM.

Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Reference or Source
Pseudomonas fluorescens		
WCS365	Wild-type biocontrol strain, efficient competitive colonizer of tomato, potato, and wheat roots	Dekkers et al. 1998a Geels and Schippers 1983
PCL1500	Tn*5lacZ* derivative of WCS365, not impaired in competitive root colonization, Km^r	van der Bij et al. 1996
FAJ2060	WCS365 mutant with an impaired *cheA* gene constructed by single homologous recombination, Km^r	This study
F113	Wild-type biocontrol strain	Shanahan et al. 1992
PCL1510	Tn*5lacZ* derivative of F113, not impaired in competitive root-tip colonization, Km^r	This study
FAJ0758	F113 mutant with an impaired *cheA* gene after double homologous recombination, Km^r	This study
SBW25	Wild-type biocontrol strain	Rainey and Bailey 1996
FAJ0761	SBW25 mutant with an impaired *cheA* gene constructed by double homologous recombination, Km^r	This study
OE28.3	Wild-type strain, colonizer of wheat roots	De Mot and Vanderleyden 1991
PCL1513	Tn*5lacZ* derivative of OE28.3, not impaired in competitive root-tip colonization	This study
FAJ2061	OE28.3 mutant with an impaired *cheA* gene constructed by single homologous recombination, Km^r	This study
PCL1524	Tn*5* derivative of *P. fluorescens* WCS365, lacking flagella	Camacho 2001
Escherichia coli		
S17-1	MM294, RP4-2 Tc::Mu-Km::Tn*7* chromosomally integrated	Simon et al. 1983
Plasmids		
pCIB100	pLRKΔ211 (ColE1) with pSUP5011 mobilization segment. Carries Tn5*lacZ* (Km^r). The *lacZ* is constitutively expressed in *Pseudomonas* spp.	Lam et al. 1990
pCR2.1	Cloning vector for *Taq*-amplified PCR products, Km^r, Ap^r	Invitrogen
pSUP202	Mobilizable pBR325 derivative, Tc^r, Cm^r, Ap^r	Simon et al. 1983
pUC4K	pUC4 derivative with aminoglycoside 3′-phosphotransferase gene (*aph*) from Tn*903*, Km^r	Amersham Pharmacia Biotech

Also the other amino acids found in the tomato root exudates, namely L-histidine, L-phenylalanine, glycine, L-arginine, L-threonine, L -asparagine, and L-glutamine, initiated a response at concentrations as low as 10 mM, except L-phenylalanine, which initiated no response at all.

DISCUSSION

Characterization of *cheA* mutants of various *P. fluorescens* strains.

We have shown previously that motility is an important trait for competitive tomato root-tip colonization (Dekkers et al. 1998b; de Weger et al. 1987, 1997; Simons et al. 1996). To test the hypothesis that chemotaxis toward root exudate components is the reason behind this phenomenon, we disrupted the *cheA* gene controlling flagella-mediated swimming toward chemo-attractants in four root-colonizing *P. fluorescens* strains (Fig. 3) and tested their colonizing (Tables 2 through 5) and chemotactic (Fig. 2; Table 6) behavior.

Chemotaxis is mediated by a two-component regulatory system consisting of a sensor kinase, CheA, and a response regulator, CheY. Chemoreceptors, methyl-accepting proteins (MCPs), are transmembrane signal transducers located in the cytoplasmic membrane. They monitor the concentration of the corresponding chemicals in the environment. Via methylation of the MCPs, a signal is transduced, and autophosphorylation of CheA takes place. Subsequently, P-CheA donates the phosphate group to CheY, and P-CheY will interact with the flagellar motor. Whenever the signal drops below a certain threshold, CheY will be phosphorylated, and clockwise rotation will occur. Subsequently, the bacteria will start to tumble, which is required to change swimming direction (Adler 1969; Aizawa et al. 2000). If this signal rises above the threshold value, CheY will be dephosphorylated and counter clockwise rotation will occur, resulting in a run of the bacterial cell.

