MPMI Vol. 11, No. 1, 1998, pp. 45–56. Publication no. M-1997-1027-01R.

A Two-Component System Plays an Important Role in the Root-Colonizing Ability of *Pseudomonas fluorescens* Strain WCS365

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Accepted 7 October 1997.

We describe the characterization of a novel Tn*5lacZ* colonization mutant of the efficiently colonizing *Pseudomonas fluorescens* strain WCS365, mutant strain PCL1210, which is at least 300- to 1,000-fold impaired in colonization of the potato root tip after co-inoculation of potato stem cuttings with a 1:1 mixture of mutant and parental cells. Similarly, the mutant is also impaired in colonization of tomato, wheat, and radish, indicating that the gene involved plays a role in the ability of *P. fluorescens* WCS365 to colonize a wide range of plant species. A 3.1-kb DNA fragment was found to be able to complement the observed mutation. The nucleotide sequence of the region around the Tn*5lacZ* insertion showed three open reading frames (ORFs). The transcriptional start site was determined. The operon is preceded by an integration host factor (IHF) binding site consensus sequence whereas no clear –10 and –35 sequences are present. The deduced amino acid sequences of the first two genes of the operon, designated as *colR* and *colS*, show strong similarity with known members of two-component regulatory systems. ColR has homology with the response regulators of the OmpR-PhoB subclass whereas ColS, the product of the gene in which the mutation resides, shows similarity to the sensor kinase members of these two-component systems. Hydrophobicity plots show that this hypothetical sensor kinase has two transmembrane domains, as is also known for other sensor kinases. The product of the third ORF, Orf222, shows no homology with known proteins. Only part of the *orf222* gene is present in the colonization-complementing, 3.1-kb region, and it therefore does not play a role in complementation. No experimental evidence for a role of the ColR/ ColS two-component system in the suspected colonization traits chemotaxis and transport of exudate compounds could be obtained. The function of this novel two-component system therefore remains to be elucidated. We conclude that colonization is an active process in which an environmental stimulus, through this two-component system, activates a so far unknown trait that is crucial for colonization.

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Nucleotide and/or amino acid sequence data have been submitted to the DDBJ/EMBL/GenBank data base under accession number Y09798.

Infections by microbial pathogens are very common in plants and are responsible for the loss of approximately one third of the crop yield (Lugtenberg et al. 1994). Many plant diseases are treated with chemical fungicides. However, several of these chemicals become a threat to health and to the environment when they contaminate drinking water. Therefore, most governments have introduced a policy of banning several of the available chemical pesticides. This policy has increased the interest in microbiological control of plant diseases by the use of plant growth promoting rhizobacteria (PGPRs) (Schroth and Hancock 1982; Schippers et al. 1987; Lugtenberg et al. 1991). One group of PGPRs that receives considerable attention is that of the fluorescent *Pseudomonas* spp. The mechanisms of action of these pseudomonads have recently been extensively reviewed (Thomashow and Weller 1996). PGPRs usually attack pathogens by the production of antifungal factors (AFFs). In order to effectively protect the plant root, these AFFs must be present on the whole root system, especially on the younger parts that are most susceptible to infections. Therefore, the delivery system of these AFFs, i.e., rhizosphere colonization, is an important biocontrol trait in PGPR strains. Colonization often appears to be even the limiting factor for biocontrol (Schippers et al. 1987; Weller 1988).

We initiated a program to identify the major bacterial traits involved in colonization. Initially, we have isolated mutants in traits that, we suspected, play a major role in colonization. By comparison of the colonization behavior of wild-type and mutant strains we have shown that the ability of *Pseudomonas* to produce flagella (de Weger et al. 1987b), synthesize the O-antigen of lipopolysaccharides (LPS) (de Weger et al. 1989), and synthesize amino acids (Simons et al. 1997) is crucial for colonization. Moreover, auxotrophy for vitamin B1 and a slightly increased generation time were also correlated with poor colonization ability (Simons et al. 1996). However, this approach of studying suspected traits is limited by our knowledge of bacterial physiology. Therefore, we have now started with the more objective approach to randomly mutagenize the effectively colonizing strain *P. fluorescens* WCS365, which is one of the most efficient root colonizers of potato (Geels and

Schippers 1983a; Brand et al. 1990; Glandorf 1992; Simons et al. 1996). The resulting individual mutant cells were screened in competition with those of the parental strain for their ability to colonize the root tip after inoculation of sterile potato stem cuttings or germinated seeds. Following isolation and characterization of the corresponding wild-type genes, this approach can lead to the identification of novel colonization signals and traits.

RESULTS

Preliminary characterization of the colonization-defective mutant PCL1210.

Mutant strain PCL1210 was originally isolated as a putative colonization mutant by screening on potato plantlets in the compact system as described in Materials and Methods (Lugtenberg and de Weger 1992; Brand et al. 1990; Glandorf 1992). We therefore tested mutant strain PCL1210 for the known colonization traits of motility, presence of O-antigen of LPS, ability to produce amino acids and vitamin B1, and high growth rate in rich liquid medium in competition with the parental strain (results not shown). In addition, cell envelope protein patterns of the two strains were compared in order to test whether PCL1210 is indeed a derivative of strain *P. fluorescens* WCS365. In all these tests the behavior of mutant PCL1210 was indistinguishable from that of the parental strain (data not shown).

Subsequently, mutant strain PCL1210 was retested twice for its ability to colonize the root tip after inoculation of potato stem cuttings with a 1:1 mixture of cells of mutant PCL1210 and its parental strain *P. fluorescens* WCS365. The results (Table 1) show that the mutant strain PCL1210 is statistically significantly and at least 300- to 1,000-fold impaired in its ability to colonize the root tip in competition with its parent. The mutant also colonizes the higher parts of the root system, close to the green parts of the plant, 100-fold less efficiently than the parent in competition experiments with the parental strain (results not shown).

When potato stem cuttings were inoculated with cells of mutant PCL1210 alone, the cells colonized the root tip as effectively as the parental strain (results not shown). This result was confirmed by scanning electron microscopy, which also showed that the mutant was, like the wild type, able to form micro-colonies (results not shown).

To test the colonization behavior of mutant PCL1210 on different plants, germinated seeds of radish, tomato, and wheat as well as sterile potato stem cuttings were inoculated with a 1:1 mixture of mutant and wild type and root tip colonization was determined in the standard sand column. The results (Table 1) show that on all tested plant species the mutant is strongly impaired in colonization.

Table 1. Root tip colonization of mutant strain PCL1210 after 1:1 co-inoculation with the wild-type strain *Pseudomonas fluorescens* WCS365 in gnotobiotic assays[z]

	Root tip colonization [$\log_{10}$ (CFU + 1/cm) root tip]	
Colonization conditions	**WCS365**	**PCL1210**
Compact sand column		
Potato (experiment 1)	5.4 a	0.2 b
Potato (experiment 2)	5.4 a	1.1 b
Standard sand column		
Potato	4.5 a	0.2 b
Tomato	4.1 a	0.7 b
Wheat	4.5 a	2.5 b
Radish	3.9 a	1.4 b

[z] In every experiment 10 plants were inoculated and individually processed. Experiments were performed at least two times. When values in the same row are followed by a different letter, they are significantly different at $P = 0.05$ on nonparametric multiple comparisons by Wilcoxon-Mann-Whitney (Sokal and Rohlf 1981). Detection limit is 2.4 log CFU/ml.

