Identification of Chemotaxis Sensory Proteins for Amino Acids in *Pseudomonas fluorescens* Pf0-1 and Their Involvement in Chemotaxis to Tomato Root Exudate and Root Colonization

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*Pseudomonas fluorescens* Pf0-1 showed positive chemotactic responses toward 20 commonly-occurring L-amino acids. Genomic analysis revealed that *P. fluorescens* Pf0-1 possesses three genes (Pf01_0124, Pf01_0354, and Pf01_4431) homologous to the *Pseudomonas aeruginosa* PA01 ptcA gene, which has been identified as a chemotaxis sensory protein for amino acids. When Pf01_4431, Pf01_0124, and Pf01_0354 were introduced into the ptcA ptcB ptcC triple mutant of *P. aeruginosa* PA01, a mutant defective in chemotaxis to amino acids, its transformants showed chemotactic responses to 18, 16, and one amino acid, respectively. This result suggests that Pf01_4431, Pf01_0124, and Pf01_0354 are chemotaxis sensory proteins for amino acids and their genes were designated ctaA, ctaB, and ctaC, respectively. The ctaA ctaB ctaC triple mutant of *P. fluorescens* Pf0-1 showed only weak responses to Cys and Pro but no responses to the other 18 amino acids, indicating that CtaA, CtaB, and CtaC are major chemotaxis sensory proteins in *P. fluorescens* Pf0-1. Tomato root colonization by *P. fluorescens* strains was analyzed by gnotobiotic competitive root colonization assay. It was found that ctaA ctaB ctaC mutant was less competitive than the wild-type strain, suggesting that chemotaxis to amino acids, major components of root exudate, has an important role in root colonization by *P. fluorescens* Pf0-1. The ctaA ctaB ctaC triple mutant was more competitive than the cheA mutant of *P. fluorescens* Pf0-1, which is non-chemotactic, but motile. This result suggests that chemotactants other than amino acids are also involved in root colonization by *P. fluorescens* Pf0-1.

**Key words:** plant growth-promoting rhizobacteria, chemotaxis, methyl-accepting chemotaxis protein, *Pseudomonas fluorescens*, root colonization

Certain strains of *Pseudomonas fluorescens* exert beneficial effects on plants (3, 13, 17). These strains are able to enhance plant growth indirectly by preventing the growth or action of plant-pathogenic microorganisms. *P. fluorescens* F113 protects sugar beet seedlings from damping-off disease caused by the fungal pathogen *Pythium ultimum* by producing the antifungal metabolite 2,4-diacetylphloroglucinol, hydrogen cyanide, and extracellular protease (1, 22). *P. fluorescens* strain 2-79 is able to suppress wheat take-all produced by *Gaeumannomyces graminis* var. *tritici* (1, 22). *P. fluorescens* WCS365 is a biocontrol agent against the antifungal metabolite 2,4-diacetylphloroglucinol, hydrophilic periplasmic membrane-spanning region, a hydrophilic periplasmic

et al. showed that a hypermotile mutant of *P. fluorescens* F113, was more competitive for rhizosphere colonization than the wild-type strain and exhibited improved biocontrol activity against the pathogenic fungus *F. oxysporum* and the pathogenic oomycete *Phytophthora cactorum* compared with the wild-type strain (3). de Weert et al. demonstrated that cheA mutant of *P. fluorescens* WCS365, which is non-chemotactic but motile, colonized the tomato root tip less efficiently than the wild-type strain in the competitive root colonization assay (6).

Plant root exudates contain various organic compounds. Major components of tomato root exudate are amino acids (glutamic acid, aspartic acid, leucine, isoleucine, and lysine as major components [25]), organic acids (especially citric acid, malic acid and succinic acid [10]), and sugars (glucose and xylose as major components [14]). Previous studies demonstrated that *P. fluorescens* strains exhibit chemotactic responses toward plant seed and root exudates and their components (6, 20, 26, 30); therefore, it is supposed that chemotaxis to components of plant root exudates is involved in effective root colonization.

