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The *sss* Colonization Gene of the Tomato-*Fusarium oxysporum* f. sp. *radicis-lycopersici* Biocontrol Strain *Pseudomonas fluorescens* WCS365 Can Improve Root Colonization of Other Wild-type *Pseudomonas* spp. Bacteria

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We show that the disease tomato foot and root rot caused by the pathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici* can be controlled by inoculation of seeds with cells of the efficient root colonizer *Pseudomonas fluorescens* WCS365, indicating that strain WCS365 is a biocontrol strain. The mechanism for disease suppression most likely is induced systemic resistance. *P. fluorescens* strain WCS365 and *P. chlororaphis* strain PCL1391, which acts through the production of the antibiotic phenazine-1-carboxamide, were differentially labeled using genes encoding autofluorescent proteins. Inoculation of seeds with a 1:1 mixture of these strains showed that, at the upper part of the root, the two cell types were present as microcolonies of either one or both cell types. Microcolonies at the lower root part were predominantly of one cell type. Mixed inoculation tended to improve biocontrol in comparison with single inoculations. In contrast to what was observed previously for strain PCL1391, mutations in various colonization genes, including *sss*, did not consistently decrease the biocontrol ability of strain WCS365. Multiple copies of the *sss* colonization gene in WCS365 improved neither colonization nor biocontrol by this strain. However, introduction of the *sss*-containing DNA fragment into the poor colonizer *P. fluorescens* WCS307 and into the good colonizer *P. fluorescens* F113 increased the competitive tomato root tip colonization ability of the latter strains 16- to 40-fold and 8- to 16-fold, respectively. These results show that improvement of the colonization ability of wild-type *Pseudomonas* strains by genetic engineering is a realistic goal.

Commercial application of fluorescent *Pseudomonas* spp. as biocontrol agents against a large group of plant pathogens can be hampered by their inconsistent performance in the field (Schroth and Hancock 1982). These inconsistencies are often accompanied by inefficient colonization of the plant root by the biocontrol strain (Schippers et al. 1987), suggesting that colonization is often the limiting factor for biocontrol.

To study colonization, we selected *P. fluorescens* strain WCS365 (Geels and Schippers 1983a, 1983b) as the most efficient root colonizer among a series of biocontrol strains (Brand et al. 1991). This strain is not antagonistic against a large number of fungal pathogens on antagonistic test plates under laboratory conditions (T. F. C. Chin-A-Woeng, *unpublished data*). However, strain WCS365 induces induced systemic resistance (ISR) in *Arabidopsis thaliana* ecotype Columbia, which thereby is protected against the pathogen *P. syringae* pv. *tomato* strain DC3000 (Gerrits and Weisbeek 1996). Therefore, we tested whether *P. fluorescens* WCS365 is also able to control tomato foot and root rot, the plant disease studied in our previous work (Chin-A-Woeng et al. 1998; Lugtenberg et al. 2000).

In order to unravel the molecular basis of root colonization, *P. fluorescens* WCS365 has been used for the generation of colonization mutants (Dekkers et al. 1998a, 1998b, 1998c; Lugtenberg and Dekkers 1999; Simons et al. 1997). One of the colonization mutants, strain PCL1233, was complemented for colonization by a wild-type DNA fragment containing a multicistronic transcription unit that comprises at least six open reading frames (ORFs) (Dekkers et al. 1998b). The fifth ORF of this DNA fragment, which is homologous to the site-specific recombinases Sss (Hofte et al. 1994), XerC (Colloms et al. 1990), FimB, and FimE (Dorman and Higgins 1987; Klemm 1986), was shown to be crucial for colonization (Dekkers et al. 1998b). This gene is thought to play a role in DNA rearrangements that regulate the transcription of a gene or a set of genes involved in the biosynthesis of cell surface components (Dekkers et al. 1998b). In a more general perspective, it is thought that lack of DNA rearrangements in mutant PCL1233 results in cells that are locked in a state unfavorable for competitive colonization (Dekkers et al. 1998b).

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In this article, we show that WCS365 is able to control tomato foot and root rot and describe how its colonization behavior on the tomato root is influenced by the presence of the antibiotic-producing biocontrol strain *P. chlororaphis* PCL1391. Moreover, we describe the effect of inactivated *col* genes, including *sss*, on biocontrol. Finally, we describe how the introduction of the *sss* operon into other *Pseudomonas* spp. affects the colonization abilities of these wild-type strains.

RESULTS

Biocontrol by *P. fluorescens* WCS365 and by a mixture with *P. chlororaphis* strain PCL1391.