Isolation of the wild-type colonization locus.

Total DNA of mutant PCL1210 was digested with the restriction enzyme *Sal*I, which divides the Tn*5lacZ* (Lam et al. 1990) present in mutant PCL1210 into two fragments. One of these fragments contains an intact kanamycin resistance gene that, after ligation of the total DNA of mutant PCL1210 into pIC20H (Marsh et al. 1984), allows selection for the presence of kanamycin resistance encoding plasmids. The resulting plasmid, pMP5203, harbors a large part of Tn*5lacZ* but also a 1.5-kb flanking region containing part of the gene that is mutated by the Tn*5lacZ* insertion (Fig. 1). The 1.5-kb flanking fragment was used in further experiments. An *Eco*RI-*Eco*RI fragment (see Figures 1 and 2) of pMP5203 was cloned into pME3049 (Voisard et al. 1994), which behaves as a suicide plasmid in *Pseudomonas*. The resulting plasmid, pMP5204, was transferred by triparental mating to the wild-type strain *P. fluorescens* WCS365. A single homologous recombination event led to insertion of pMP5204 into the chromosomal DNA, and yielded a kanamycin (pME3049) resistant *P. fluorescens* WCS365 strain. The chromosomal DNA of this strain was isolated and a restriction enzyme digestion with *Bam*HI was performed followed by ligation and transformation of the digestion mixture. After selection for kanamycin resistance,

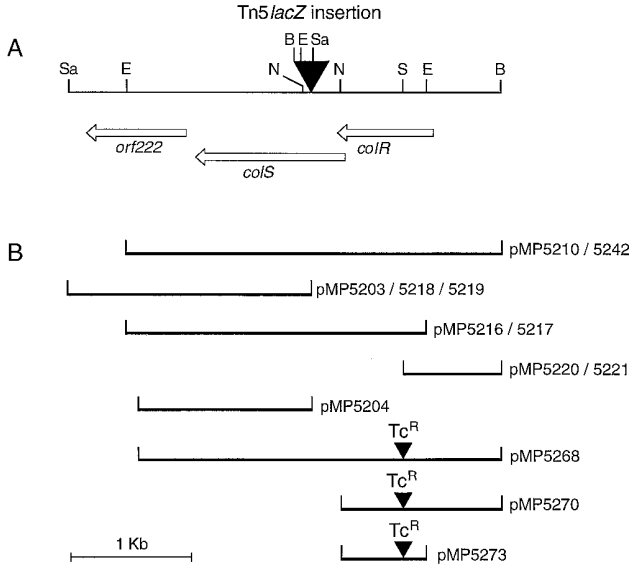


Fig. 1. Restriction map of the larger DNA fragment isolated from *Pseudomonas fluorescens* with the help of pME3049 (pMP5210). **A,** Open arrows indicate direction and size of the three open reading frames that were found. Location of the Tn*5lacZ* insertion in the genome of PCL1210 is indicated. **B,** Fragments that were used for complementation (pMP5242) or single-strand-DNA sequencing are shown. B, *Bam*HI; E, *Eco*RI; N, *Nco*I; S, *Sma*I; Sa, *Sal*I.

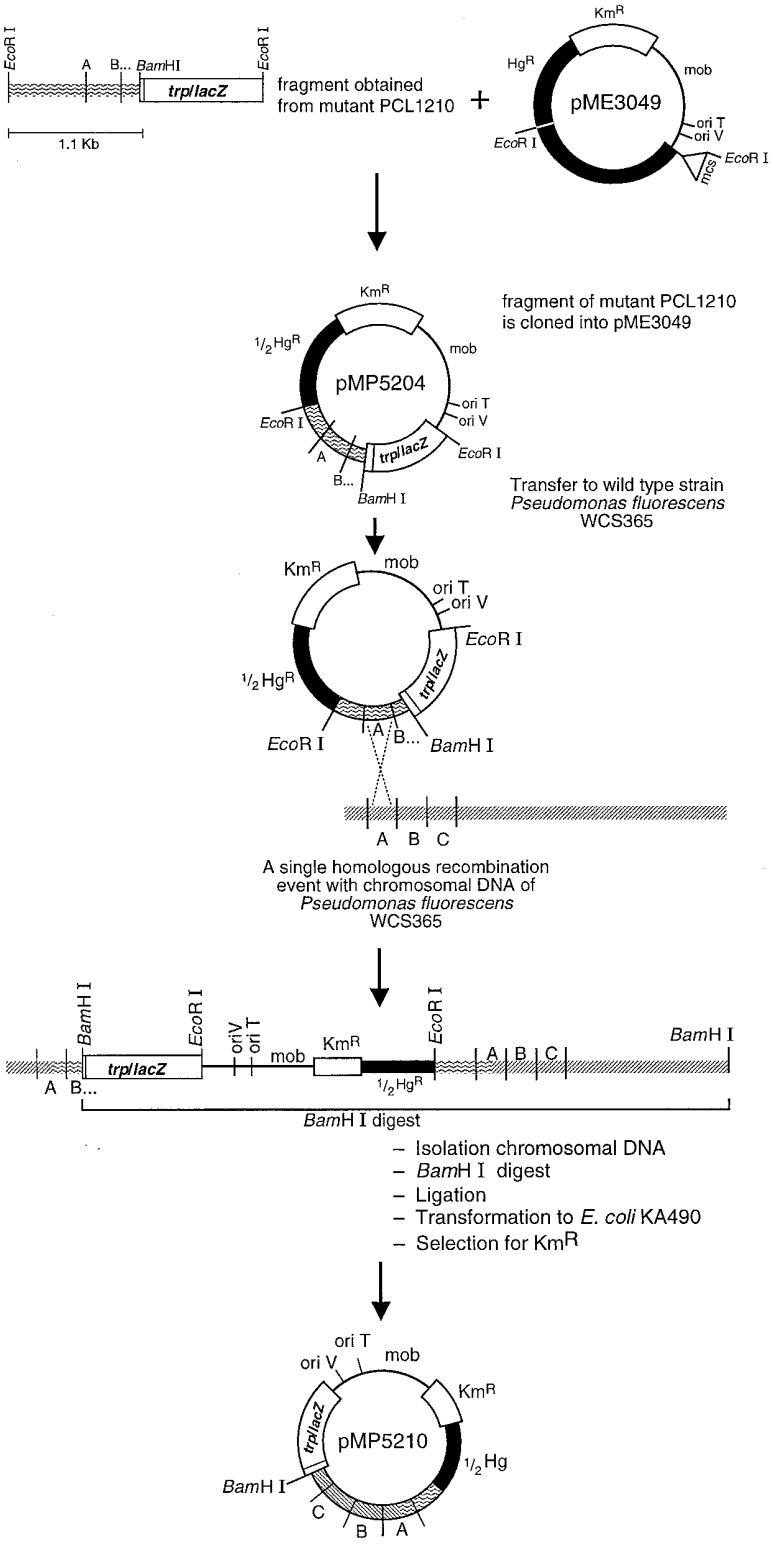


Fig. 2. Isolation of a larger DNA fragment of PCL1210 with the help of plasmid pME3049 (Voisard et al. 1994). An *Eco*RI-*Eco*RI fragment containing a part of Tn*5lacZ* and a part of the gene mutated in PCL1210 was ligated into pME3049. The resulting construct, pMP5204, was brought back to the wild-type *P. fluorescens* strain WCS365 by triparental mating, followed by selection for a single homologous recombination event. The chromosomal DNA containing the pME3049 plasmid was subsequently digested with *Bam*HI, ligated, and transformed into *Escherichia coli.* The resulting plasmid pMP5210 contains a fragment of mutant PCL1210 spanning the Tn*5lacZ* insertion, which is theoretically approximately in the middle (Voisard et al. 1994; Schnider et al. 1995).