Methyl-accepting chemotaxis proteins (MCPs) are chemotaxis sensory proteins responsible for the detection of chemotactic ligands (11). MCPs are membrane-spanning homodimers and typical features of MCPs are as follows: a positively charged N terminus followed by a hydrophobic membrane-spanning region, a hydrophilic periplasmic
domain, a second hydrophobic membrane-spanning region and a hydrophilic cytoplasmic domain (7). Chemotactic ligands bind to periplasmic domains of MCPs and their binding initiates chemotaxis signaling. The diverse ligand specificities among MCPs reflect amino acid sequence diversities of periplasmic domains of MCPs. The C-terminal cytoplasmic domains of MCPs are relatively conserved. A 44-amino-acid domain. MCPs from phylogenetically diverse bacteria have specificities among MCPs reflect amino acid sequence diversities of periplasmic domains of MCPs. The C-terminal cytoplasmic domains of MCPs are relatively conserved. A 44-amino-acid domain. MCPs from phylogenetically diverse bacteria have specificities among MCPs reflect amino acid sequence diversities of periplasmic domains of MCPs. The C-terminal cytoplasmic domains of MCPs are relatively conserved. A 44-amino-acid domain. MCPs from phylogenetically diverse bacteria have specificities among MCPs reflect amino acid sequence diversities of periplasmic domains of MCPs. The C-terminal cytoplasmic domains of MCPs are relatively conserved. A 44-amino-acid domain. MCPs from phylogenetically diverse bacteria have specificities among MCPs reflect amino acid sequence diversities of periplasmic domains of MCPs. The C-terminal cytoplasmic domains of MCPs are relatively conserved. A 44-amino-acid domain. MCPs from phylogenetically diverse bacteria have specificities among MCPs reflect amino acid sequence diversities of periplasmic domains of MCPs. The C-terminal cytoplasmic domains of MCPs are relatively conserved. A 44-amino-acid domain. MCPs from phylogenetically diverse bacteria have specificities among MCPs reflect amino acid sequence diversities of periplasmic domains of MCPs. The C-terminal cytoplasmic domains of MCPs are relatively conserved. A 44-amino-acid domain. MCPs from phylogenetically diverse bacteria have specificities among MCPs reflect amino acid sequence diversities of periplasmic domains of MCPs. The C-terminal cytoplasmic domains of MCPs are relatively conserved. A 44-amino-acid domain. MCPs from phylogenetically diverse bacteria have specificities among MCPs reflect amino acid sequence diversities of periplasmic domains of MCPs. The C-terminal cytoplasmic domains of MCPs are relatively conserved. A 44-amino-acid domain. MCPs from phylogenetically diverse bacteria have specificities among MCPs reflect amino acid sequence diversities of periplasmic domains of MCPs. The C-terminal cytoplasmic domains of MCPs are relatively conserved. A 44-amino-acid domain. MCPs from phylogenetically diverse bacteria have specificities among MCPs reflect amino acid sequence diversities of periplasmic domains of MCPs. The C-terminal cytoplasmic domains of MCPs are relatively conserved.

**Materials and Methods**

**Bacterial strains, plasmids, and growth conditions**

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* JM109 (18) and S17-1 (23) were used for plasmid construction and transconjugation, respectively. *P. fluorescens*, *Pseudomonas aeruginosa* and *E. coli* strains were grown with shaking in 2× YT medium (18) supplemented with appropriate antibiotics. *P. aeruginosa* and *E. coli* strains were cultured at 37°C, while *P. fluorescens* strains were grown at 28°C.

**Chemotaxis assay**

The computer-assisted capillary assay method was carried out as described previously (16). Cells in a 10 μL suspension were placed on a coverslip, and the assay was started by placing the coverslip upside down on the U-shaped spacer to fill the chemotaxis chamber with the cell suspension. Cells were videotaped over 3 min. Digital image processing was used to count the number of bacteria accumulating toward the mouth of a capillary containing a known concentration of an attractant plus 1% (w/v) agarose. The strength of the chemotactic response was determined by the number of bacterial cell per frame. The chemotaxis buffer was 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid) buffer (pH 7.0).

