# Increased Uptake of Putrescine in the Rhizosphere Inhibits Competitive Root Colonization by *Pseudomonas fluorescens* Strain WCS365

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**Sequence analysis of the chromosomal Tn*5lacZ* flanking regions of the *Pseudomonas fluorescens* WCS365 competitive root colonization mutant PCL1206 showed that the Tn*5lacZ* is inserted between genes homologous to *bioA* and *potF*. The latter gene is the first gene of the *potF*$_1$*F*$_2$*GHI* operon, which codes for a putrescine transport system in *Escherichia coli*. The position of the Tn*5lacZ* suggests an effect on the expression of the *pot* operon. A mutation in the *potF*$_1$ gene as constructed in PCL1270, however, had no effect on competitive root colonization. The rate of uptake of [1,4-$^{14}$C]putrescine by cells of mutant PCL1206 appeared to be increased, whereas cells of strain PCL1270 were strongly impaired in the uptake of putrescine. Dansylation of tomato root exudate and subsequent thin-layer chromatography showed the presence of a component with the same R$_f$ value as dansyl–putrescine, which was identified as dansyl–putrescine by mass spectrometric analyses. Other polyamines such as spermine and spermidine were not detected in the root exudate. Growth of mutant strains, either alone or in competition with the wild type, was tested in media containing putrescine, spermine, or spermidine as the sole nitrogen source. The results show that mutant PCL1206 is strongly impaired in growth on putrescine and slightly impaired on spermine and spermidine. The presence of the polyamines had a similar effect on the growth rate of strain PCL1270 in the presence of putrescine but a less severe effect in the presence of spermine and spermidine. We conclude that an increased rate of putrescine uptake has a bacteriostatic effect on *Pseudomonas* spp. cells. We have shown that putrescine is an important tomato root exudate component and that root-colonizing pseudomonads must carefully regulate their rate of uptake because increased uptake causes a decreased growth rate and, therefore, a decreased competitive colonization ability.**

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The efficient colonization of the plant root system to deliver antifungal metabolites is an important and often limiting factor in the biocontrol of soilborne diseases by bacteria (Bull et al. 1991; Schippers et al. 1987; Weller et al. 1988). Colonization of plant surfaces by microbes can play a crucial role in the biocontrol of plant diseases (Chin-A-Woeng et al. 2000) and is likely to play an important role in processes such as rhizoremediation, phytostimulation, and biofertilization (Lugtenberg and Dekkers 1999). Identification of root colonization traits will therefore lead to a better understanding of these processes.

Traits identified as important for root colonization are motility (De Weger et al. 1987), the presence of the O antigen of lipopolysaccharide (De Weger et al. 1989; Dekkers et al. 1998c), synthesis of amino acids (Simons et al. 1997), growth rate and synthesis of vitamin B1 (Simons et al. 1996), a two-component system (Dekkers et al. 1998a), and site-specific recombination (Dekkers et al. 1998b). Moreover, a role for NADH–ubiquinone oxidoreductase in competitive colonization was suggested (Dekkers et al. 1998b). The root colonization gene *sss*, which encodes a site-specific recombinase (Dekkers et al. 1998b), has been used recently to improve root colonization of other wild-type *Pseudomonas* spp. strains (Dekkers et al. 2000).

In a screening procedure aimed at the isolation of root colonization mutants of the efficient root-colonizing *Pseudomonas fluorescens* strain WCS365 (Geels and Schippers 1983), strain PCL1206 was isolated because it was 10- to 50-fold impaired in competitive root colonization on tomato following seedling inoculation with a 1:1 mixture of mutant and parental strains (Simons et al. 1996). Furthermore, its impaired root-colonizing ability was observed on the roots of radish and wheat (Dekkers et al. 1998c), indicating a broad host range. Here, we report the molecular characterization of mutant PCL1206.

## RESULTS

### Genetic characterization of competitive root colonization mutant PCL1206.

In order to identify the chromosomal sequences flanking the Tn*5lacZ* in mutant PCL1206, total chromosomal DNA was isolated and digested with *Sal*I. The digested DNA fragments were subcloned into pIC20H (Marsh et al. 1984) and transformed into *Escherichia coli* DH5α cells. Selection for kanamy-

cin resistance resulted in the isolation of plasmid pMP5252. Plasmid pMP5252 harbors a *Sal*I insert, which consists of part of the Tn*5lacZ* containing the Km$^r$ gene and a chromosomal DNA fragment of approximately 2.5 kb. In order to obtain a chromosomal clone to identify a larger region around the Tn*5lacZ*, a lambda FIX II genomic library of strain WCS365 was screened. The DNA probe used for this screening was obtained with the chromosomal DNA insert of pMP5252 as a template for a polymerase chain reaction (PCR) with primers oMP508 and oMP509. The resulting PCR fragment consisted of 0.47 kb of the first open reading frame (ORF) downstream of the Tn*5lacZ* insertion and was cloned into pIC20H with *Pst*I and *Kpn*I, resulting in pMP5401. Screening of the genomic library resulted in the isolation of a DNA fragment of approximately 11 kb, which was cloned into a pBluescript vector (Stratagene, La Jolla, CA, U.S.A.), resulting in plasmid pMP5403.