pMP5210 was isolated and appeared to contain a 3.1-kb DNA fragment of the wild-type strain. (Figs. 1 and 2).

The 3.1-kb fragment was cloned into pWTT2081, a plasmid that is stable in the rhizosphere (van der Bij et al. 1996). The resulting plasmid, pMP5242, appeared to complement the mutant's colonization defect in competition with the parental strain (see Table 2).

Generation of a new colonization mutant with homologous recombination.

These experiments were performed to verify that the mutation as observed in mutant PCL1210 is due to the Tn*5lacZ* insertion and not to a mutation elsewhere. Plasmid pMP5273 (see Fig. 1), which contains a part of the *colS* gene as well as a part of the *colR* gene, and a tetracycline resistance gene, was introduced into the wild-type *P. fluorescens* WCS365. After selection for single or double homologous recombination, the newly generated mutant, PCL1251, was tested for its competitive colonization abilities (see Table 2). Competitive colonization experiments showed that PCL1251, like PCL1210, is statistically significantly impaired in its colonization ability, compared with the parental strain WCS365.

Nucleotide sequence analysis.

The *Sal*I-*Sal*I DNA fragment of PCL1210, present in pMP5203, was also used for sequence analysis (Sanger et al. 1977) to determine the exact position of the Tn*5lacZ* insertion in the DNA fragment. This showed that the isolated 3.1-kb DNA fragment present in pMP5210 was identical to the Tn*5lacZ* flanking fragment in pMP5203. A number of overlapping DNA fragments of the complementing region (see Figure 1) were cloned into M13mp19, and 3,347 bp were sequenced. Computer analysis with the CODON PREFERENCE program, which is part of the University of Wisconsin GCG software, was used to identify possible protein-encoding regions. This analysis revealed three potential open reading frames (ORFs) designated as *colR*, *colS*, and *orf222*, respectively (Fig. 1). Both *colR* and *colS* are preceded by a Shine-Dalgarno sequence, 5 bp AGAGG for *colR* and 6 bp GGAGG for *colS*. The third ORF contains a Shine-Dalgarno sequence (GGAGG) located 9 bp upstream of the translational start site.

Table 2. Potato root tip colonization of the complemented mutant PCL1210 and a newly generated colonization mutant of *Pseudomonas fluorescens* WCS365 after 1:1 co-inoculation with the wild-type strain in gnotobiotic assays[z]

	Root tip colonization [$\log_{10}$ (CFU + 1/cm) root tip]	
Strains tested	**WCS365**	**PCL1210**
WCS365 vs PCL1210 (parent vs original colonization mutant	5.9 a	3.5 b
PCL1240 vs PCL1242 (parent vs complemented mutant)	5.4 a	4.6 a
PCL1500 vs PCL1251 (parent vs the newly generated colonization mutant)	5.6 a	2.9 b

[z] In every experiment 10 plants were inoculated and individually processed. Experiments were performed at least two times. When values in the same row are followed by a different letter, they are significantly different at $P = 0.05$ on nonparametric multiple comparisons by Wilcoxon-Mann-Whitney (Sokal and Rohlf 1981). Detection limit is 2.4 log CFU/ml.

The putative transcription start sites on the 5′ end of *colR* were determined, and neither –10 nor –35 consensus sequences are clearly present. However, a very good putative integration host factor (IHF) binding site (Goosen and van de Putte 1995) is present, which overlaps the putative –35 sequence when the second transcription start site is considered as the actual start site. Binding studies with purified *Escherichia coli* IHF showed, however, that the purified IHF is not binding to the IHF binding site.

The first ORF, *colR*, is predicted to encode protein containing 227 amino acids with a molecular mass of 25.387 kDa. The second ORF, *colS*, in which the Tn*5lacZ* is inserted, can encode a protein of 435 amino acids with a predicted molecular mass of 47.564 kDa. The third ORF, *orf222*, could encode a protein of 222 amino acids with a predicted molecular mass of 25.807 kDa.

Comparison of the deduced amino acid sequences of the three ORFs with known protein sequences.

The amino acid sequence deduced from the first ORF, ColR, shows highest similarity with RcaC (Chiang et al. 1992) (identity 40%, similarity 59%) and PmrA (Roland et al. 1993) (identity 39%, similarity 60%), two regulatory proteins known as response regulators (Fig. 3). ColS shows homology with histidine kinase proteins such as BasS (Nagasawa et al. 1993) (identity 30.5%, similarity 50%) and PmrB (Roland et al. 1993) (identity 29%, similarity 52%) (Fig. 4). Although the codon usage bias of the last part of the DNA sequence of *orf222* (results not shown) is not typical for *Pseudomonas*, we cannot exclude that *orf222* encodes a protein. However, the 222-amino-acid sequence deduced from *orf222* shows no homology with known proteins.

With the prediction method of Kyte and Doolittle (1982), which is part of the University of Wisconsin GCG software, a hydrophobicity plot was drawn of the deduced amino acid sequence of ColS. This result predicts two hydrophobic regions at the N-terminal part of the protein (Fig. 4D).

Homology with ColR/ColS in other strains.

In order to test whether other *Pseudomonas* strains have any homology with the *colR* and *colS* genes, the chromosomal DNA of *Pseudomonas* spp. WCS134, WCS379, *P. fluorescens* WCS315, *P. putida* strains WCS085 (Geels and Schippers 1983a; de Weger et al. 1986) and RC1 (Bolton et al. 1989), *P. fluorescens* strains WCS307 and WCS374, and *P. putida* strain WCS358 (Geels and Schippers 1983b) was isolated. No clear hybridization with the chromosomal DNA of the tested strains was observed when an *Nco*I-*Eco*RI DNA fragment (Fig. 1) containing the response regulator, or an *Eco*RI-*Nco*I DNA fragment (Fig. 1) containing part of the sensor kinase and part of *orf222* was used as a radioactive probe (results not shown).

Is mutant PCL1210 impaired in chemotaxis toward, or in uptake of, major exudate nutrients?