Table 2. Competitive tomato root-tip colonization ability of *cheA* mutants of four *Pseudomonas fluorescens* strains[y]

Competing strains	Competitive root-tip colonization [$\log_{10}$ (CFU+1/cm) root tip][z]	
	Wild type	*cheA* mutant
WCS365 vs. FAJ2060	4.8 ± 0.9a	1.9 ± 1.7b
OE28.3 vs. FAJ2061	4.7 ± 1.8a	3.6 ± 2.4b
SBW25 vs. FAJ0761	4.9 ± 0.5a	2.9 ± 1.2b
F113 vs. FAJ0758	5.1 ± 0.9a	2.2 ± 1.9b

[y] Strains were tested in a gnotobiotic sand system. Mutant strains were inoculated on seedlings in a 1:1 ratio with the wild-type strain. Plant roots were analyzed after 7 days.

[z] In every experiment, 10 plants were inoculated and individually processed. When values in the same row are followed by a different letter, they are significantly different at $P = 0.05$ on nonparametric multiple comparisons using the Wilcoxon-Mann-Whitney test. X-gal was added for plate selection.

In the *cheA*-containing motility and chemotaxis region on the chromosome of both *P. putida* PRS2000 (Ditty et al. 1998) and *P. aeruginosa* PAO1 (Kato et al. 1999), two putative motility genes (*motA* and *motB*, encoding components of the proton-driven flagellar motor) are present downstream of *cheB*. From the high organizational conservation of this DNA region among *Pseudomonas* species and from analysis of the corresponding gene cluster in *P. fluorescens* PfO-1, the gene order *cheA-cheB-motA-motB* can be expected for the *P. fluorescens* strains used in this study. We have identified an equivalent locus in *P. fluorescens* OE28.3 (GenBank accession number AF481091) (Fig. 3), and in silico analysis revealed it to be highly conserved in *P. fluorescens* PfO-1, *P. syringae* pv. *syringae* DC3000, and *P. putida* KT2440 as well.

The *cheA* gene targeted in this study directs flagella-mediated swimming toward chemo-attractants. Genome analysis has revealed the coexistence of multiple chemosensory systems in *Pseudomonas* spp. *P. aeruginosa* PAO1 has three additional loci with *che*-like genes that encode (probable) chemosensory pathways (Stover et al. 2000). One of these loci is required for type IV pili-mediated twitching motility (Darzins 1994; Kearns et al. 2001), and similar loci can be found in the draft genomic sequences of *P. fluorescens* PfO-1, *P. syringae* DC3000, and *P. putida* KT2440. No function has yet been assigned to the two other putative chemosensory systems (Bourret et al. 2002). Locus PA3708-PA3702 of *P. aeruginosa* PAO1 is highly conserved in *P. fluorescens* PfO-1, *P. syringae* DC3000, and *P. putida* KT2440 and has an equivalent in *P. fluorescens* OE28.3 as well (R. De Mot, *unpublished data*). A counterpart for the second uncharacterized *P. aeruginosa* locus, PA0180-PA0173, appears to be absent from these fluorescent *Pseudomonas* spp.

Since the constructed mutants can only be used to test our hypothesis when the *cheA* mutations have no effect on (putative) downstream motility genes, we studied their motility using microscopic video analysis. The results indicate that the *cheA* mutants are motile but impaired in chemotaxis because of the lower tumbling frequency. In addition, using swim plate assays, it was shown for *P. fluoresens* WCS365 that its *cheA* mutant FAJ2060 is motile, in contrast to its nonmotile mutant, PCL1524 (Fig. 1). In conclusion, the *cheA* mutations in the constructed *cheA* mutants do not affect motility. Smooth swimming behavior is expected for cells that do not phosphorylate CheY due to a defective CheA protein, as was ob-