**DNA manipulation**

Standard procedures were used for plasmid DNA preparations, restriction enzyme digestions, ligations, transformations, and agarose gel electrophoresis (18). PCR reactions were carried out using KOD Plus DNA polymerase (Toyobo, Tokyo, Japan) according to the manufacturer’s instructions. Oligonucleotides used for PCR are listed in Table 2. *P. aeruginosa* was transformed by electroporation as described previously (15). Plasmids were introduced to *P. fluorescens* strains by transconjugation using *E. coli* S17-1 (23).

**Plasmid construction and construction of deletion mutants of *P. fluorescens**
Pf0-1

The Pf01 0124, Pf01 0354, and Pf01 4431 genes were amplified from *P. fluorescens* Pf0-1 genome by PCR using PFL01f/1

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Table 1. Bacterial strains and plasmids used in this study

| Strain or plasmid       | Relevant characteristics                                           | Reference or source |
|-------------------------|------------------------------------------------------------------|---------------------|
| Pseudomonas fluorescens |                                                                   |                     |
| Pβ-1                    | wild-type strain                                                | 5                   |
| FL0124                  | Pβ-1 derivative, ActaA (Pfl01_4431) ActaC (Pfl01_0354)           | This study          |
| FL0354                  | Pβ-1 derivative, ActaA (Pfl01_4431) ActaB (Pfl01_0124) ActaC (Pfl01_0354) | This study          |
| FL4431                  | Pβ-1 derivative, ActaB (Pfl01_0124) ActaC (Pfl01_0354)           | This study          |
| FLD3                    | Pβ-1 derivative, ActaA ActaB ActaC (Pfl01_2038)                   | This study          |
| PB1-ΔcheA               | Pβ-1 derivative, ΔcheA                                          | This study          |
| PB1R1Rf                 | Pβ-1 derivative, spontaneous rifampicin-resistant mutant         | This study          |
| FLD3R1Rf                | FLD3 derivative, spontaneous rifampicin-resistant mutant, ActaA ActaB ActaC | This study          |
| Pseudomonas aeruginosa  |                                                                   |                     |
| PAO1                    | wild-type strain                                                | 9                   |
| PCT2                    | PAO1 derivative, ApclB ApclC                                      | 31                  |
| Escherichia coli        |                                                                   |                     |
| JM109                   | recA1, endA1, gyrA96, thi-1, hsdR17 (mcrA), e14+ (mcrB), supE44, relA1, Mlac-proABF*[traD36, proAB, lacI, lacZ ΔM15] | 18                  |
| S17-1                   | MM294 derivative, RP4-2 Tc::Mu-Km::Tn7 chromosomally integrated | 23                  |
| Plasmids                |                                                                   |                     |
| pUCP18                  | Escherichia-Pseudomonas shuttle vector; Cb'                       | 21                  |
| pFLCP01                 | pUC18 with a 2.2 kb PCR fragment containing ctaB (Pfl01_0124); Cb' | This study          |
| pFLCP02                 | pUC18 with a 2.1 kb PCR fragment containing ctaC (Pfl01_0354); Cb' | This study          |
| pFLCP03                 | pUC18 with a 2.2 kb PCR fragment containing ctaA (Pfl01_4431); Cb' | This study          |
| pK18mobsacB             | Km' pUC18 derivative, lacZa, mob site, sacB                       | 19                  |
| pUGMF001                | pK19mobsacB with a 1.2-kb PCR fragment upstream of ctaB (Pfl01_0124) and a 1.1-kb PCR fragment downstream of ctaB (Pfl01_0124); Km' | This study          |
| pUGMF02                 | pK19mobsacB with a 1.3-kb PCR fragment upstream of ctaC (Pfl01_0354) and a 1.4-kb PCR fragment downstream of ctaC (Pfl01_0354); Km' | This study          |
| pUGMF03                 | pK19mobsacB with a 1.3-kb PCR fragment upstream of ctaA (Pfl01_4431) and a 1.2-kb PCR fragment downstream of ctaA (Pfl01_4431); Km' | This study          |