Sequence analyses of the chromosomal clones revealed that the Tn*5lacZ* is inserted between two ORFs that are transcribed in the same direction (Fig. 1). The first ORF shows 76% identity and 86% similarity at the amino acid level with the *bioA* homolog of *Pseudomonas aeruginosa* PAO1 (Stover et al. 2000) coding for an aminotransferase. Because the Tn*5lacZ* is inserted downstream of the stop codon of this ORF, the insertion does not affect the transcription of the *bioA* homolog. The second ORF has 57% identity and 75% similarity at the amino acid level with the *potF* gene from *E. coli* (Pistocchi et al. 1993). This gene encodes a periplasmic putrescine binding protein and is part of a transport system for putrescine. This transport system can be classified in the ATP-binding cassette (ABC) superfamily in the subclass of the polyamine/opine/phosphonate uptake transporter family. Downstream of this ORF, named *potF*$_1$, a second ORF with 56% identity and 74% similarity at the amino acid level with the *potF* gene of *E. coli* was found and tentatively named *potF*$_2$. Downstream of *potF*$_1$ and *potF*$_2$, ORFs were revealed with amino acid sequence homology to *potG* (59% identity, 71% similarity), *potH* (56% identity, 75% similarity), and *potI* (57% identity, 70% similarity), respectively. In *E. coli*, these genes code for proteins that form a putrescine transport system (Pistocchi et al. 1993). No additional ORF was located in the first 400 bp located downstream of the *potI* homolog. Upstream of the *bioA* gene, another ORF transcribed in the same direction was identified. This ORF has homology to the *glnA* gene of *P. aeruginosa* PA01 coding for a glutamine synthetase homolog (Stover et al. 2000). Homology studies with the complete genome of the *P. aeruginosa* gene bank revealed a similar *pot* operon with an identical organization of genes (results not shown). In contrast to the *E. coli pot* operon, *P. aeruginosa* and *P. fluorescens* WCS365 contain two highly homologous *potF* genes, which are located next to each other. The identity/similarity between the components of the WCS365 *pot* operon and the *P. aeruginosa pot* operon are 75/83%, 52/56%, 67/71%, 64/67%, and 62/66% for the *potF*$_1$, *potF*$_2$, *potG*, *potH*, and *potI* genes, respectively.

Analyses of the five ORFs encoding the PotF$_1$, PotF$_2$, PotG, PotH, and PotI proteins revealed frames of 370, 365, 380, 306, and 274 amino acids, respectively, corresponding with calculated molecular weights of 40,015, 39,977, 42,227, 34,268, and 29,728, respectively.

Alignment of the amino acid sequences of PotF$_1$ and PotF$_2$ from PCL1206 resulted in the identification of several highly conserved domains. Comparison of these domains with the amino acid sequence described for the *E. coli* PotF protein (Vassylyev et al. 1998) showed the presence of conserved N-terminal domains consisting of residues Trp, Ser, Asp, and Tyr in both proteins, which are involved in the binding of putrescine in *E. coli*. In *E. coli*, Trp-244, Asp-247, Phe-276, and Asp-278 are described as C-domain residues involved in the binding of putrescine (Vassylyev et al. 1998). The sequences of PotF$_1$ and PotF$_2$ of strain WCS365 also contain those residues in these regions. Two conserved cysteine residues also are present in both protein sequences, which indicates a disulfide bridge because these amino acids are located in the same region as in the *E. coli* PotF. The protein-processing site, which is described in the *E. coli* PotF that is located between amino acids Ala-26 and Ala-27, could be located in WCS365 between Ala-28 and Asp-29 in PotF$_1$ and Ala-25 and Ala-26 in PotF$_2$, according to the prediction program Signal P (Nielsen et al. 1997).

When we compared the hydrophobicity patterns of the five proteins of the *pot* operon (window length of 7), no clear hydrophobic regions were detected in PotF$_1$, PotF$_2$, and PotG (data not shown). In PotH and PotI, however, six putative transmembrane segments were identified (data not shown). The region linking the fourth and the fifth transmembrane segment contained the conserved residues EAA(M/S)DLG, which are described in the *E. coli* PotH and PotI proteins as the putative binding residues for the PotG protein (Pistocchi et al. 1993).

### Construction of a mutation in the *potF*$_1$ gene of *P. fluorescens* WCS365.

To be able to test the phenotype of a mutation in the *pot* operon, a mutant of WCS365 was constructed with a homologous recombination within the first *potF* gene. For that purpose, a *Sma*I–*Sma*I fragment of 600 bp was isolated from pMP5252 containing the central part of the *potF*$_1$ gene. This fragment was first cloned in pIC20H (Marsh et al. 1984), resulting in pMP5290, and subsequently cloned as an *Eco*RI–*Sal*I fragment to pMP5285, which is a derivative of pME3049 (Voisard et al. 1994) obtained after digestion with *Hin*dIII and subsequent religation. The resulting plasmid, pMP5291, was integrated in the WCS365 chromosome, resulting in mutant strain PCL1270. The stability of the insertion was tested after a competitive root tip colonization assay and appeared to be 100% stable. With the use of *Eco*RI, plasmid pMP5291 could be rescued together with flanking chromosomal DNA from the homologous recombinant PCL1270, resulting in pMP5299. Sequence analysis of pMP5299 showed that, indeed, pMP5291 is inserted in the *potF*$_1$ gene (results not shown).

### Competitive tomato root tip colonization between *P. fluorescens* WCS365 and PCL1270.

Colonization of tomato root tips after inoculation of seedlings with a 1:1 mixture of PCL1270 and PCL1500, a Tn*5lacZ*
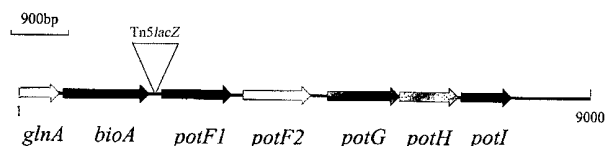


**Fig. 1.** Chromosomal location of the Tn*5lacZ* in the competitive root colonization-defective mutant PCL1206. Arrows indicate the size and direction of open reading frame transcriptions. Indicated genes are homologs of *glnA*, glutamine synthetase; *bioA*, aminotransferase; *potF*$_1$, putrescine binding protein; *potF*$_2$, putrescine binding protein; *potG*, nucleotide binding protein; *potH*, transmembrane protein; *potI*, transmembrane protein.
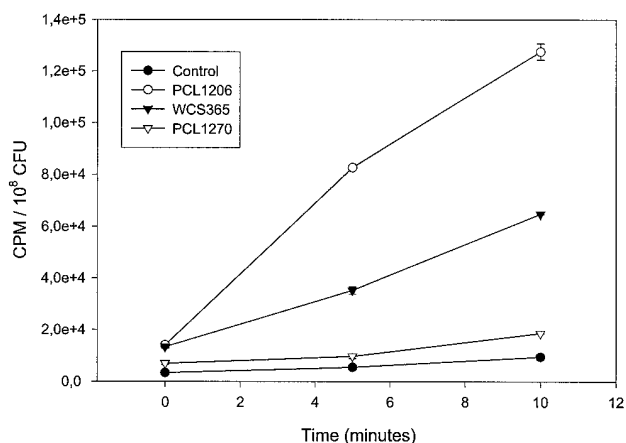
**Fig. 2.** [1,4-$^{14}$C] putrescine uptake by *Pseudomonas fluorescens* WCS365 and its mutant derivatives PCL1206 and PCL1270. Cells were diluted to $10^8$ CFU/ml in BM medium, without a nitrogen source. [1,4-$^{14}$C]-labeled putrescine was added to a final concentration of 50 µM. Cells were collected on membrane filters and washed, and the radioactivity was quantified with liquid scintillation counting. Results are the mean of duplicate samples. The results of two other experiments revealed similar data.