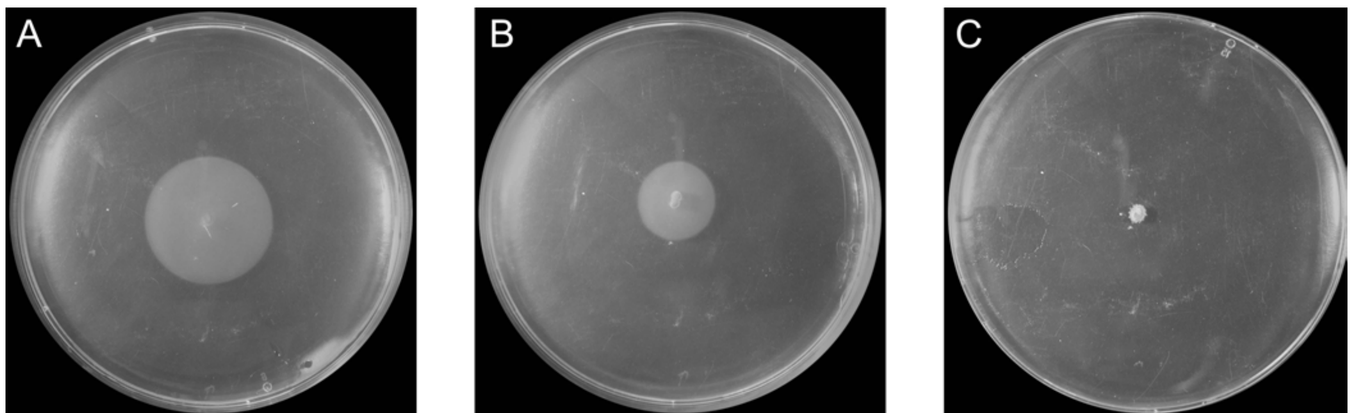

Fig. 1. Motility assay on minimal basic medium. Medium was solidified with 0.3% agar and succinic acid (0.01%) added as a carbon source. The plates were inoculated with **A,** *Pseudomonas fluorescens* WCS365; **B,** its *cheA* mutant FAJ2060; and **C,** its nonmotile mutant PCL1524.

served previously for *cheA* mutants of *P. putida* (Ditty et al. 1998) and *P. aeruginosa* (Kato et al. 1999).

Effect of *cheA* mutations on tomato root colonization.

To study the behavior of the *cheA* mutants in the rhizosphere of tomato, seedlings were inoculated with a 1:1 mixture of the parental strain and the respective mutant or with a suspension of each strain alone and allowed to grow in a gnotobiotic sand system or in nonsterile potting soil. Although the *cheA* mutants are not impaired in their colonizing ability of tomato roots in sand after inoculation alone on the seedlings (data not shown), all *cheA* mutants appeared to be heavily impaired in their competitive colonization behavior under gnotobiotic conditions (Table 2) as well as in potting soil (Table 3). It should be noted that, in the latter case, a large (approximately 10^8 CFU/g) indigenous microbial population was present. The ability of colonization mutants to colonize root tips to the same extent as the parental strain after inoculation alone was observed earlier for most colonization defective mutants (Lugtenberg et al. 2001). In order to further compare the behavior of *cheA* mutants during competitive root colonization with that of the parental strain, we followed colonization through time for all four strains and their corresponding *cheA* mutants (Table 4) and spatial distribution along the colonized roots (Table 5) for *P. fluorescens* strain WCS365 and its *cheA* mutant, FAJ2060. A time-course of colonization showed that, 2 to 3 days after the root system starts to develop when root systems are still short, the mutants are losing the competition (Table 4). Apparently as soon as the root starts to develop, *cheA* mutants in contrast to wild-type cells are not able to keep track of the growing root under competitive conditions.

Analysis of the spatial distribution of bacteria along the root during competitive colonization showed that, after 7 days of growth, the *cheA* mutant FAJ2060 is present, although in much lower cell numbers, on the upper and middle parts of the roots, whereas it is hardly detectable on the root tip (Table 5). These results indicate that decreased abilities of *cheA* mutants for proliferation, survival, or both on higher root parts are responsible for their poor competitive root-tip colonization. The poor chemotactic ability of *cheA* mutants toward major root exudate components (discussed below) is the likely reason for this defect.

Chemotaxis toward tomato root exudate and its individual components.

Since *P. fluorescens* WCS365 is the best competitive root-tip colonizer of the tested strains, this strain, its *cheA* mutant FAJ2060, and its nonmotile mutant PCL1524 were used in the chemotaxis drop assay of Fahrner and associates (1994) also described by Grimm and Harwood (1997). This drop assay was used to analyze taxis toward tomato root exudate as well as toward the known individual components of the tomato root and seedling exudate (Table 6).