The observation that colonization mutant PCL1210 is impaired in the functioning of a two-component system prompted us to reinvestigate the possibility that the mutation affects one of the already known colonization traits in which two-component systems are known to play a role. Chemotaxis could play a role as part of the established colonization trait motility. Similarly, transport of exudate carbon sources could

play a role in the suspected colonization trait high growth rate. Chemotaxis is regulated by the CheY/A two-component regulatory system (Swanson et al. 1994) and the transport of di- and tri-carboxylic acids is also regulated by two-component systems (Stock et al. 1989, 1990). The chemotactic abilities of *P. fluorescens* WCS365 and of mutant PCL1210 were compared on swarm plates containing the identified tomato root exudate compounds malic acid, fumaric acid, citric acid, succinic acid, glucose, ribose, galactose, and fructose (M. Simons, *personal communication*). However, no difference was observed between the chemotactic abilities of the wild type and the mutant (results not shown).

Since impaired transport will result in impaired growth, and since measurement of growth on many substrates is technically much more feasible than measurement of transport, we measured growth rates at high and low substrate concentrations. Generation times of wild-type strain *P. fluorescens* WCS365 and mutant PCL1210, as judged from measurements of OD_{620} values of cells grown in liquid medium containing 0.2% of the carbon sources mentioned above, were indistinguishable. Growth in competition in BM containing tomato root exudate as the carbon source showed no differences between the parental and the mutant strain. These results indicate that impaired transport of the major exudate carbon sources is not the reason for the mutant's poor colonization ability.

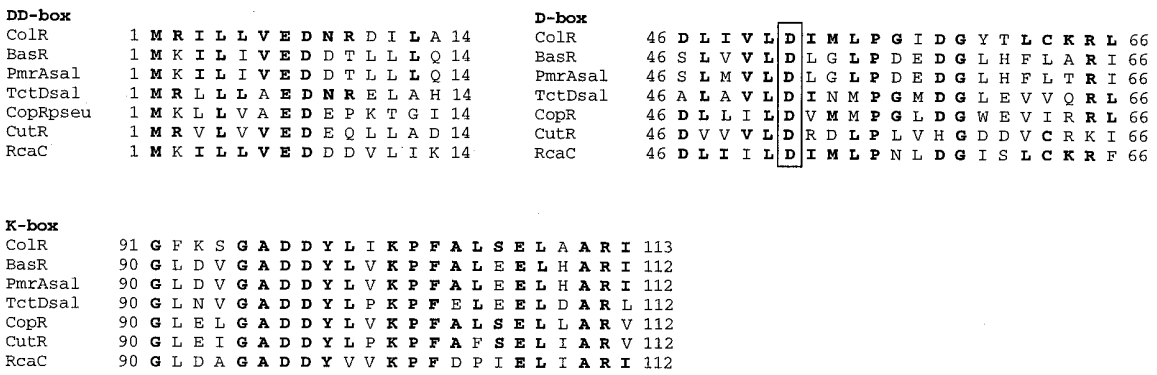
Finally, the Biolog system, which indicates the ability of a bacterial strain to oxidize 95 different carbon sources (Bochner 1989), was used to test whether *P. fluorescens* WCS365 and mutant PCL1210 differ in their behavior toward these carbon sources. The results (not shown) did not indicate any differences between the two strains.

DISCUSSION

Characterization of mutant PCL1210 as a novel colonization mutant.

Colonization often is the limiting step in biocontrol of plant diseases caused by pathogenic fungi (Schippers et al. 1987; Weller 1988). In this paper we describe the analysis of mutant PCL1210, a derivative of the efficient root colonizer *P. fluorescens* WCS365, which is impaired in its ability to colonize

A



B

	Percentage similarity with ColR	Percentage identity with ColR
BasR	59	38
PmrAsal	60	39
TctDsal	58	39
CopR	61	38.5
CutR	60	37.5
RcaC	59	40

C

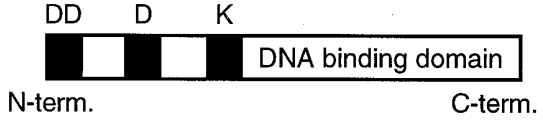


Fig. 3. Amino acid sequence similarity in the three conserved regions (Stock et al. 1989; Albright et al. 1989) of the response regulator ColR. **A,** Sequences of the response regulator ColR, BaeS (Nagasawa et al. 1993), and CpxA (Rainwater and Silverman 1990; Plunkett et al. 1993) from *Escherichia coli*, PmrAsal (Roland et al. 1993) and TctDsal (Widenhorn et al. 1989) from *Salmonella typhimurium*, and CopR from *Pseudomonas syringae* (Mills et al. 1993). CutR was isolated from *Streptomyces lividans* (Tseng and Chen 1991) and RcaC from the cyanobacterium *Fremyella diplosiphon* (Chiang et al. 1992). Conserved aspartic acid residue that is phosphorylated by a sensor kinase is boxed. All identical residues are shown in bold. **B,** Overall similarity and identity between ColR and the other regulatory proteins. **C,** An overview of the homologous domains within the regulator protein.

roots of various plants. Mutant PCL1210 appeared to be indistinguishable from the parental strain with respect to the known colonization traits of motility (de Weger et al. 1987b), production of the O-antigen of LPS (de Weger et al. 1989), amino acids (Simons et al. 1997), and vitamin B1, and high growth rate (Simons et al. 1996). Competitive growth in SSM in competition with the wild type showed that the mutant is as fit as the wild type under the tested conditions. Therefore, mutant PCL1210 must be impaired in a novel colonization trait.

A

H-box

ColS	**F**	T	S	**D**	**V**	**S**	**H**	**E**	**L**	**R**	**T**	**P**	**L**	M	**V**	**L**	A	T
BasS	**F**	T	A	**D**	**V**	A	**H**	**E**	**L**	**R**	**T**	**P**	**L**	A	G	V	R	L
PmrB	**F**	T	A	**D**	**V**	A	**H**	**E**	**L**	**R**	**T**	**P**	**L**	S	G	V	R	L
CpxA	L	L	**S**	**D**	I	**S**	**H**	**E**	**L**	**R**	**T**	**P**	**L**	T	R	**L**	Q	.
PfeS	L	L	R	T	L	**S**	**H**	**E**	**L**	**R**	**T**	**P**	**L**	A	R	**L**	R	.
BaeS	F	M	A	D	I	**S**	**H**	**E**	**L**	**R**	**T**	**P**	**L**	A	V	**L**	R	G

N-box

ColS	T	L	Y	**N**	**A**	**T**	F	**L**	T	A	V	**M**	G	**N**	**L**	**L**	**R**	**N**	**A**	**L**	H	**Y**
BasS	V	Q	G	D	**A**	**T**	L	**L**	R	M	L	L	R	**N**	**L**	**V**	**E**	**N**	**A**	H	R	**Y**
PmrB	V	R	G	D	**A**	**T**	L	**L**	R	M	L	L	R	**N**	**L**	V	E	**N**	**A**	H	R	**Y**
CpxA	L	Y	G	**N**	P	N	A	**L**	E	S	A	L	E	**N**	I	V	**R**	**N**	**A**	L	R	**Y**
PfeS	V	E	V	H	L	D	S	**L**	A	Q	A	**M**	E	**N**	**L**	**L**	**R**	**N**	**A**	I	R	H
BaeS	V	F	G	D	R	D	R	**L**	M	Q	L	F	N	**N**	**L**	**L**	E	**N**	S	L	R	**Y**