derivative of WCS365 that is not impaired in root tip colonization (Van der Bij et al. 1996), showed no significant difference in log colony forming units (CFU) per centimeter of root tip between the two strains. In two independent experiments, the bacterial numbers depicted as log CFU per centimeter of root tip for PCL1270 and PCL1500 were 3.6 ± 0.5 and 3.3 ± 0.6, respectively, and 4.1 ± 0.4 and 4.0 ± 0.4, respectively (each result is the mean value of 10 plants).

## Rate of uptake of [1,4-$^{14}$C]putrescine by WCS365 and its mutant derivatives PCL1206 and PCL1270.

The rate of uptake of putrescine by mutants PCL1206 and PCL1270 was compared with that of the wild-type strain. The rate of [1,4-$^{14}$C]putrescine uptake by PCL1206 is increased, whereas the uptake rate by PCL1270 cells was very low (Fig. 2). Similar results were obtained in standard BM medium (Lugtenberg et al. 1999) (results not shown). Dilution platings of the cultures on King's B (KB) (King et al. 1954) agar plates showed no changes in the number of CFU during the experiment.

## Thin-layer chromatography (TLC) analysis of dansylated tomato root exudate.

Silica TLC separation of the dansylated polyamines cadaverine, putrescine, spermidine, and spermine resulted in spots with
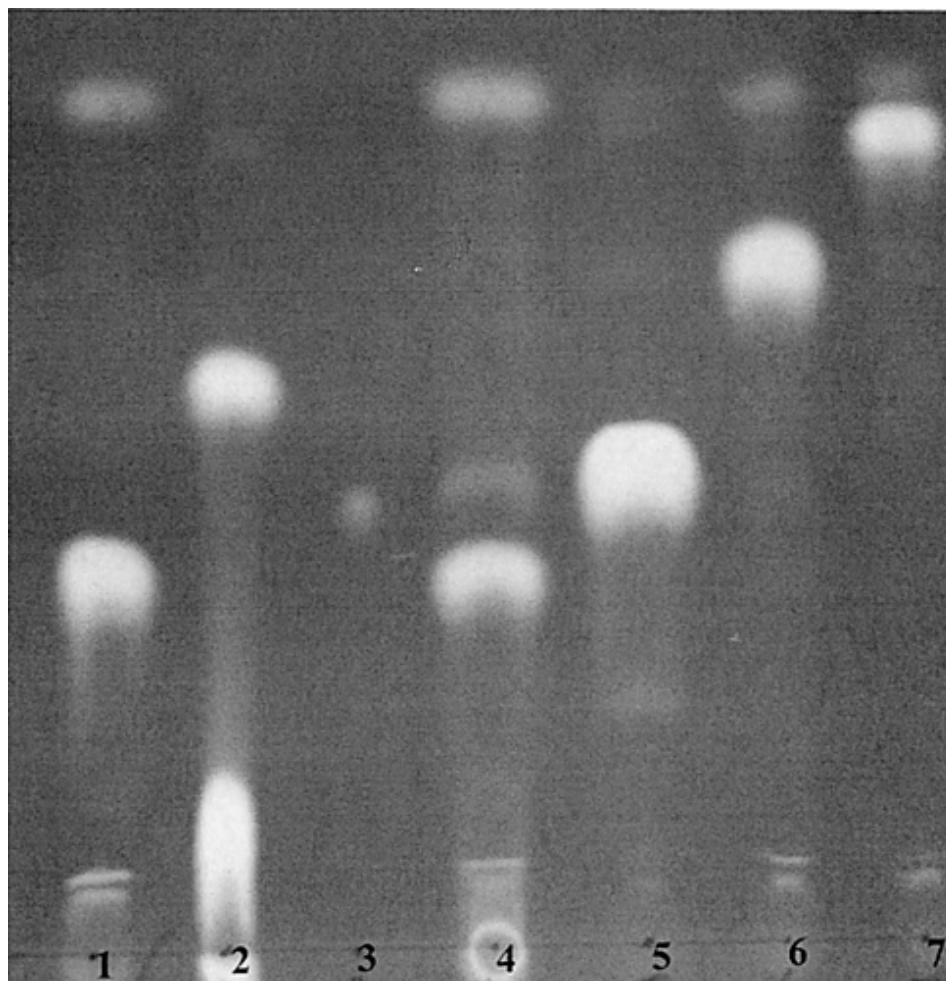


**Fig. 3.** Silica thin-layer chromatographic separation of dansylated tomato root exudate sample. Dansylated samples of ammonium sulfate (lane 1), cadaverine (lane 2), reference dansyl–putrescine (lane 3), tomato root exudate (lane 4), putrescine (lane 5), spermidine (lane 6), and spermine (lane 7) are shown.

different $R_f$ values (Fig. 3, lanes 2, 5, 6, and 7). TLC separation of the dansylated amino acids aspartic acid, glutamic acid, leucine, and lysine resulted in spots with $R_f$ values different from those of the polyamines (data not shown).

Dansylation of tomato root exudate, followed by silica TLC fractionation, showed that in the exudate, a compound is present with a $R_f$ value of 0.53 (Fig. 3, lane 4), which is the same as the $R_f$ value obtained for standard dansyl–putrescine (Fig. 3, lane 3). A second spot has the same $R_f$ value (0.17) as dansylated ammonium and several dansylated amino acids. TLC analysis of dansylated plant nutrient solution (PNS) medium in which the exudate was collected did not result in detectable components (data not shown). No significant spots in the root exudate sample were present at $R_f$ values corresponding to dansylated cadaverine, spermidine, and spermine.