Previous studies on tomato exudate have shown that its major components are organic acids (with citric [55.2%], malic [15.3%], and lactic [10%] acids as the major components) (A. H. M. Wijfjes, *unpublished data*), amino acids (especially isoleucine, leucine, lysine, aspartic acid, and glutamic acid) (Simons et al., 1997; A. H. Wijfjes et al., *unpublished data*), and sugars (with glucose [37%] and xylose [33%] as major components) (Lugtenberg et al. 1999). In our chemotaxis study, 5 out of 10 organic acids and all major amino acids but none of the sugars initiated a response of WCS365 cells (Table 6). Nonmotile mutants, *cheA* mutants, and the chemotaxis buffer (control) did not initiate a response. In order to identify the best chemo-attractant, various concentrations of the individual components (filter sterile) in a 4- to 100-mM range were tested. Results show that, among the organic acids, only malic acid could be detected by WCS365 cells at 10 mM. At this concentration, all amino acids except L-lysine were detected. L-isoleucine appeared to be the best chemo-attractant (Table 6) since its minimal concentration for detection (4 mM) was 2.5-fold lower than that for malic acid. When we compare the concentration-dependent chemotactic responses of cells to malic acid and L-isoleucine (with their concentrations in the exudate being approximately 70 and 2.2 μM, respectively), we can suggest that malic acid is one of the most important chemo-attractants in the tomato rhizosphere. In addition, we suggest that citric acid, present in the exudate at approximately 115 μM and active as a chemo-attractant in a concentration as low as 20 mM, represents another major chemo-attractant in this rhizosphere.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

All *P. fluorescens* strains and their chemotatic mutants (Table 1) were grown in liquid KB (King et al. 1954) at 28°C

Table 3. Competitive tomato root tip colonization ability of *cheA* mutants of four *Pseudomonas fluorescens* strain in nonsterile potting soil[y]

	Competitive root-tip colonization [$\log_{10}$ (CFU+1/cm) root tip][z]	
Competing strains	**Wild type**	***cheA* mutant**
WCS365 vs.FAJ2060	2.9 ± 1.6a	1.9 ± 1.3b
OE28.3 vs. FAJ2061	2.6 ± 1.9a	2.0 ± 1.3b
SBW25 vs. FAJ0761	3.5 ± 0.5a	2.5 ± 2.2b
F113 vs. FAJ0758	2.8 ± 1.6a	1.6 ± 1.4b

[y] Mutant strains are inoculated on seedlings in a 1:1 ratio with the wild-type strain. Plant roots were analyzed after 7 days.

[z] In every experiment, 10 plants were inoculated and individually processed. When values in the same row are followed by a different letter, they are significantly different at $P = 0.05$ on nonparametric multiple comparisons using the Wilcoxon-Mann-Whitney test.

Table 4. Time-course of competitive tomato-root-tip colonization of *cheA* mutants of four *Pseudomonas fluorescens* strains[y]

	Competitive root-tip colonization[z] [$\log_{10}$ (CFU+1/cm) root tip]	
Competing strains	**Wild type**	***cheA* mutant**
WCS365 vs. FAJ2060		
Day 1	5.3 ± 0.1a	5.2 ± 0.1b
Day 2	5.2 ± 0.2a	4.9 ± 0.2b
Day 3	5.6 ± 0.0a	4.5 ± 0.3b
Day 4	5.4 ± 0.3a	3.3 ± 0.6b
OE28.3 vs. FAJ2061		
Day 1	5.8 ± 0.0a	5.7 ± 0.1b
Day 2	5.4 ± 0.7a	5.3 ± 0.6b
Day 3	5.5 ± 0.1a	4.9 ± 0.3b
Day 4	4.7 ± 0.7a	4.0 ± 0.5b
SBW25 vs. FAJ0761		
Day 1	5.8 ± 0.1a	5.7 ± 0.2b
Day 2	5.4 ± 0.0a	5.0 ± 0.3b
Day 3	5.3 ± 0.0a	3.1 ± 0.2b
Day 4	4.8 ± 0.2a	3.0 ± 0.3b
F113 vs. FAJ0758		
Day 1	5.8 ± 0.1a	5.6 ± 0.1b
Day 2	5.7 ± 0.1a	5.2 ± 0.3b
Day 3	5.7 ± 0.1a	4.3 ± 0.4b
Day 4	5.4 ± 0.1a	3.3 ± 0.1b

[y] In a gnotobiotic sand system. Mutant strains were inoculated on seedlings in a 1:1 ratio with the wild-type strain.