Cb', carbenicillin resistance; Km', kanamycin resistance.
PFL01r, PFL02r, and PFL03r primer sets, and DPFL01Uf/DPFL01Ur and DPFL01Df/DPFL01Dr was conducted to amplify 1.2-kb regions upstream and downstream of backbone of Pf0-1 genome, respectively. Amplified upstream and downstream regions were digested with dIII-HindIII and XhoI, respectively, and ligated with the backbone of Pf0-1 genome, respectively. Amplified upstream and downstream regions were digested with HindIII-XhoI and XhoI-EcoRI, respectively, and ligated with the backbone of HindIII-EcoRI-digested pK18mobsacB (19) to obtain pUGMF01. PCR using primer sets DPFL02Uf/DPFL02Ur and DPFL02Dr/DPFL02Df was conducted to amplify a 1.4-kb upstream region and a 1.3-kb downstream region of Pf0-1 genome, respectively. Amplified upstream and downstream regions were digested with SaltI-BamHI and Spil-SalI, respectively, and ligated with the backbone of Spil-BamHI-digested pK18mobsacB to obtain pUGMF02. PCR using primer sets DPFL03Uf/DPFL03Ur and DPFL03Df/DPFL03Dr was conducted to amplify a 1.3-kb upstream region and a 1.2-kb downstream region of Pf0-1 genome, respectively. Amplified upstream and downstream regions were digested with HindIII-Xhol and Xhol-EcoRI, respectively, and ligated with the backbone of HindIII-EcoRI-digested pK18mobsacB (19) using suicide plasmids pUGMF01, pUGMF02, and pUGMF03, respectively. Unmarked gene deletion was confirmed by PCR using PCR primers specific to upstream and downstream sites of each gene.

Preparation of tomato root exudate

Exudate was prepared from a plant species, tomato (Solanum lycopersicum cv. Oogatahukuju). Tomato seeds were sterilized by autoclaved sand column. Tomato seeds (S. lycopersicum cv. Oogatahukuju) were sterilized as described at "Preparation of Pf0-1 genome" section. To synchronize germination, seeds were placed at the center of a growth tube and 5 mm below the surface of quartz sand. Bacterial cells were grown for 14 h in 2× YT medium, centrifuged (3,300×g, 2 min), washed three times with PNS, and adjusted to 10^6 CFU mL^{-1} in PNS. For the colonization assay, 100 µL bacterial cell suspensions were added to the edge of a plant growth tube. For the competitive colonization assay, 100 µL of 1:1 (v/v) mixture of the tested strain and the competitor were mixed and inoculated at the edge of a plant growth tube. The plant growth tubes were incubated in a climate-controlled growth chamber (NK System, Osaka, Japan) at 28°C, and 16 h of daylight. After 18 days, root exudates were collected and evaporated to dryness at 45°C under a vacuum, dissolved in 1 mL water, and sterilized by membrane filtration (0.45-µm pore size).

Selection for rifampicin resistance mutants

Spontaneous rifampicin-resistant mutants of Pf0-1 were generated by spreading bacterial cells, grown overnight in 2× YT, onto 2× YT agar plates containing 20 µg mL^{-1} rifampicin. The plates were incubated at 28°C for 20 h to form colonies. The resulting rifampicin-resistant colonies were then streaked on 2× YT agar containing 50 µg mL^{-1} rifampicin, and Rif' strains were subsequently maintained on this medium. One mutant showing a growth rate similar to that of the wild-type strain was selected and designated P0-1-Rif. Similarly, a rifampicin-resistant mutant of Pf0-1 obtained was designated PFL03-Rif.