## Structural identification
## of putrescine as a root exudate component.

In order to discern whether the fluorescent TLC spot with $R_f$ value 0.53 from the dansylated root exudate is dansyl–putrescine, the fluorescent TLC spot obtained from the dansyl–putrescine standard and the root exudate spot with the similar $R_f$

value were scraped off the TLC plate. Subsequently, the fluorescent material was extracted and the extract was analyzed with electrospray (ES) mass spectrometry and ES–collision induced dissociation (CID) tandem mass spectrometry.

The ES mass spectrum of standard dansylated putrescine yielded, as the base peak, a singly charged protonated molecule at $m/z$ 555.0, which corresponds to the mass of putrescine bearing two dansyl groups, as expected for a diamine. This peak is accompanied by further peaks also arising from dansyl–putrescine at $m/z$ 577.1 (M+Na$^+$), 278.3 (M+2H$^{2+}$), and 300.3 (M+2Na$^{2+}$). The spectrum obtained from the fluorescent root exudate spot also contains peaks at $m/z$ 555.0 and 300.4, although the intensities of these peaks are lower than those in the spectrum from the standard. These observations are consistent with those obtained on TLC and indicate that putrescine is present in the root exudate. To identify the root exudate spot and thus confirm this conclusion, ES–CID tandem mass spectrometry was performed.

The product ion spectra of the singly charged parent ions at $m/z$ 555 were recorded from the standard putrescine and root exudate spot, yielding very similar spectra (Fig. 4A and B). As expected from the literature describing the mass spectrometric
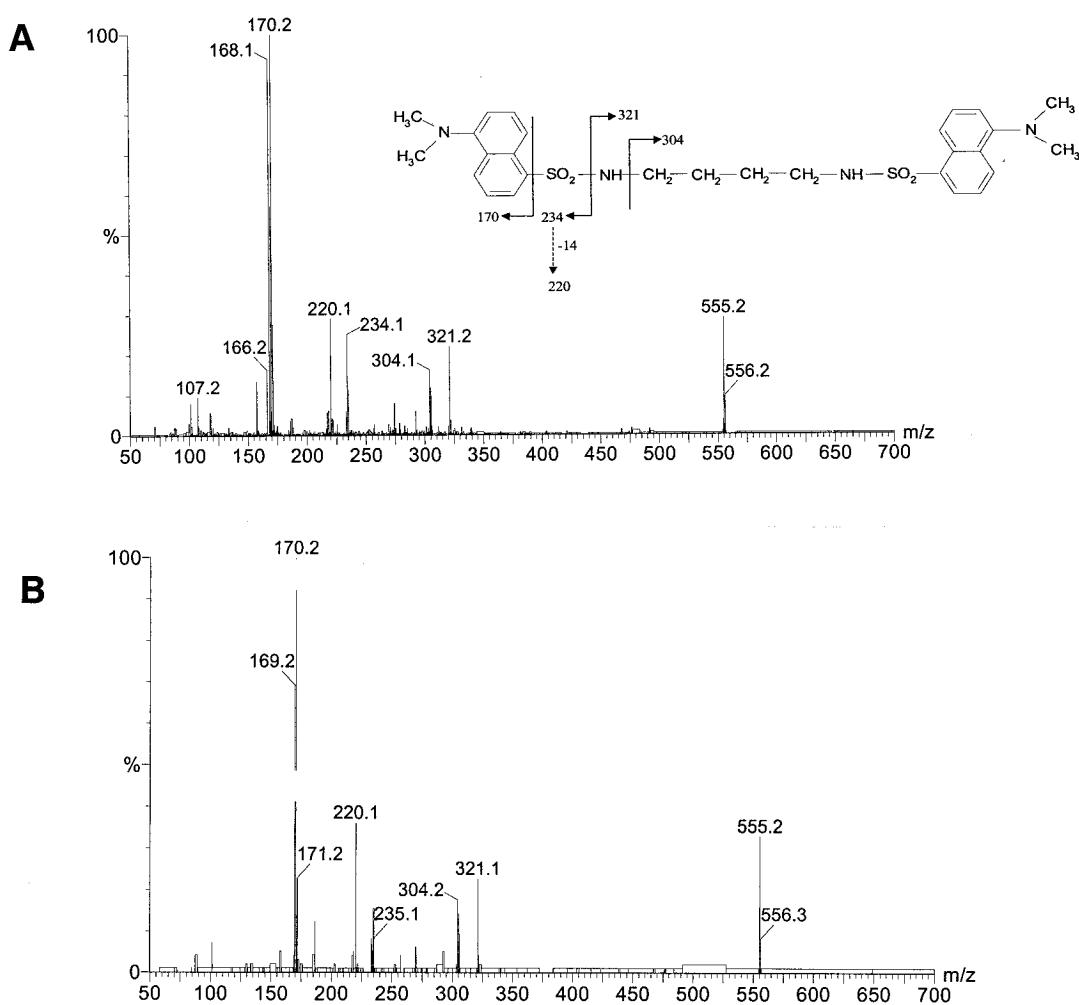


**Fig. 4.** Structural identification of dansylated putrescine present in dansylated tomato root exudate. **A,** Fragmentation scheme and electrospray (ES)–collision induced dissociation (CID) tandem mass spectrometry (MS) spectrum of standard dansylated putrescine and **B,** ES-CID tandem MS spectrum of the dansylated root exudate spot.

behavior (under electron ionization, fast atom bombardment, ES, and CID conditions) of a range of dansylated compounds (Dalvie and O'Donnel 1998; Feistner 1995), the spectra obtained are dominated by ions that arise from the dansyl derivatizing group. The base peak in the CID spectra is $m/z$ 170, which corresponds to the dimethyl aminonaphthalene group (Fig. 4A, insert). This peak is flanked by ions at $m/z$ 169 and 171, which are described as being characteristic of dansylated amines (Walter et al. 1987). In addition to this major peak, other peaks are observed at $m/z$ 321, 304, 234, and 220. The first and third of these arise from the cleavage between the dansyl sulfur and the putrescine nitrogen atoms (Fig. 4A, insert), with charge retention on the dansyl–putrescine [M+H-dansyl; (M+H-234)]$^+$ or the dansyl moieties, respectively. The peak at $m/z$ 304 arises on the loss of a dansyl amine group such as [M+H-dansyl-NH$_3$; (M+H-234-17)]$^+$ (Fig. 4A, insert), whereas the peak at $m/z$ 220 is the result of methyl loss from the $m/z$ 234 ion. The very close similarity of the CID spectra and the demonstration that the spots correspond to dansyl–putrescine shows that putrescine is, indeed, present in the root exudate.