[z] In every experiment, 10 plants were inoculated and individually processed. When values in the same row are followed by a different letter, they are significantly different at $P = 0.05$ on nonparametric multiple comparisons using the Wilcoxon-Mann-Whitney test.

under vigorous shaking. When indicated, BM was used (Lugtenberg et al. 2001). Medium was solidified with 1.8% agar (Select Agar; Gibco BRL, Life Technologies, Paisley, U.K.) and when appropriate, kanamycin (Sigma-Aldrich BV, Zwijndrecht, The Netherlands) or 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (SphaeroQ, Leiden, The Netherlands) was added in final concentrations of 50 µg/ml or 40 µg/ml, respectively. *Escherichia coli* was grown at 37°C on solidified LB medium (Sambrook et al. 1989). To determine growth rates of the individual strains, the optical density at 620 nm (OD_{620}) was followed during growth. For growth of wild types and mutants in competition, cultures grown overnight were diluted to an OD_{620} of 0.1, and mutant and wild type were mixed in a 1:1 ratio. After growth overnight, the culture was diluted 1,000-fold in fresh KB, samples were diluted and plated on KB/X-gal, and the ratio between the Tn*5lacZ*-tagged wild type (blue colonies) and mutant (white colonies) was determined.

Generation of *cheA* mutants via homologous recombination.

Two polymerase chain reaction (PCR) primers (5′-CGCCG-ATGAAGAAATCCTCCAGGA-3′ and 5′-GTCAGCGGCAC-CTTGATGACGATC-3′) were designed on the basis of the *P. putida cheA* (Ditty et al. 1998) and the *P. aeruginosa cheA* sequences (Kato et al. 1999). For each of the four strains under study, a fragment of the expected length (1.8 kb) was amplified by PCR using an Eppendorf Mastercycler thermocycler. Lysed colonies were used as a source of template DNA, and 10% dimethylsulfoxide was added to the reaction mixture to prevent primer mismatching. PCR reaction conditions were: 45 s at 94°C, 45 s at 60°C, and 1 min at 72°C.

The respective *cheA* fragments were purified from agarose gels, cloned with the T/A cloning kit (Invitrogen, Merelbeke, Belgium) in the pCR2.1 vector (Invitrogen), and sequenced on both strands with an automated A.L.F. sequencer (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). These fragments were subsequently cloned in pSUP202, a suicide vector for *Pseudomonas* strains (Simon et al. 1983). The F113 and SBW25 *cheA* fragments were *Bam*HI and *Xho*I digested and then cloned in the pSUP202 *Bam*HI and *Sal*I sites. The OE28.3 and WCS365 *cheA* fragments were *Hin*dIII and *Xho*I digested and then inserted in the pSUP202 *Hin*dIII and *Sal*I sites. In all cases, the pSUP202 *Sal*I site was inactivated, which allowed insertion of the *aph*-cassette (*Sal*I fragment conferring kanamycin resistance) from pUC4K (Amersham Pharmacia Biotech) in the *Sal*I sites of the *cheA* fragments from strains F113, SBW25, and WCS365. Due to the presence of two *Sal*I sites in the *cheA* fragment from strain OE28.3, an internal fragment was replaced with the *aph* cassette.

The four *Pseudomonas* strains were transformed with the suicide vector carrying the respective interrupted *cheA* fragments, and double homologous recombinants were screened by replica plating (loss of the plasmid-encoded chloramphenicol resistance). For strains SBW25 and F113, double homologous recombinants were obtained. The corresponding mutants were designated FAJ0761 and FAJ0758, respectively. The *cheA* mutants selected for strains OE28.3 (FAJ2061) and WCS365 (FAJ2060) were the result of a single recombination event. Despite repeated attempts, mutants resulting from a double recombination event could not be obtained for these strains. Because an internal *cheA* fragment lacking the 5′ and 3′ parts of the coding region was used for insertion of the *aph*-cassette, single recombination equally resulted in inactivation of the chromosomal *cheA* gene.