Gnotobiotic root colonization assays

Forty grams of quartz sand (0.1 to 0.3 mm) was placed in glass tubes (22 mm inner diameter, 25 mm outer diameter, 12 cm length) and compacted by gentle shaking. The open end of the tube was plugged with a silicone resin stopper. The tube was then autoclaved for 15 min at 121°C. Ten milliliters of PNS (plant nutrient solution) was cooled, consisting of 1.25 mM Ca(NO_3)_2·2H_2O, 1.25 mM KNO_3, 0.5 mM MgSO_4·7H_2O, 0.25 mM KH_2PO_4, and trace elements (in mg L^{-1}): Fe (as FeEDTA), 4.6; B, 0.5; Zn, 0.05; Cu, 0.02; Mo, 0.01, was added to an autoclaved sand column. Tomato seeds (S. lycopersicum cv. Oogatahukuju) were sterilized as described at "Preparation of tomato root exudate" section. To synchronize germination, seeds were plated on Petri dishes containing PNS solidified with 1.5% (w/v) Bacto Agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubated overnight in the dark at 4°C, followed by incubation at 28°C for 2 days. A germinated seed was aseptically placed at the center of a growth tube and 5 mm below the surface of quartz sand. Bacterial cells were grown for 14 h in 2× YT medium, centrifuged (3,300×g, 2 min), washed three times with PNS, and adjusted to 10^6 CFU mL^{-1} in PNS. For the colonization assay, 100 µL bacterial cell suspensions were added to the edge of a plant growth tube. For the competitive colonization assay, 100 µL of 1:1 (v/v) mixture of the tested strain and the competitor were mixed and inoculated at the edge of a plant growth tube. The plant growth tubes were incubated in a climate-controlled growth chamber (28°C, 16 h daylight) to allow the plantlets to grow. After 7 days of growth in the plant growth tubes, the root systems of tomato were mostly unbranched. The root tip (1 to 2 cm length) was removed and shaken vigorously to obtain the root. The root was placed in 0.5 mL of PNS to remove bacteria. The bacterial suspension was diluted and 100 µL was plated on 2× YT agar plates. For the competitive colonization assay, the bacterial suspension was spread on 2× YT agar plates with and without rifampicin. For statistical analysis, the nonparametric Wilcoxon-Mann-Whitney test was used (27).

Results

Chemotactic responses toward amino acids by Pf0-1

Amino acids are one of the major tomato exudate components (25) and strong chemoattractants of Pf0-1 strains (6, 26, 28); therefore, we focused on the present study on the identification of a chemotaxis sensory protein(s) for amino acids in Pf0-1. We first measured chemotactic responses of Pf0-1 toward each of twenty commonly-occurring l-amino acids by the computer-assisted capillary assay (16). Pf0-1 exhibited chemotactic responses toward all twenty amino acids (Table 3). In particular, Cys, Gln, Gly, Ile, Lys,
Amino Acid Chemotaxis Sensors in P. fluorescens

Met, Phe, Pro, and Ser were strong chemoattractants of P. fluorescens Pf0-1, while it showed weak chemotactic responses toward Glu and Trp.

Identification of chemotaxis sensory proteins for amino acids

In Pseudomonas aeruginosa PAO1, PctA, PctB, and PctC have been identified as MCPs for amino acids (12, 31). There is 46–70% identity among amino acid sequences of periplasmic domains of these MCPs. To search for the PctA homologue of P. fluorescens Pf0-1, BLASTP analysis was performed on a protein database of P. fluorescens Pf0-1 by using the 250 amino acid sequence of the putative periplasmic domain of PctA (residues 28 to 277) as a query sequence. BLASTP search found that three proteins, Pfl01_4431, Pfl01_0124, and Pfl01_0354, showed the highest similarity to the query sequence (65%, 58%, and 46% identity, respectively). They possessed HCD in the C-terminal regions and have been annotated as MCPs on the basis of homology. Pfl01_4431, Pfl01_0124, and Pfl01_0354 are 59, 56, and 44% identical to the periplasmic domain of PctB (residues 27 to 277), and 49, 45, and 51% identical to that of PctC (residues 27 to 280).