On the basis of the comparison on TLC with the putrescine standard, we estimate that the concentration of putrescine present in the root exudate is 10 to 20 µg per ml. Because the exudate of 10 seedlings was collected in 10 ml of PNS solution, the exudate of one plant contains approximately 10 to 20 µg of putrescine after 14 days of growth.

### Growth of *P. fluorescens* WCS365 and its derivatives PCL1206 and PCL1270 on putrescine, spermine, and spermidine as the sole nitrogen source.

In order to test whether the mutations in PCL1206 and PCL1270 affect the ability to use putrescine and other polyamines as the sole nitrogen source, the growth of the mutants and the parental strain in media with one of the various polyamines as the sole source of nitrogen was compared. For wild-type WCS365, the growth rate on putrescine as the sole nitrogen source was similar to the growth rate on the commonly used nitrogen source ammonium sulfate, with generation times of approximately 100 min. The growth rates of mutants PCL1206 and PCL1270 in modified BM media with putrescine as the sole nitrogen source were lower than that of wild-type strain WCS365 when strains were grown alone (generation times of approximately 250 min). The lag phases of the mutants also were longer than that of the wild type. In the case of PCL1206, growth on the polyamines spermine and spermidine as sole nitrogen source also was slightly decreased because of an extended lag phase, whereas no differences in growth rates on these polyamines were observed for PCL1270.

Competitive growth between PCL1206 and WCS365 in liquid BM medium with putrescine as the sole nitrogen source showed that PCL1206 is out competed by WCS365 (Fig. 5A). Competition experiments with spermine and spermidine as the sole nitrogen sources showed less stringent effects (Fig. 5B and C). Mutant PCL1270 also loses in competition with the wild-type strain derivative PCL1500 when grown on putrescine as the sole nitrogen source (Fig. 5D). Competitive growth on spermine and spermidine showed no differences during the first 25 h. Differences were observed after 25 h, however, when PCL1270 seemed to reach a stationary phase earlier than PCL1500 (data not shown).

## DISCUSSION

*P. fluorescens* WCS365 mutant strain PCL1206 is impaired in its ability to colonize the root tips of tomato, wheat, and radish plants when tested in competition with its wild type (Dekkers et al. 1998b). In order to elucidate the trait, which causes the decrease in colonizing ability, we identified the chromosomal region flanking the Tn*5lacZ* insertion. The transposon appeared to be inserted between two ORFs (Fig. 1). The first ORF has homology to *bioA* of *P. aeruginosa* PA01 (Stover et al. 2000), whereas the second ORF shows high homology with *potF* of *E. coli* (Pistocchi et al. 1993). Because the stop codon of the *bioA* gene is located 10 bp upstream of the Tn*5lacZ* insertion in PCL1206, this gene was not affected. Sequencing of the DNA fragment downstream of the first *potF* gene showed that, after a second *potF* gene, *potG*, *potH*, and *potI* homologs also are present (Fig. 1).

In *E. coli*, the *pot* operon consists of the *potFGHI* genes, which encode an ABC putrescine transport system in which all four proteins are required for optimal transport activity (Pistocchi et al. 1993). The *potF* gene codes for a periplasmic putrescine binding protein. Vassylyev et al. (1998) described the crystal structure of the PotF protein, which consists of two similar, globular domains, between which putrescine is bound. Additionally, the residues important for the binding of putrescine and protein folding are present in the two PotF proteins of WCS365, indicating a similar function. The *potG* gene codes for a nucleotide binding protein, whereas *potH* and *potI* code for transmembrane proteins (Pistocchi et al. 1993). The WCS365 sequences of *potH* and *potI* also contain putative transmembrane domains and, in both proteins, a putative domain for the binding of the PotG protein is present.

In *E. coli* the promoter region of the *pot* operon is described as being located 155 bp upstream of the *potF* gene (Pistocchi et al. 1993). By interfering with a probable promoter region, the position of the Tn*5lacZ* insertion in PCL1206 is suggested to affect transcription of the *pot* genes. In PCL1206, no conserved promoter sequences were located in the 59-bp intergenic region between *bioA* and the *potF*$_1$.

Uptake of polyamines can be repressed, as shown in *E. coli* for the *potABCD* system (Furuchi et al. 1991), a second system for the uptake of polyamines. This system has a high affinity for spermidine and a low affinity for putrescine. Antognoni et al. (1999) suggested the presence of two regions near the transcriptional initiation site that are important in the transcriptional inhibition of the operon through the binding of the precursor of the PotD protein to these DNA regions.

Because of the mutation in PCL1206, tomato root exudate was analyzed for the presence of polyamines, and dansylation was used to detect polyamines. We used this technique to distinguish the different amino acids, known to be present in the root exudate (Simons et al. 1997) and the polyamines putrescine, spermine, spermidine, and cadaverine. Putrescine was the only polyamine that we could detect in this exudate (Figs. 3 and 4). As far as we are aware, there are no reports which show the presence of putrescine or other polyamines in root exudates. The amount of putrescine in root exudate is approximately 60 nmol per plant after 14 days of plant growth, whereas glutamic acid, the most abundant amino acid in tomato root exudate, is estimated to be present in 4.4 nmol per plant after 7 days of plant growth (Simons et al. 1997).