DF-box

ColS	**V**	**E**	**D**	**S**	**G**	V	**G**	**I**	P	**E**	**E**	**K**	**R**	**E**	A	M	**F**	**E**	**P**	**F**	**V**	**R**	G	N
BasS	**V**	**E**	**D**	E	**G**	P	**G**	**I**	D	**E**	S	**K**	C	G	E	L	S	K	A	**F**	**V**	**R**	M	D
PmrB	**V**	**E**	**D**	E	**G**	P	**G**	**I**	D	**E**	S	**K**	C	G	K	L	S	**E**	A	**F**	**V**	**R**	M	D
CpxA	**V**	D	**D**	D	**G**	P	**G**	V	S	P	E	D	R	**E**	Q	I	**F**	R	**P**	**F**	Y	**R**	T	D
PfeS	L	Q	**D**	Q	**G**	P	**G**	V	A	**E**	D	Q	L	**E**	R	I	**F**	L	P	Y	Q	**R**	L	D
BaeS	F	A	**D**	S	A	P	**G**	V	S	D	D	Q	L	Q	K	L	**F**	**E**	R	**F**	Y	**R**	T	E

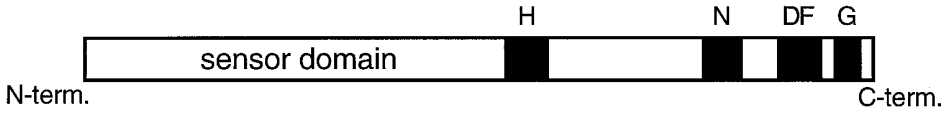
G-box

ColS	**G**	L	**G**	**L**	**G**	**L**	**S**	L	**V**	Q	**R**	**I**	C	**E**	.	N	**Q**	**G**	**W**	T
BasS	**G**	I	**G**	**L**	**G**	**L**	**S**	I	**V**	S	**R**	**I**	T	Q	L	H	H	**G**	Q	F
PmrB	**G**	I	**G**	**L**	**G**	**L**	**S**	I	**V**	S	**R**	**I**	T	Q	L	H	**Q**	**G**	Q	F
CpxA	**G**	T	**G**	**L**	**G**	L	A	I	**V**	E	T	A	I	Q	Q	H	R	**G**	**W**	.
PfeS	**G**	F	**G**	**L**	**G**	**L**	A	I	A	R	**R**	A	I	**E**	L	Q	G	**G**	R	.
BaeS	**G**	S	**G**	**L**	**G**	**L**	A	I	C	L	N	I	V	**E**	A	H	N	**G**	R	.

B

	Percentage similarity with ColS	Percentage identity with ColS
BasS	52	30.5
PmrB	52	29
CpxA	53	26
PfeS	54	28
Baes	48	24.5

C



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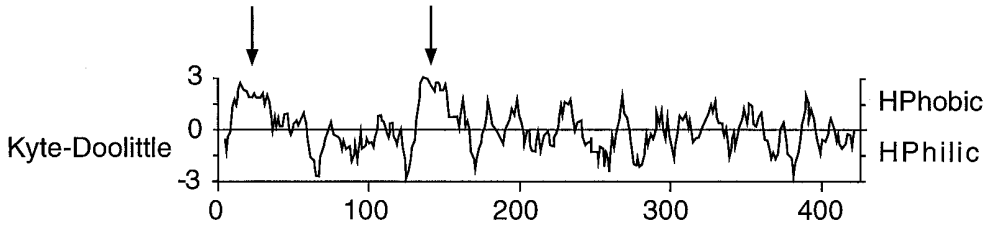

Fig. 4. Amino acid sequence similarity of the sensor kinase ColS. **A,** Similarity of four conserved regions (Stock et al. 1989; Albright et al. 1989) of the ColS sensor kinase protein with those of the sensor kinases BasS and BaeS, both described by Nagasawa et al. (1993), CpxA (Rainwater and Silverman 1990; Plunkett et al. 1993) from *Escherichia coli*, PmrB from *S. typhimurium* LT2 (Roland et al. 1993), and PfeS from *Pseudomonas aeruginosa* (Dean and Poole 1993). Site of autophosphorylation, the conserved histidine residue, is boxed. All identical residues are shown in bold. **B,** Overall similarity and identity between ColS and the other sensor kinases. **C,** Overview of the homologous domains within the sensor kinase protein. **D,** Hydrophobicity plot of the sensor kinase ColS. A hydrophobicity plot of the deduced amino acid sequence of ColS was drawn with a Kyte and Doolittle (1982) prediction model that is part of the PEPLOT program of GCG Wisconsin. Hydrophobic regions of more than 20 amino acids are marked by arrows.

Analysis of colonization behavior.

Mutant PCL1210 was initially isolated as a mutant impaired in colonization of the potato root. Using two slightly different colonization assays, we showed that mutant PCL1210, in competition with the wild-type strain *P. fluorescens* WCS365, is strongly impaired in colonization of the potato root tip (Table 1). Under these conditions competitive colonization of the upper parts of the potato root system appeared to be reduced (results not shown). When mutant PCL1210 is applied alone on the potato root its colonization behavior is indistinguishable from that of the wild type, indicating that the mutant is impaired in competitive colonization rather than in colonization per se. Furthermore, these results show that colonization is not reduced because of sensitivity of the mutant toward toxins exuded by the root. Previously described data of Glandorf (1992) have shown that the colonization of mutant PCL1210, then known as mutant 10, on potato is also impaired in field soil, especially on the lower parts of the root systems (4 to 6 cm, total length 6 cm), indicating that the impaired colonization trait also plays a role under agricultural conditions. The impaired colonization gene of mutant PCL1210 not only plays a role in the colonization of potato roots but also in the colonization of tomato, radish, and wheat roots (Table 1), indicating that the novel colonization trait plays a major role in colonization of a wide range of plant species.

The newly generated mutant PCL1251 was also tested for its colonization abilities and showed a statistically significant decreased colonization ability (see Table 2), indicating that the mutation in mutant PCL1210 is caused by a defect in *colS* and not by a mutation elsewhere.

Regulation of *colR/colS*.

Considering the lack of a promoter sequence upstream of *colS,* and the fact that the termination codon of *colR* overlaps with the ORF of *colS,* it is likely that both genes constitute one operon. The third ORF, *orf222,* which is situated 120 bp farther downstream of the *colR/colS* operon, is not preceded by any putative promoter sequences. Therefore, it is likely that this third ORF, together with *colR* and *colS,* constitutes one operon. Complementation with the 3.1-kb fragment, in which a substantial part of *orf222* is missing, showed that *orf222* is not required for complementation of mutant PCL1210. The fact that the presumed c*olR/colS, orf222* operon is not preceded by a clear σ^{70} consensus sequence suggests that expression of this operon requires the use of an alternative σ factor or the use of an additional transcription factor. The third transcription start site, which is located 222 bp upstream of the translation start site, could be a likely candidate to bind such an additional transcriptional activator. Another likely candidate to enhance transcription is the almost perfect IHF-binding site (Goosen and van der Putte 1995) located directly upstream of the transcriptional start site, overlapping the putative –35 sequence. The lack of interaction with purified *E. coli* IHF protein in binding studies may be due to the lack of one or more other factors required for formation of such a complex, or to a difference between *E. coli* and *Pseudomonas* IHF.