To investigate whether Pfl01_4431, Pfl01_0124, and Pfl01_0354 act as MCPs for amino acids, their genes were cloned into broad-host-range plasmid pUCP18 (21), the resulting recombinant plasmids were introduced into the pctA, pctB, and pctC triple mutant of P. aeruginosa PCT2 (31), and the recombinant strains were examined for chemotactic responses to amino acids. Whereas P. aeruginosa PCT2 was non-chemotactic to all amino acids tested, PCT2 (pFLCP01 [containing the Pfl01_0124 gene]), PCT2 (pFLCP02 [containing the Pfl01_0354 gene]), and PCT2 (pFLCP03 [containing the Pfl01_4431 gene]) showed chemotactic responses to 16, 1, and 18 amino acids, respectively (Table 4), demonstrating that these proteins act as MCPs for amino acids. PCT2 (pFLCP01) and PCT2 (pFLCP03) showed strong responses to 5 amino acids (Ala, Asn, Lys, Met, and Ser) and 14 amino acids (Ala, Arg, Cys, Gly, His, Ile, Leu, Lys, Met, Pro, Ser, Thr, Trp, and Val).

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**Table 3.** Chemotactic responses of P. fluorescens Pf0-1 wild-type and mutant strains toward amino acids

| Compounds | Pf0-1 | FL0124 (ΔctaA ctaC) | FL0354 (ΔctaA ctaB) | FL4431 (ΔctaB ctaC) | FLD3 (ΔctaA ctaB ctaC) |
|-----------|-------|-----------------|-----------------|-----------------|-----------------|
| Ala       | ++    | +               | +               | +               | −               |
| Arg       | ++    | +++             | +               | +               | −               |
| Asn       | ++    | +               | −               | +++             | −               |
| Asp       | ++    | −               | −               | −               | −               |
| Cys       | +++   | +++             | +++             | +++             | +               |
| Gln       | +++   | +               | −               | −               | −               |
| Glu       | +     | +               | −               | −               | −               |
| Gly       | +++   | +               | +               | +               | −               |
| His       | +++   | ++              | +               | −               | −               |
| Ile       | +++   | +++             | +               | −               | −               |
| Leu       | ++    | −               | −               | −               | −               |
| Lys       | +++   | +               | −               | ++              | −               |
| Met       | +++   | ++              | +               | +++             | −               |
| Phe       | +++   | +               | +               | +++             | −               |
| Pro       | +++   | ++              | +               | +++             | +               |
| Ser       | +++   | ++              | −               | +++             | −               |
| Thr       | +     | −               | −               | −               | −               |
| Trp       | +     | −               | −               | −               | −               |
| Tyr       | ++    | +               | +               | +               | −               |
| Val       | ++    | +++             | −               | +++             | −               |

* Videotape frames were analyzed at the initiation of observation and 1 min after the initiation. Normalized cell numbers were calculated by dividing the number of bacteria at 1 min by that at the initiation of the observation. The strength of chemotaxis is presented as the normalized cell number: +++ > 3; ++ ≤ 3–2; + ≤ 2–1.5; −, no response.

**Table 4.** Chemotactic responses of P. aeruginosa PCT2 containing P. fluorescens Pf0-1 ctaA, ctaB, or ctaC genes toward amino acids

| Compounds | PCT2 (pFLCP01) (+ctaB) | PCT2 (pFLCP02) (+ctaC) | PCT2 (pFLCP03) (+ctaA) |
|-----------|-----------------|-----------------|-----------------|
| Ala       | +++             | −               | +++             |
| Arg       | +               | −               | +++             |
| Asn       | +++             | −               | +               |
| Asp       | ++              | −               | ++              |
| Cys       | +               | −               | +++             |
| Gln       | +               | −               | +               |
| Glu       | +               | −               | −               |
| Gly       | +               | −               | −               |
| His       | −               | −               | +++             |
| Ile       | −               | −               | +++             |
| Leu       | +               | −               | +++             |
| Lys       | +++             | −               | +++             |
| Met       | +++             | +               | +++             |
| Phe       | +               | −               | +++             |
| Pro       | +               | −               | +++             |
| Ser       | +++             | −               | +++             |
| Thr       | +               | −               | +++             |
| Trp       | +               | −               | +++             |
| Tyr       | +               | −               | +++             |
| Val       | +               | −               | +++             |