Many functions of polyamines in bacterial cells have been described, i.e., in the protection of DNA, protein synthesis, cell growth, proliferation (Karahalios et al. 1998; Nastri and Algranati 1988; Tabor and Tabor 1985), resistance toward antibiotics (Diniello et al. 1998; Kashiwagi et al. 1992; McKee et al. 1991; Nastri and Algranati 1996; Taber et al. 1987), and outer membrane permeability (DeLa Vega and Delcour 1996; Iyer and Delcour 1997; Koski and Vaara 1991). These studies describe the importance of the intracellular putrescine concentration, which depends partially on biosynthesis and partially on the uptake and secretion of putrescine. This suggests that the fitness of bacterial cells, which is very important during competitive colonization of plant roots, is not optimal when putrescine uptake is increased.

Uptake studies with [1,4-$^{14}$C]putrescine show that the Tn5 insertion in the promoter region of the *pot* operon (PCL1206) results in an increase in the uptake of putrescine, whereas a mutation in the first gene of the operon (PCL1270) results in impaired putrescine uptake (Fig. 2). This can be explained by the presence of a sequence within the promoter region, which is involved in the inhibition of the transcription of the *pot* operon, the function of which is disrupted by the Tn5 transposon. In the case of WCS365, such a transcriptional inhibition system also could be present. The colonizing ability of PCL1206 if decreased,

probably as a result of increased levels of intracellular putrescine, which would have an as-yet-unknown effect on the functioning of the cell. Experiments in which the survival of PCL1206 cells was tested at different concentrations (up to 1 mM) of putrescine showed no lethal effects on PCL1206 (data not shown).

To test whether mutants PCL1206 and PCL1270 are impaired in growth on putrescine as a nitrogen source, growth was tested in liquid BM medium with putrescine as the sole nitrogen source and compared with the wild type. PCL1206 and PCL1270 are able to grow on putrescine when inoculated in this medium alone, but the lag phase is extended. This indicates that PCL1206 and PCL1270 cells can adapt to growth on putrescine, presumably by activation of other systems such as putrescine binding proteins (Kulakova et al. 1997) or other polyamine transport proteins because *P. aeruginosa* contains, in addition to the two PotF proteins situated in the *potFGHI* operon, two PotF-like proteins with 54% identity, one with 43% identity, and two with 35% identity on the amino acid level. In competition with the wild type, PCL1206 and PCL1270 are strongly impaired in growth on putrescine (Fig. 5A). PCL1206 and, to a lesser extent PCL1270, also are impaired in growth on spermine and spermidine when grown in competition with the wild type (Fig. 5B and C). The latter effect is less pronounced, however, than that observed when grown on putrescine.
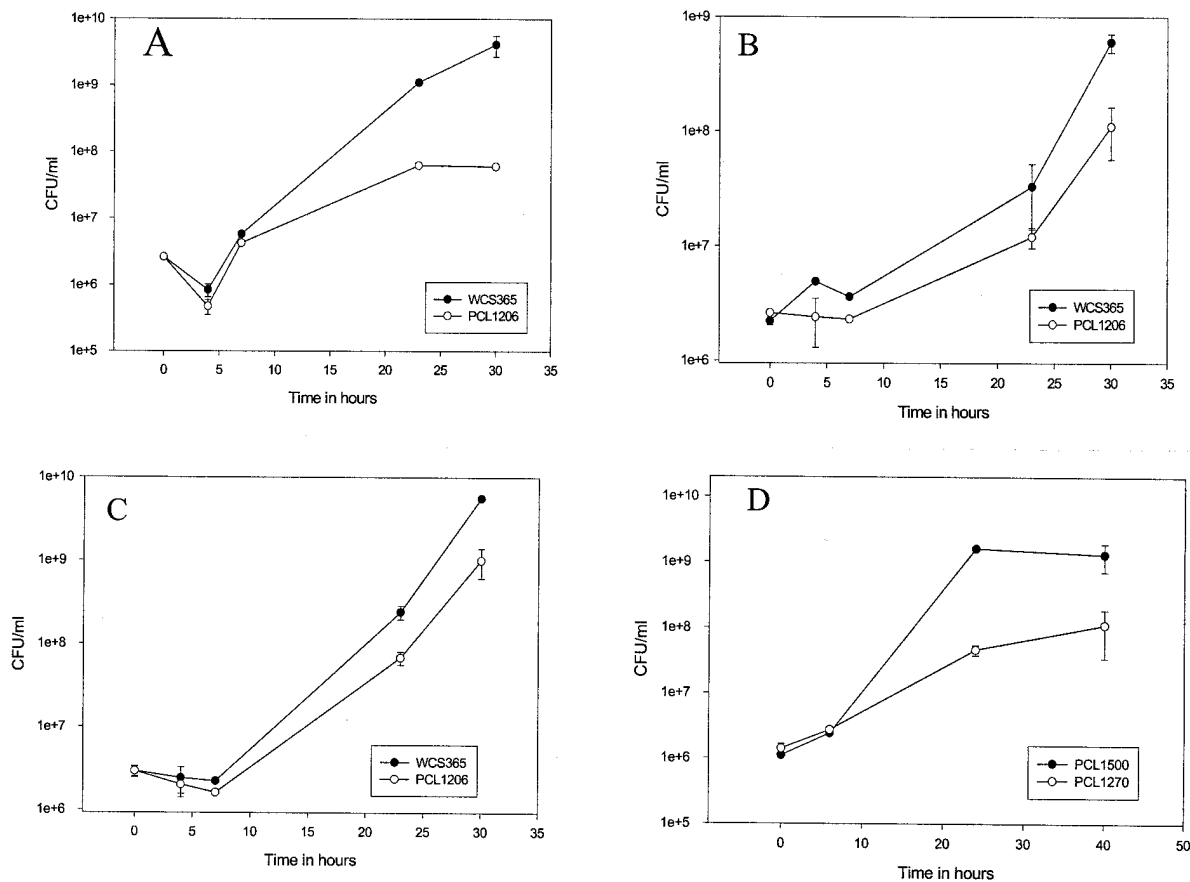


**Fig. 5.** Competitive growth between *Pseudomonas fluorescens* WCS365 (PCL1500) and its mutants, PCL1206 and PCL1270. Growth competition between WCS365 and PCL1206 was analyzed in modified BM medium with the polyamines **A,** putrescine, **B,** spermine, or **C,** spermidine as the sole nitrogen source. **D,** Competitive growth between PCL1270 and PCL1500 was analyzed in modified BM medium with putrescine as the sole nitrogen source. Cells from cultures grown overnight in standard BM medium were washed, mixed in a 1:1 ratio, and subsequently diluted to a final concentration of 10$^6$ CFU/ml. Samples were taken after various time intervals, diluted, and plated on King's B plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside to distinguish the two bacterial strains.