Possible function of ColR/ColS.

The deduced amino acid sequences of the first two ORFs (ColR and ColS) share considerable similarity with a number of proteins that are members of two-component regulatory systems. Two-component systems are able to respond to a large variety of environmental stimuli, such as changes in osmolarity (Comeau et al. 1985) and phosphate concentration (Makino et al. 1986). By autophosphorylation of a conserved histidine residue, the sensor kinase member of the two-component system is able to phosphorylate the response regulator, which in turn is able to activate a gene or a set of genes (Albright et al. 1989; Stock et al. 1989, 1990; Charles et al. 1992).

ColR, the first gene of the operon, shows homology with various response regulators that are members of these two-component systems. It has been shown (Albright et al. 1989; Stock et al. 1995) that these response regulators are highly conserved in the N-terminal region of the protein (see Figure 3). This N-terminal region contains three conserved boxes (see Figure 3). The second one contains the conserved aspartic acid residue (D51), which is phosphorylated by the second member of the two-component system, the histidine kinase protein. The C-terminal domain of ColR is shared by OmpR (Comeau et al. 1985) and PhoB (Makino et al. 1986). This subclass of response regulators shares C-terminal homology with the N-terminal region of the ToxR protein, a transcriptional activator that regulates cholera toxin production (Miller et al. 1987). These shared C-terminal domains between the different subclasses suggest that they have a similar interaction with respect to DNA binding and transcription. (Ronson et al. 1987; Charles et al. 1992).

The second ORF, ColS, is the sensor kinase member of the two-component system. These sensor kinases share a highly conserved C-terminal domain of approximately 240 amino acids (Albright et al. 1989; Stock et al. 1989, 1990; Charles et al. 1992) in which the autophosphorylated histidine residue and a number of other conserved residues are present. As shown in Figure 4A, these conserved regions are also present in ColS. Most sensor kinases are transmembrane proteins and share two hydrophobic regions in the amino terminus of the protein (Albright et al. 1989; Stock et al. 1989, 1990; Charles et al. 1992). ColS shares this specific feature with known sensor kinases (Fig. 4D). Therefore, it is likely that the amino terminal part of ColS contains a transmembrane spanning domain and is able to detect a change in the environment and that the cytoplasmic carboxy-terminus, containing the conserved histidine residue, is autophosphorylated and is responsible for the interaction with the response regulator.

BasS/BasR and BaeS are two-component systems with an unknown function isolated from *E. coli* (Nagasawa et al. 1993), the PmrA/PmrB two-component system confers resistance to cationic, hydrophobic agents and is suspected to play a role in virulence in *S. typhimurium* LT2 (Roland et al. 1993), and CpxA confers resistance to the drug amikacin in *E. coli* (Rainwater and Silverman 1990). The PfeR/PfeS two-component system is involved in the regulation of expression of the ferric enterobactin receptor and is induced by enterobactin (Dean and Poole 1993), the response regulator TctD is involved in transport of tricarboxylates (Widenhorn et al. 1989), CopR induces resistance to copper in *P. syringae* (Mills et al. 1993), and CutR is involved in copper metabolism in *S. lividans* 66 (Tseng and Chen 1991). The RcaC response regulator is involved in the control of expression of phycobiliproteins as a response in changes of light quality in cyanobacte-

ria. (Chiang et al. 1992). To us, striking similarities found with a number of response regulators and sensor kinases do not provide a clue for the role of the ColR/ColS two-component system in colonization.

Southern hybridization of chromosomal DNA of other *Pseudomonas* strains (see Results) showed no homologies when the *colR* and *colS* genes were used separately as a probe. This can either be due to the uniqueness of the two-component system or it may be caused by the low level of sequence homology shared among these genes, since only a few boxes in the regulator and sensor are highly conserved (see Figures 3C and 4C). The latter is also observed for LuxI and LuxR homologues, where identification of homologues genes is hampered by the low level of sequence homology that is shared (Salmond et al. 1995).

From these results we conclude that root colonization by *P. fluorescens* WCS365 is an active process in which a so far not identified environmental stimulus, through a two-component system, activates a so far unknown colonization trait. In future research we will attempt to identify the stimulus and the colonization trait.

MATERIAL AND METHODS

Bacterial strains, plasmids, and growth conditions.

All strains and plasmids used and their characteristics are listed in Table 3. Cells of the wild-type *P. fluorescens* strain WCS365 and of its Tn*5lacZ* derivatives were grown overnight at 28°C on solidified King B medium (King et al. 1954) or in liquid King B medium under vigorous shaking. The final concentrations of antibiotics in the medium were as follows: nalidixic acid, 15 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 40 μg/ml. Various *E. coli* strains, which were used for plasmid propagation and for transferring the plasmids to *Pseudomonas*, were grown overnight in liquid or solidified Luria broth (LB, Sambrook et al. 1989). The antibiotics used for the maintenance of the different plasmids were as described above with the exception of carbenicillin, which was present in a final concentration of 100 μg/ml. As an indicator for β-galactosidase activity, 5-bromo-4-chloro-3-indolyl-β D-galactoside (X-gal) was used in a final concentration of 40 μg/ml.

DNA modifications.

For the transfer of plasmids from *E. coli* to *Pseudomonas*, triparental mating was performed with pRK2013 as the helper plasmid (Ditta et al. 1980). Acceptor, donor, and helper bacteria from cultures grown overnight on plates were mixed on a King B plate and incubated at 28°C overnight. A sample of the mixture was suspended in sterile phosphate-buffered saline solution (PBS; 0.9% NaCl buffered with 10 mM sodium phosphate, pH 7.2) and different dilutions were plated on King B medium supplemented with the appropriate antibiotics.

Transposon mutagenesis was performed by mating *P. fluorescens* WCS365 with *E. coli* S17-1 (Simon et al. 1983), a strain harboring the suicide plasmid pCIB100 (Lam et al. 1990) containing a Tn*5* with a constitutively expressed *lacZ* gene. Transconjugants were selected on King B plates supplemented with nalidixic acid (15 μg/ml), kanamycin (50 μg/ml), and X-gal. Individual mutants were screened by coinoculation of potato stem cuttings with a 1:1 mixture of cells of the parental strain and the mutant (Simons et al. 1996).

All general DNA techniques, including restriction enzyme digestions, ligations, plasmid isolations, transformation of *E. coli*, and Southern blotting, were performed as described by Sambrook et al. (1989). Both restriction enzymes and T4 ligase were obtained from Pharmacia Biotech LKB, Woerden, The Netherlands.