Table 5. Spatial distribution of WCS365 and its *cheA* mutant FAJ2060[y]

Competing strains	Competitive root-tip colonization[z] [$\log_{10}$ (CFU+1)/root part]	
WCS365 vs. FAJ2060	**WCS365**	**FAJ2060**
Part 1	6.1 ± 0.5a	4.5 ± 1.8b
Part 2	5.6 ± 0.6a	3.3 ± 1.6b
Part 3	5.3 ± 0.7a	2.5 ± 2.1b
Part 4	4.8 ± 0.9a	2.0 ± 1.7b

[y] Competitive tomato-root colonization in a gnotobiotic sand system was tested. Mutant strains are inoculated on seedlings in a 1:1 ratio with the wild-type strain. Plant roots were judged after 7 days. Roots were divided into four parts from crown to root tip: parts 1, 2, and 3 were 2.5 cm in length and the root tip, part 4, 1 to 2 cm.

[z] In every experiment, 10 plants were inoculated and individually processed. When values in the same row are followed by a different letter, they are significantly different at $P = 0.05$ on nonparametric multiple comparisons using the Wilcoxon-Mann-Whitney test.

Motility assays.

The wild type and *cheA* mutant strains grown in LB medium until the exponential phase were transferred to 100 µl microchambers. Samples of free-swimming cells were inspected with a Nikon Optiphot phase contrast microscope. The images were transferred via a Panasonic Vidicon video camera to a Sony Umatic video recorder. Tracks were analyzed by the Hobson Backtracker image analyzing system (Jeziore-Sassoon et al. 1998).

Motility was also tested as described by Dekkers and associates (1998a). Briefly, KB medium was 20-fold diluted and semisolidified with 0.3% of agar (Select Agar, Gibco

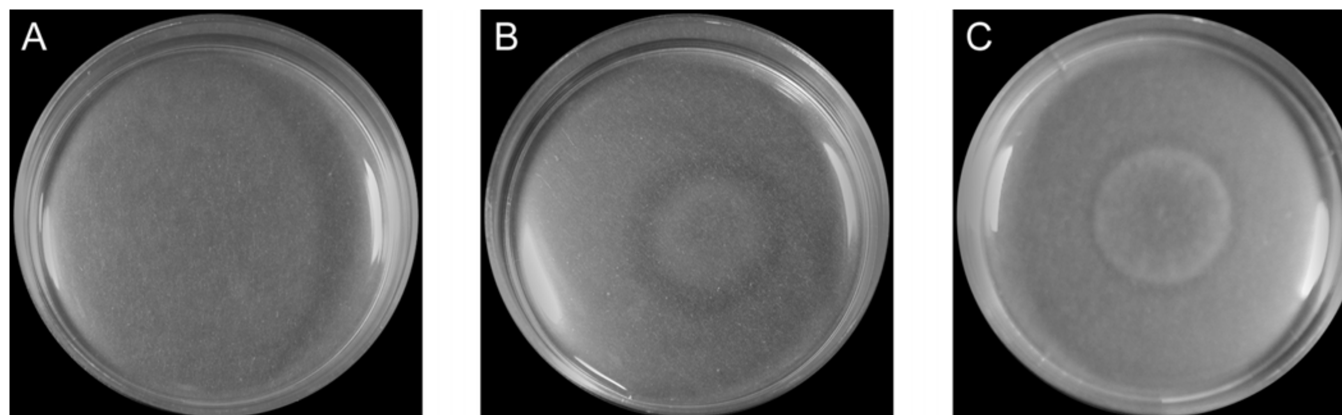

Fig. 2. Chemotactic response of *Pseudomonas fluorescens* WCS365 to organic acids in a “drop” assay. Drops applied to the center of the dish contained **A,** chemotaxis buffer; **B,** 40 mM succinic acid; and **C,** 100 mM malic acid. Photographs were taken after incubation for 30 min.

BRL) and poured into petri dishes. Samples of bacteria were applied in the middle of the agar plate, and after growth overnight, the diameter of the resulting spot was measured. Experiments were performed in triplicate. The 20-fold diluted KB was replaced with BM (Lugtenberg et al. 1999) with succinic acid as the carbon source (0.01%) in order to analyze motility under poor nutrient conditions.

Construction of *lacZ* derivatives of *Pseudomonas* strains with wild-type colonizing ability.