Because the growth rate on putrescine in a minimal medium is comparable to the growth rate on the common nitrogen source ammonium sulfate (data not shown), it can be concluded that putrescine can function as a suitable nitrogen source for strain WCS365. Mutant PCL1270, however, shows no defects in its colonization ability when tested in competition with its parental strain, WCS365.

The impaired colonization ability of PCL1206 can be explained by its decreased ability to optimally regulate the uptake of putrescine from the tomato root exudate, suggesting that the putrescine concentration in the rhizosphere is sufficient to inhibit the colonization ability of PCL1206.

Summarizing, we have shown, for what we believe is the first time, that (i) putrescine is a component of tomato root exudate; (ii) *Pseudomonas* spp. contains genes involved in putrescine uptake and are highly homologous to the *pot* operon of *E. coli*; (iii) a mutation in the *potF* gene does not result in impaired competitive root colonization; and (iv) a mutation in the promoter region of the *pot* operon results in the increased uptake of putrescine, which leads to decreased competitive colonization ability and indicates that regulated uptake is an important trait for competition of *Pseudomonas* spp. bacteria in the tomato rhizosphere.

## MATERIALS AND METHODS

### Bacterial growth conditions.

The bacterial strains used in this study are listed in Table 1. *Pseudomonas* spp. strains were cultured at 28°C in liquid KB medium under vigorous aeration. *E. coli* strains were grown at 37°C in Luria-Bertani medium (Sambrook et al. 1989) under vigorous aeration. Media were solidified with 1.8% Bacto agar (Difco, Detroit, MI, U.S.A.) and, when appropriate, supplemented with kanamycin and carbenicillin to final concentrations of 50 and 100 μg per ml, respectively. The indicator 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; Duchefa, Haarlem, The Netherlands) was added to a final concentration of 40 μg per ml to distinguish mutants PCL1206 and PCL1500 (blue colonies) as well as mutant PCL1270 and wild-type strain WCS365 (white colonies).

Growth on the polyamines putrescine, spermine, or spermidine (Sigma-Aldrich Chemie, Steinheim, Germany) as nitrogen sources was performed in modified BM medium (Lugtenberg et al. 1999) with succinic acid as the standard carbon source and in which the standard nitrogen source, ammonium sulfate, was replaced by one of the respective polyamines to a final concentration of 2.0 mM. For competitive growth, cells from an overnight culture in standard BM medium were washed with phosphate buffered saline (PBS). Subsequently, the cells were mixed in a 1:1 ratio and diluted to a final concentration of $10^6$ CFU/ml. During growth, samples were taken after various time intervals and dilutions were plated on KB plates supplemented with X-Gal.

### Tomato root tip colonization assay.

Root colonization assays were performed in the gnotobiotic system, as described by Simons et al. (1996). Briefly, tomato seedlings were inoculated with a 1:1 mixture of two bacterial

**Table 1.** Bacterial strains and plasmids used

| Strains and plasmids | Relevant characteristics | Reference or source |
|---|---|---|
| *Pseudomonas fluorescens* | | |
| WCS365 | Efficient root colonizing strain isolated from potato roots. | Dekkers et al. 1998a; Geels and Schippers 1983; Simons et al. 1996 |
| PCL1206 | Tn5*lacZ* mutant of WCS365 impaired in competitive root colonization ability. | Dekkers et al. 1998c |
| PCL1270 | WCS365 derivative mutated in the *potF*$_1$ gene; made by homologous recombination. | This study |
| PCL1500 | Tn5*lacZ* derivative of WCS365 not impaired in root colonization. | Van der Bij et al. 1996 |
| *Escherichia coli* | | |
| DH5α | *endA1*, *gyrSA96*, *hrd17*(*rK-mK−*), *supE44*, and *recA1* used for propagation of plasmids. | Boyer and Roulland-Dussoix 1969 |
| Plasmids | | |
| pMP5252 | pIC20H (Marsh et al. 1984) containing a 2.5-kb *Sal*I chromosomal fragment of PCL1206 and a part of the Tn5*lacZ* harboring the Km$^r$ gene. | This study |
| pMP5285 | pME3049 (Voisard et al. 1994) derivative (suicide plasmid for *Pseudomonas* spp.) used for homologous recombination; Km$^r$, Cb$^r$. | This study |
| pMP5290 | pIC20H containing an 0.6-kb *Sma*I insert of the *potF*$_1$ gene. | This study |
| pMP5291 | pMP5285 containing the 0.6-kb fragment of the *potF*$_1$ gene from pMP5290 cloned with *Eco*RI–*Sal*I. | This study |
| pMP5299 | pMP5285 containing a chromosomal 4.2-kb fragment from PCL1270 obtained after digestion with *Eco*RI and religation; Km$^r$ | This study |
| pMP5401 | pIC20H containing an 0.47-kb *Pst*I–*Kpn*I polymerase chain reaction product of the central part of the *potF*$_1$ gene. | This study |
| pMP5403 | pBluescript KS (Stratagene, La Jolla, CA, U.S.A.) containing an 11-kb *Not*I insert of WCS365 chromosomal DNA. | This study |

suspensions and planted in a gnotobiotic quartz sand system containing plant nutrient solution, without a carbon source, but with $NO_3$ as the nitrogen source. After growth for 7 days, the root tip was isolated and bacteria were removed from the root by vortexing. Subsequently, the number of CFU was determined by dilution plating. Each plant test was performed with 10 plants, and statistics performed with the Wilcoxon-Mann-Whitney test for mixed inocula (Sokal and Rohlf 1981).