For the isolation of the fragment located next to the Tn*5lacZ* insertion, and of the total wild-type gene, the method described by Schnider et al. (1995) and Voisard et al. (1994) was followed. Total chromosomal DNA of mutant PCL1210 was digested with *Sal*I, which preserves the kanamycin resistance gene present on the Tn*5lacZ*. After digestion of the total chromosomal DNA, all fragments were ligated into pIC20H (Marsh et al. 1984) and a selection was made for kanamycin-resistant colonies. After plasmid isolation, the region that is situated next to the Tn*5lacZ* insertion can be isolated. An *Eco*RI-*Eco*RI fragment (see Figures 1 and 2) was isolated from the larger *Sal*I-*Sal*I digest (pMP5203) and this fragment was cloned into pME3049, resulting in plasmid pMP5204. By triparental mating this suicide plasmid was introduced into *P. fluorescens* WCS365 and a selection was made for a single homologous recombination. Digestion of the total chromosomal DNA with *Bam*HI resulted in plasmid pMP5210, which contains the gene fragment of interest (see Figure 2). Complementation was carried out by subcloning the 3.1-kb fragment of pMP5210 (see Figure 1) into the stable plasmid pWTT2081 (van der Bij et al. 1996), resulting in plasmid pMP5242.

For single-stranded DNA sequencing, different fragments of pMP5210 (see Figure 1) were cloned into M13 vector tg130/131 or M13 mp18/19 (Kieny et al. 1983). Single-stranded DNA sequencing was performed according to Sanger et al. (1977) with (α-^{35}S)dATP (Amersham International, Little Chalfont, England). T7 and the deaza sequencing kit (Pharmacia) were used according to the manufacturer's guidelines. Primers used for sequencing were obtained from Isogen Bioscience, Maarssen, The Netherlands.

IHF binding studies were performed according to Goosen et al. (1996). For primer extension experiments, mRNA was isolated from an overnight culture of *P. fluorescens* WCS365 according to van Slogteren et al. (1983). To label the primer with (α-^{32}P)dATP (Amersham), polynucleotide kinase (Promega Corporation BNL, Leiden, The Netherlands) was used. Primer extension was performed with approximately 10 μg of mRNA with moloney murine leukemia virus reverse transcriptase (M-MLV reverse transcriptase) (Promega) at a temperature of 51°C.

Generation of a new colonization mutant with homologous recombination.

Plasmid pMP5268, which contains a tetracycline resistance cassette inserted in the *colR* gene (see Figure 1), was digested with *Nco*I and *Bgl*II. Both sticky ends were filled in with the Klenow fragment and the blunt ends were subsequently religated, resulting in plasmid pMP5270. Plasmid pMP5270 was digested with *Sph*I and *Eco*RI, the *Eco*RI sticky end was filled in with the Klenow fragment and the blunt ends were religated, resulting in plasmid pMP5273. This plasmid contains a part of the *colS* gene and a part of the *colR* gene in which a tetracycline resistance gene is inserted. With electroporation, plasmid pMP5273 was introduced into the wild-type *P. fluo-*

Table 3. Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Characteristics[z]	Reference/source
Pseudomonas spp.		
WCS134	Wild-type strain used for hybridization	Geels and Schippers 1983a; de Weger et al. 1986
WCS379	Wild-type strain used for hybridization	Geels and Schippers 1983a; de Weger et al. 1986
P. putida		
RC1	Wild-type strain used for hybridization	Bolton et al. 1989
WCS085	Wild-type strain used for hybridization	de Weger et al. 1986
WCS315	Wild-type strain used for hybridization	de Weger et al. 1986
WCS358	Wild-type strain used for hybridization	Geels and Schippers 1983b
P. fluorescens		
WCS365	Biocontrol strain in a *Fusarium oxysporum* f. sp. *radicis-lycopersici*-tomato system (B. Kroon, *personal communication*). Efficient colonizer of the potato root.	Geels and Schippers 1983a
WCS307	Wild-type strain used for hybridization	Geels and Schippers 1983b
WCS374	Wild-type strain used for hybridization	Geels and Schippers 1983b
PCL1500	Tn*5lacZ* (Lam et al. 1990) derivative of *P. fluorescens* WCS365 that behaves as its wild type in a gnotobiotic assay. Nal^r, Km^r	van der Bij et al. 1996
PCL1210	Tn*5lacZ* derivative of *P. fluorescens* WCS365 that is impaired in colonization. Nal^r, Km^r	Lugtenberg and de Weger 1992
PCL1240	*P. fluorescens* WCS365 harboring plasmid pWTT2081. Tc^r	This paper
PCL1242	Mutant PCL1210 complemented with plasmid pMP5242. Tc^r	This paper
PCL1251	Newly generated mutant with an insertion in the *colR* and *colS* genes (see Materials and Methods). Tc^r	This paper
E. coli		
HB101	*supE44 hsdS20 (r_B-m_B-) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl -1.* Used for transformation and propagation of plasmids.	Woodcock et al. 1989
XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac-F′[proAB+lacI^q lacZ M15 Tn10(tet^r)].* Used for transformation and propagation of the M13 phagemids.	Stratagene, La Jolla, CA
DH5α	*endA1 gyrSA96 hrdR17(rK- mK-) supE44 recA1.* Used for transformation and propagation of plasmids.	Boyer and Roulland-Dussoix 1969
S17-1	MM294, RP4-2:Mu-Km::Tn*7* chromosomally integrated	Simon et al. 1983
Plasmids		
pRK2013	Helper plasmid for triparental mating	Ditta et al. 1980
pME3049	Suicide plasmid used for picking up wild-type genes, Km^r	Voisard et al. 1994
pIC20H	Cb^r	Marsh et al. 1984
pWTT2081	Plasmid stable in the rhizosphere and used for complementation of the mutants, copy number 4 to 9 copies /cell. Tc^r	van der Bij et al. 1996
pMP5203	pIC20H that contains a *Sal*I-*Sal*I fragment of the chromosomal region of PCL1210 flanked by a Tn*5lacZ* fragment containing Km^r. Cb^r, Km^r	This paper
pMP5204	pME3049 that contains a 1.5-kb *Eco*RI-*Eco*RI fragment of pMP5203 (see Figure 2) used for homologous recombination with *P. fluorescens* WCS365. Km^r	This paper
pMP5210	pME3049 containing a 3.1-kb fragment of *P. fluorescens* WCS365 obtained by homologous recombination and spanning the Tn*5lacZ* insertion. Km^r	This paper
pMP5216/17	M13tg130 (Kieny et al. 1983) containing a 2.5-kb *Eco*RI-*Eco*RI fragment in two orientations of pMP5210 (see Figure 2) used for single-strand sequencing.	This paper
pMP5218/19	M13mp18/19 containing a 2-kb *Sal*I-*Bam*HI fragment of pMP5203 (see Figure 2) used for single-strand-sequencing	This paper
pMP5220/21	M13mp18/19 containing a 0.8-kb *Bam*HI-*Sma*I fragment of pMP5110 (see Figure 2) used for single-strand sequencing	This paper
pMP5242	pWTT2081 containing the total 3.1-kb complementing fragment of pMP5210 that is used to complement mutant PCL1210. Tc^r	This paper
pMP5268	3.1-kb complementing kb fragment of pMP5210 cloned into pIC20H and digested with *Sma*I. Subsequently, a Tc cassette obtained from pWTT2081 is inserted in this *Sma*I site (see Figure 1).	This paper
pMP5270	pMP5268 digested with *Nco*I and *Bgl*II; the sticky ends are filled in and the plasmid is religated (see Figure 1).	This paper
pMP5273	pMP5270 digested with *Sph*I and *Eco*RI; the sticky end is filled in and the plasmid is religated.(see Figure 1).	This paper

[z] Abbreviations: Nal, nalidixic acid; Cb, carbenicillin; Km, kanamycin; Tc, tetracycline.

rescens WCS365. This plasmid behaves as a suicide plasmid in *P. fluorescens* WCS365 and a selection was made for homologous recombination (single or double) by selection for tetracycline resistance. The newly generated mutant, PCL1251, was subsequently checked by Southern hybridization for the presence of plasmid pMP5273 in the genome (results not shown) and tested for its competitive colonization abilities (see Table 2).