* Chemotactic responses are the same as in Table 3.
respectively, while PCT2 (pFLCP02) exhibited moderate responses only to Met. No common structural features are found among side chains of amino acids to which both PCT (pFLCP01) and PCT2 (pFLCP03) responded. Based on these results, the Pf01_4431, Pf01_0124, and Pf01_0354 genes were designated ctaA, ctaB, and ctaC (cta: chemotactic transducer of amino acids).

We constructed a ctaA ctaB ctaC triple mutant of *P. fluorescens* Pf0-1 (*P. fluorescens* FLD3) to assess the possibility of chemotaxis sensory protein(s) other than CtaA, CtaB, and CtaC. The triple mutant FLD3 showed moderate responses only toward Cys and Pro (Table 3). We then examined ctaB ctaC, ctaA ctaC, and ctaA ctaB double mutants of *P. fluorescens* Pf0-1 (*P. fluorescens* FL4431, FL0124, and FL0354, respectively) for their chemotaxis to amino acids to investigate the role of each MCP in amino acid chemotaxis in *P. fluorescens* Pf0-1. The ctaA ctaB double mutant showed a strong response to Met and Cys and weak or moderate responses to Arg, Gly, Pro, and Thr (Table 3). Since *P. fluorescens* FLD3 showed moderate responses to Cys and Pro, Met is the main chemotactant of CtaC. The ctaB ctaC and ctaA ctaC double mutants showed strong responses to several amino acids and their response patterns were similar to those of PCT2 (pFLCP03) and PCT2 (pFLCP03), respectively. These results suggest that CtaA and CtaB play the major roles in amino acid chemotaxis in *P. fluorescens* Pf0-1 (Fig. 1).

Of the major root exudate components other than amino acids, malic acid and succinic acid were strong attractants to *P. fluorescens* Pf0-1 (data not shown); therefore, we examined *P. fluorescens* FLD3 for its ability to respond to malic acid and succinic acid. It showed chemotactic responses to malic acid and succinic acid comparable to those by the parental strain (data not shown), suggesting that CtaA, CtaB, and CtaC are not involved in the detection of malic acid and succinic acid.

**Chemotaxis of *P. fluorescens* strains to tomato root exudate**

*P. fluorescens* Pf0-1 wild-type and mutant strains were tested for chemotaxis to tomato root exudate to assess the involvement of MCPs for amino acids in chemotaxis to the root exudate. *P. fluorescens* Pf0-1 wild-type strain was strongly attracted by tomato root exudate, while ctaA ctaB ctaC triple mutant showed much decreased responses (Fig. 2). The double mutants showed stronger responses to root exudate than the triple mutant, but weaker responses than the *P. fluorescens* Pf0-1 wild-type strain. In particular, ctaA ctaB double mutant showed the weakest responses among the double mutants. This result suggests that amino acids are the major chemotactants of *P. fluorescens* Pf0-1 in tomato root exudate. It also suggests that CtaA, CtaB, and CtaC are responsible for chemotaxis to root exudate to various degrees and that CtaC is less responsible than CtaA and CtaB.

**Root colonization analysis**

In order to investigate the importance of chemotaxis to amino acids in the root colonization process, we examined *P. fluorescens* Pf0-1, spontaneous rifampicin resistant mutant of Pf0-1 (Pf0-1Rif), the ctaA ctaB ctaC triple mutant, and its spontaneous rifampicin-resistant mutant (FLD3Rif) as well as double mutants for their root-colonizing ability by the gnotobiotic root colonization system. We also tested the cheA mutant of Pf0-1, which is a general non-chemotactic mutant, with the root colonization assay to confirm the report by de Weert *et al.* that flagella-driven chemotaxis is an important trait for tomato root colonization by *P. fluorescens* (6). We confirmed that there were no significant differences in growth in LB medium between mutants and the wild type Pf0-1. When germinated tomato seedlings were inoculated with single strains, all mutants colonized the tomato root to the same extent as the wild-type strain (Fig. 3A). We then carried out competitiveness assays between chemotaxis mutants and the wild-type strain by inoculating seedlings with a 1:1 mixture. Because Pf0-1Rif and FLD3Rif competed fully with Pf0-1 and FLD3, respectively (data not shown), we used Pf0-1Rif and FLD3Rif as competitor strains in competitive colonization assays to distinguish the competing strains from