Tn5::*lacZ* insertion mutants were generated by biparental mating of a wild-type *Pseudomonas* strain (WCS365, OE28.3, or F113) with *E. coli* S17-1 harboring plasmid pCIB100 (Lam et al. 1990) as described previously by Simons and associates (1996). Tn*5LacZ* insertion mutants obtained were analyzed for their competitive root-colonizing ability against their wild type in the gnotobiotic system, and appropriate mutants showing wild-type colonization ability were used in the experiments described here. For *Pseudomonas* strain SBW25, no *lacZ* derivative could be obtained with wild-type colonizing ability.

Table 6. Induction of chemotaxis by tomato root exudate and by individual root exudate components[x]

Chemo-attractant	WCS365 (wild type)	FAJ2060 ($cheA^-$)	PCL 1524 (nonmotile)
Tomato root exudate[y]	+	–	–
Organic acids			
Malic acid (10)[z], pyroglutamic acid (20)	++	–	–
Citric acid (20), succinic acid (40), fumaric acid (20)	+	–	–
Lactic acid, oxalic acid, *t*-aconitic acid, propionic acid, ketoglutaric acid	–	–	–
Sugars			
Glucose, fructose, maltose, ribose, xylose and sucrose	–	–	–
Amino acids			
L-Aspartic acid (9), L-glutamic acid (9), L- isoleucine (4), L-leucine (8), L-lysine (20)	++	–	–

[x] Cells were pregrown on semisolid minimal basic medium with 1% succinic acid. Putative chemo-attractants were tested individually. The response indicated with ++, +, and – refers to a concentration in the 10 µl drop of 100 mM. ++ = fast response, after 30 min; + = significant response; – = no response.

[y] Tested at 50-fold concentrated.

[z] The lowest concentration (mM) in the 10 µl drop that induced a significant response is given in parentheses. All other amino acids detected in the root exudate (Simons et al. 1997) were tested. Except for L-phenylalanine, all amino acids initiated a response from WCS365 cells.

Root-tip colonization assays in a gnotobiotic sand system and in soil.

Root colonization assays were performed as described by Simons and associates (1996), using the gnotobiotic system containing sterile sand to which 10% (vol/wt) plant nutrient solution (PNS) (Hoffland et al. 1989) was added to moisten the sand. This PNS solution does not contain any C or N compounds. When colonizing ability in potting soil was tested, the sterile sand column was replaced by nonsterile potting soil (Intratuin, Voorschoten, The Netherlands). For competitive colonization experiments, sterile germinated tomato seeds (*Lycopersicon esculentum* Mill. cv. Carmello; Novartis Seeds B.V., Enkhuizen, The Netherlands) were inoculated with a 1:1 mixture of a parental strain and tagged with *lacZ* (except *Pseudomonas fluorescens* SBW25) and the corresponding mutant strain. The seedlings were placed in the tubes and allowed to grow in a climate-controlled growth chamber at 18°C, 70% relative humidity, and 16 h of daylight. After 7 days, roots were isolated, cut into parts that always included the 1-cm-long root tip with adhering sand, and bacteria were removed from the root parts by shaking in 1 ml phosphate buffered saline. The ratio between parental and mutant cells was determined by plating dilutions on solidified KB medium supplemented with kanamycin (only for strains containing a Tn*5lacZ* or *aph* cassette) and X-gal (detection limit 100 CFU/ml). For *P. fluorescens* SBW25, plates with and without kanamycin were used to determine the ratio between wild-type cells and *cheA* mutant cells. When soil columns were used, the plates were supplemented with cycloheximide to a final concentration of 100 µg/ml (Sigma, St Louis) to prevent fungal growth.

All results were statistically analyzed using the nonparametric Wilcoxon-Mann-Whitney test (Sokal and Rohlf 1981). To avoid log 0 cases, calculations were carried out using log (CFU + 1)/cm root tip.

Isolation of tomato root exudate.

Tomato root exudate was isolated as described by Simons and associates (1997). Briefly, 100 sterile seedlings were placed in 100 ml of PNS and were allowed to grow in a climate-controlled growth chamber at 18°C, 70% relative humidity, and 16 h of daylight. After 7 days, root exudate was collected in 50 ml aliquots and was freeze dried using a freeze-dry apparatus from Snijders Scientific (Tilburg, The Netherlands).