**DNA techniques, sequencing, and plasmid construction.**

All general DNA techniques, Southern blotting, and hybridizations were performed as described by Sambrook et al. (1989). Generation of a mutation by single homologous recombination was performed according to Dekkers et al. (1998c). Nucleotide sequencing was performed by Base Clear (Leiden, The Netherlands) or with the Thermo Sequenase kit, according to the methods described by the supplier (Amersham Life Science, Cleveland, OH, U.S.A.). Sequences were analyzed with DNAman software (Lynnon Biosoft, Quebec, Canada) and sequence homology searches were performed with the National Center for Biotechnology Information (Bethesda, MD, U.S.A.) gene bank (BLAST searches). In order to obtain a chromosomal DNA fragment spanning the Tn5lacZ insertion site, a Lambda FIX II genomic library of wild-type strain WCS365, constructed by Stratagene, was screened according to the instruction manual provided by the manufacturer. The DNA probe used for this screening was obtained after a PCR reaction with primers oMP508 (CGCGGTACC-CTGTTCAGGCGGACGACAAAG) and oMP509 (AAA-CTGCAGGATCATCTCGGTCGGCGAATC).

**[1,4-$^{14}$C]Putrescine uptake assay.**

Cells from an overnight culture were inoculated in fresh KB medium and grown at 28°C to an optical density at 620 nm of 0.6, washed with PBS, and subsequently diluted to $10^8$ CFU/ml in standard BM medium or in BM medium without the nitrogen source ammonium sulfate. During the experiment, cultures were incubated at 28°C. The addition of [1,4-$^{14}$C]putrescine (Sigma-Aldrich Chemie) to a final concentration of 50 μM started the experiment, and 200 μl samples in duplo were taken after various time intervals. Cells were collected on membrane filters (0.45-μm cellulose nitrate filter; Sartorius, Göttingen, Germany) and washed twice with 1 ml of PBS. The radioactivity in the cells was quantified after the addition of liquid scintillation fluid Ultima gold (Packard Bioscience, Groningen, The Netherlands) with a 1214 Rackbeta liquid scintillation counter (LKB Wallac, Turku, Finland).

**TLC analysis of dansylated polyamines.**

Tomato root exudates were collected after growth of sterile seedlings during 2 weeks in PNS, as described by Simons et al. (1997). Exudate was concentrated 20-fold and, subsequently, 200 μl was used for dansylation, as described by Scherer and Kneifel (1983). Briefly, to 200 μl of the concentrated root exudate, 300 μl of a $Na_2CO_3$ solution (100 mg per ml), and 800 μl of a dansyl chloride solution (7.5 mg per ml; Sigma-Aldrich Chemie) in dry acetone were added. The mixture was incubated for 10 min at 60°C. Subsequently, 100 μl of a proline solution (50 mg per ml; Sigma-Aldrich Chemie) was added and the mixture was incubated at 60°C for 10 min. Samples containing sterile water, PNS, ammonium sulfate (20 mM), L-aspartic acid (40 mM), cadaverine (200 mM), L-glutamic acid (60 mM), leucine (15 mM),

L-lysine (60 mM), putrescine (15 mM), spermidine (3.5 mM), and spermine (2.5 mM), all supplied by Sigma-Aldrich Chemie, were dansylated in the same way as the root exudate sample. After dansylation, samples were cooled to 4°C and extracted with 100 μl of GC-grade toluene (Fluka, Zwijndrecht, The Netherlands). A volume of 10 μl of the extract was analyzed by TLC with silica 60G kieselgel TLC plates (Merck, Darmstadt, Germany). As a reference, 10 μl of a 0.7 mM dansyl–putrescine solution (Sigma-Aldrich Chemie) in dry acetone was used. The chromatogram was developed in a mixture of triethylamine/chloroform (1:5, vol/vol) (Merck) and subsequently observed under UV light at 366 nm.

For mass spectrometric analysis, the silica corresponding to the spot of the root exudate sample running at the same $R_f$ value as the dansyl–putrescine reference was scraped from the TLC plate and extracted with toluene. After centrifugation, the toluene extract was transferred to a fresh vial and the toluene was evaporated. Samples were kept in the dark and subsequently analyzed with mass spectrometry.

**Mass spectrometry analysis.**

ES mass spectrometry was performed with the PerSeptive Biosystems "Mariner" Biospectrometry workstation (Framingham, MA, U.S.A.). Samples in solution were introduced into the low-pressure region of the mass spectrometer at a flow rate of 120 μl per h via a syringe pump (WPI, Sarasota, FL, U.S.A.). Samples were dissolved in 100 μl of acetonitrile/water solution (50/50 vol/vol, incorporating 0.1% formic acid). A 10-μl aliquot of this solution was diluted to 100 μl by the addition of acetone. The capillary was held at a potential of 3,000 V, and spectra were integrated every 3 s over $m/z$ 50 to 700. Spectra were acquired for 3 min under the control of Biospec Data Explorer software (PE Biosystems). A five-point calibration was performed with a 2-pmol per μl solution of DL-tryptophan, 4-acetamidophenol, des-arg-bradykinin and neurotensin.

ES–MS and ES–CID tandem mass spectrometry was performed with a hybrid quadrupole time-of-flight (Q-ToF) tandem mass spectrometer (Micromass; Altrincham, U.K.) equipped with a Z-spray sample introduction system in a nanoflow electrospray ionization source. Samples were delivered to the ion source with a fused silica capillary with a continuous flow sample introduction system and a syringe pump (Harvard Apparatus, South Natick, MA, U.S.A.) that delivered the sample solution at a rate of 1 μl per min. The capillary was held at a potential of 3,500 V, and the cone was held at 25 V. The mass spectrometer was operated in the positive ion mode with a source temperature of 80°C. The quadrupole was used in the $R_f$-only mode. ToF mass spectra were integrated every 2.3 s over $m/z$ 50 to 700 and recorded with collision energy that varied from 4 to 30 eV. Spectra were acquired under control of Masslynx software (Micromass), version 3.3.

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