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**Fig. 1.** Representation of 20 commonly-occurring L-amino acids detected by chemotaxis sensory proteins. This Venn diagram is constructed based on chemotactic responses by FL0124, FL0354, and FL4431 (Table 3).

**Fig. 2.** Chemotactic responses to tomato root exudate by *P. fluorescens* Pf0-1 (closed circles), FL0124 (open circles), FL0354 (open squares), FL4431 (closed squares), and FLD3 (open triangles). Digital image processing was used to count the number of bacteria around the mouth of a capillary containing exudates and 1% (w/v) agarose. One videotape frame was analyzed at each time point. The chemotactic response is presented as the normalized cell number. The normalized cell number was calculated by dividing the number of bacteria at each time point by that at the initiation of the observation. Vertical bars represent the standard deviations of measurements done in triplicate experiments.

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Fig. 3. Tomato root tip colonization by *P. fluorescens* strains, (A) alone and (B) in competition with Rif\(^{r}\) mutants. After 7 days, root systems were sampled. Bar indicates standard deviation. The total CFU numbers at the root tip in Fig. 3B were the same level as in Fig. 3A. There were significant \((P<0.05)\) differences in colonization between Pf0-1Rif and ΔcheA, Pf0-1Rif and FLD3, FLD3Rif and ΔcheA, FLD3Rif and Pf0-1, and FLD3Rif and FL0124.
tested strains. As previously shown by de Weert et al. (6),
the non-chemotactic cheA mutant was a very poor competitor
and showed more than 10-fold reduced ability to colonize
tomato roots (Fig. 3B). The ctaA ctaB ctaC triple mutant
exhibited higher competitive colonization ability than the
cheA mutant, but it still showed an approximately 2-fold
impaired colonization ability in the competitive colonization
assay. This result indicates that chemotaxis to amino acids
plays a role in the root colonization process by P. fluorescens.
FL0124 (ctaA ctaC double mutant) and FL4431 (ctaB ctaC
double mutant) showed almost 2-fold superior colonization
ability than FLD3Rif, while FL0354 (ctaA ctaB) showed only
1.3-fold superior colonization ability than FLD3Rif. We then
examined competitiveness between ΔcheA (cheA mutant) and
FLD3Rif (ctaA ctaB ctaC triple mutant). As shown in Fig.
3B, FLD3Rif is more competitive than cheA mutant.

Discussion

There are two classes of MCP for amino acids in bacteria.
One class includes Tar and Tsr of E. coli and Salmonella
enterica serovar Typhimurium, and the other includes P.
aeruginosa PAO1 PctA. Tsr is an MCP for the attractants
Ser, Ala and Gly, while Tar is an MCP for attractants Asp.
Pseudomonas aeruginosa impaired colonization ability in the competitive colonization
assay. This result indicates that chemotaxis to amino acids
plays a role in the root colonization process by P. fluorescens.

Additionally, ctaABC mutant was more competitive for
root colonization than cheA mutant (Fig. 3B), suggesting that
chemoattractants other than amino acids are involved in root
colonization. Since P. fluorescens PFO-1 exhibits marked
responses to organic acids (especially succinic acid and malic
acid) (data not shown), we suppose that chemotaxis to organic
acids such as succinic acid and malic acid is also involved
in root colonization by P. fluorescens. We are now searching
MCPs for organic acids in P. fluorescens PFO-1 to investigate
the involvement of chemotaxis to organic acids in root
colonization.

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