Growth of mutant PCL1210 cells in competition with parental cells.

Overnight cultures of the mutant and the wild type in liquid SSM (Meyer and Abdallah 1978) were washed in sterile PBS and subsequently diluted in SSM to a final optical density value at 620 nm of 0.1. Equal volumes of these cultures were mixed. The mixture was allowed to grow for 24 h diluted 1,000-fold. This procedure was conducted five times. The ratio between the wild-type and the mutant cells was determined at various time intervals by plating diluted samples on solidified King B medium containing X-gal. White and blue colonies represent wild-type and mutant cells, respectively.

Colonization experiments on plants.

Overnight cultures in King B medium were washed once in sterile PBS and the bacteria of the individual strains were diluted in sterile PBS to a final concentration of 10^7 CFU/ml. These suspensions were mixed 1:1 (vol/vol) and the mixture was used to inoculate sterile potato stem cuttings or germinated seeds of tomato, radish, or wheat. The colonization experiments were designed to test the ability of a strain to move with or toward the growing root tip. When sterile potato stem cuttings were worked with, the procedure of de Weger et al. (1994) for colonization was used with the following modifications. Three milliliters of sterile PNS (plant nutrient solution), containing 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM $MgSO_4$, 1 mM KH_2PO_4, and micronutrients (Hoffland et al. 1989) was added to a quartz sand column that was applied into the glass tube in a dry form. This results in a compact, moisturized sand column. After sterilization, 2 ml of water agar (sterile water containing 1% agar) was put on top of the compact sand column to prevent desiccation and to enable us to plant the fragile inoculated stem cuttings into the agar layer.

When germinated seeds were used for colonization experiments, the sand column used was slightly different. We refer to this system as the standard sand column. The glass tube of this recently described gnotobiotic system (Simons et al. 1996) was filled with sterile sand that had been mixed with sterile PNS (10% PNS) prior to deposition in the glass tube. The inoculated seedling was subsequently planted on top of the aerated sand column (Simons et al. 1996).

After 14 days of growth in the gnotobiotic compact system described by de Weger et al. (1994) or 7 days of growth in the standard system described by Simons et al. (1996), the plants were removed from the sand tube and the bacteria that had been able to reach the root were isolated as follows. The root tips (1 to 2 cm) were removed and shaken vigorously in the presence of adhering sand particles in 1.0 ml of PBS on an Eppendorf shaker for 15 min to remove the bacteria. The bacterial suspensions thus obtained were plated on King B plates containing X-gal, with a spiral plater (Spiral Systems, Cincinnati, OH). After incubation at 28°C for 2 days, the colonies were distinguished by their yellow/white (wild type) and blue (mutant) appearance on plate. The number of CFU per cm of root tip were calculated (Davies and Whitbread 1989), and the data transformed to $\log_{10}$ (CFU/cm+1) values (Loper and Schroth 1984), after which estimates of the mean and standard deviation were calculated. Experiments were performed in 10-fold, and conducted twice. Statistical comparison of strains in mixed inocula was done with the nonparametric Wilcoxon-Mann-Whitney test (Sokal and Rohlf 1981). For analysis of mutant behavior from mixed inocula, only plates with more than 30 colonies per plate (5×10^3 CFU/cm) were counted. Zeroes were included for analysis of single inocula (Kloepper and Beauchamp 1992). Calculation of $\log_{10}$ (CFU/cm+1) was chosen to avoid non-existent $\log_{10}$ (0) situations.

To determine the number of mutant and parental bacteria on the upper parts of the root system, the isolated roots were divided into three parts of equal length and the number and ratio of mutant and wild type present on these root segments were determined.

Growth on various carbon sources.

The Biolog system (Bochner 1989) (Biolog, Hayward, CA) was used according to the manufacturer's guidelines to determine differences in the ability of mutant and wild type to oxidize 95 different C-sources, some of which may be present in exudate and therefore may play a role in colonization.

To determine whether the two-component system is involved in the uptake of various C-sources present in root exudate, we compared the growth curves of the wild type and the mutant in minimal media supplemented with the following C-sources: malic acid, fumaric acid, succinic acid, citric acid, glucose, ribose, fructose, maltose, and galactose. The C-sources used for this experiment are present in the tomato seed exudate (M. Simons, *unpublished;* Lugtenberg et al. 1996).

For the measurement of growth rate at high substrate concentrations, wild-type and mutant cells of an overnight culture grown in BM (basic medium; i.e., SSM without succinic acid, M. Simons, *unpublished*) supplemented with 0.2% succinic acid, were washed, and diluted to approximately 10^7 CFU/ml in BM in which succinic acid was replaced by 0.2% of various other C-sources described to be present in tomato root exudate. The cells were incubated at 28°C on a rotary shaker and the OD_{620} values of these suspensions were measured every 60 min for 9 h.

Growth at low substrate concentrations was performed in BM supplemented with tomato root exudate as the carbon source. Tomato root exudate was obtained by incubating approximately 7 sterile germinating tomato seeds for 8 days in 5 ml of sterile PNS. The root exudate samples thus isolated were tested for sterility and the sterile samples were pooled. BM supplemented with tomato root exudate was inoculated with 10^2 to 10^3 CFU/ml of a 1:1 mixture of the washed cells of the wild type and the mutant. Increases in bacterial populations were measured every 24 h by dilution plating of samples on solidified King B medium supplemented with X-gal.

Motility, chemotaxis, and isolation of LPS and cell envelopes.

Motility was measured with semisolid (0.35% agar) plates containing 20-fold diluted King B. Chemotaxis toward various

carbon sources was measured with semisolid plates (0.35% agar) containing BM medium supplemented with various carbon sources, as described above, and X-gal (Armstrong et al. 1967).

Isolation of cell envelopes and analysis of LPS ladder patterns and membrane protein patterns were performed according to de Weger et al. (1987a).

ACKNOWLEDGMENTS

We thank D. Haas for the supply of the pME3049 plasmid and N. Goossen, Gorlaeus Laboratories, Leiden University, Leiden, The Netherlands, for supply of the purified IHF protein and technical assistance. Furthermore, we thank Thomas Chin-A-Woeng for electron microscopy data and Ineke Brand for the isolation of mutant PCL1210. The investigations were partly funded by the European Union Biotech program BIO2-CT93.0196.

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