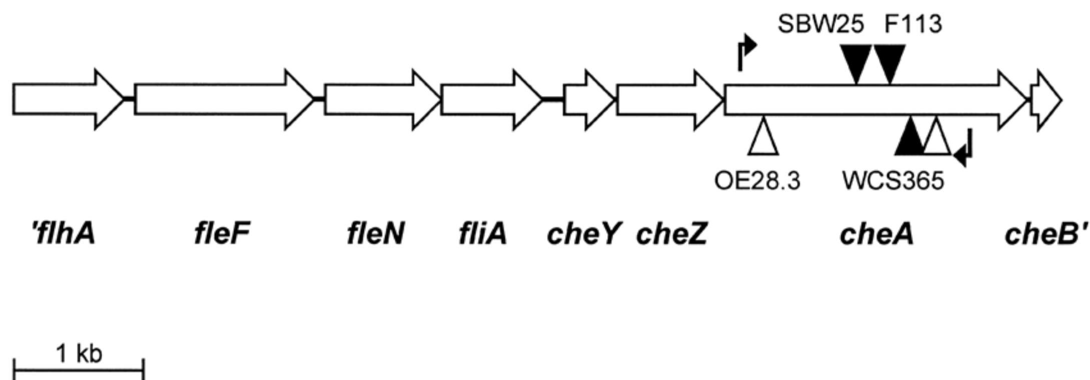

Fig. 3. Organization of the *Pseudomonas fluorescens* OE28.3 genomic DNA region carrying motility genes (*'flhA-fliA*) and chemotaxis genes (*cheY-cheB'*). Hooked arrows indicate the position of the primers used to amplify an internal *cheA* fragment of *P. fluorescens* OE28.3, F113, SBW25, and WCS365. Labeled arrowheads mark the insertion sites of the kanamycin resistance cassette (*aph*) in the respective fragments used to construct *cheA* mutants of strains F113 (same orientation as *cheA*) and OE28.3, SBW25, and WCS365 (opposite orientation). For strain OE28.3, an internal fragment (bordered by two empty arrowheads) of *cheA* was replaced with the cassette.

Root exudate was concentrated by dissolving the obtained freeze-dried material in 1.0 ml of sterile water.

Chemotaxis experiments.

Chemotaxis experiments were performed using the "drop" assay by Fahrner and associates (1994) as described by Grimm and Harwood (1997) with slight modifications. Briefly, cells grown overnight in KB were diluted 100 times into 150 ml of BM containing 1% succinic acid. When cells reached the early logarithmic phase (OD_{600} of 0.12), 40-ml samples were resuspended in 12 ml of chemotaxis buffer (100 mM potassium phosphate [pH 7.0] per 20 μM EDTA).

An aqueous solution of 1% hydroxypropylmethylcellulose (Sigma-Aldrich), formulated to give a viscosity of about 4,000 cP in a 2% aqueous solution, was added to the cell suspension to give a final volume of 15 ml. The resulting cell suspension was transferred to a 60-mm-diameter petri dish, where it formed an approximately 3-mm-thick layer. Concentrated (50-fold) root exudate or individual exudate components (Table 6) (all provided by Sigma-Aldrich) were added to the center of the dish as a 10-μl drop in concentrations of 0.1 M or lower. After incubation for 0.5 to 2 h at room temperature, the plates were inspected for the appearance of a clear zone surrounding the bacteria attracted to the attractant, which was used as the criterion for a chemotactic response toward the added component.

Isolation of a genomic fragment of *P. fluorescens* OE28.3 containing *cheA*.

The 1,800-kb PCR-amplified *cheA* fragment of strain OE28.3 was used to probe a genomic phage library of strain OE28.3 (De Mot et al. 1992) by plaque hybridization. For the genomic fragment thus isolated, overlapping deletion clones were generated using the EZ::TN Plasmid-Based Deletion Machine (Epicentre Biozym, Landgraaf, The Netherlands) and sequenced using an automated A.L.F. sequencer (Amersham Pharmacia Biotech).

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Catholic University of Leuven, Agricultural and Applied Biological Sciences Faculty PGPR and biodegradation web page: http://www.agr.-kuleuven.ac.be/dtp/cmpg/research/pgprandbiodegradation.html.

The Department of Energy and University of California Joint Genome Institute website: http:/www.jgi.doe.gov/JGI_microbial/html/index.html.

The Institute for Genomic Research BLAST Search Engine for Unfinished Microbial Genomes web page: http://tigrblast.tigr.org/ufmg/.