Biocontrol experiments performed in an *Fusarium oxysporum* f. sp. *radicis-lycopersici*-tomato system showed that *P. fluorescens* WCS365 (Table 1) in all three experiments suppressed the disease in a statistically significant way (Table 2). *P. fluorescens* WCS365 showed similar biocontrol activity to PCL1391, a *P. chlororaphis* strain recently described to control tomato foot and root rot (Chin-A-Woeng et al. 1998). Considering the observation that both strains occupy similar sites on the tomato root and that both are efficient colonizers (Lugtenberg and Dekkers 1999), we studied how the strains behave on the root after they had been applied on the seedling as a mixture. In order to distinguish the strains, they were genetically marked with variants of green fluorescent protein.

Germinated tomato seedlings were inoculated with a 1:1 mixture of red fluorescent (DsRed) or enhanced cyan fluorescent protein (ECFP)-marked WCS365, and enhanced green (EGFP) or yellow (EYFP) fluorescent protein-marked *P. chlororaphis* PCL1391 (Bloemberg et al. *in press*). Using confocal laser microscopy on 1-week-old roots of sterile tomato plantlets, we observed that bacterial cells are mainly present as elongated stretches on indented areas, such as junctions between epidermal cells and the deeper parts of the epidermis on the root surface and on root hairs (Fig. 1). The highest numbers of bacteria and microcolonies were found at the root base (Fig. 1), whereas numbers of both bacterial cells and mi-

Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Characteristics and references
Pseudomonas fluorescens	
WCS365	Isolated from potato (Geels and Schippers 1983a, 1983b). Efficient colonizer of potato roots (Brand et al. 1991; Glandorf 1992) and tomato roots (Simons et al. 1996). Causes induced systemic resistance in *Arabidopsis thaliana* ecotype Columbia against *Pseudomonas syringae* pv. *tomato* (Gerrits and Weisbeek 1996). Biocontrol strain in a *Fusarium oxysporum* f. sp. *radicis-lycopersici*-tomato system (this article).
WCS307	Isolated from potato (Geels and Schippers 1983b). Poor colonizer of potato roots (Brand et al. 1991; Glandorf 1992) and tomato roots (Simons et al. 1996).
F113	Biocontrol strain in a *Pythium ultimum*-sugar beet system (Shanahan et al. 1992). Good colonizer of tomato roots (Simons et al. 1996).
PCL1209	Mutant of *P. fluorescens* strain WCS365 impaired in the synthesis of the O-antigen of lipopolysaccharide and in competitive tomato root tip colonization (Dekkers et al. 1998a).
PCL1210	Mutant of *P. fluorescens* strain WCS365 impaired in competitive tomato root tip colonization, in which a mutation in a two-component system is responsible for the mutant phenotype (Dekkers et al. 1998a).
PCL1232	*P. fluorescens* WCS365 derivative harboring plasmid pMP5215 (Dekkers et al. 1998b) used for colonization and biocontrol experiments (this article).
PCL1233	Mutant of *P. fluorescens* strain WCS365 impaired in competitive tomato root tip colonization in which a mutation in a site-specific recombinase is responsible for the mutant phenotype (Dekkers et al. 1998b).
PCL1269	Mutant of *P. fluorescens* strain WCS365 impaired in motility and in competitive tomato root tip colonization (this article).
PCL1391	Wild-type *P. chlororaphis*, producing phenazine-1-carboxamide and biocontrol strain of tomato foot and root rot caused by *F. oxysporum* f. sp. *radicis-lycopersici* (Chin-A-Woeng et al. 1998).
PCL1500	*lacZ* derivative of *P. fluorescens* WCS365, which is as efficient as the parental strain in colonizing the tomato root tip (van der Bij et al. 1996).
PCL1502	PCL1500, a Tn*5lacZ* (Lam et al. 1990) derivative of *P. fluorescens* WCS365 harboring the empty vector pWTT2081, which is stable in the rhizosphere (van der Bij et al. 1996).
PCL1510	*lacZ* derivative of *P. fluorescens* F113 (this article).
PCL1511	*lacZ* derivative of *P. fluorescens* F113 harboring the empty vector pWTT2081 (this article).
PCL1512	Derivative of biocontrol strain *P. fluorescens* F113, which harbors the *sss*-containing plasmid pMP5215 (this article).
PCL1516	*lacZ* derivative of *P. fluorescens* WCS307 harboring the empty vector pWTT2081 (this article).
PCL1517	*P. fluorescens* WCS307 harboring the *sss*-containing plasmid pMP5215 (this article).
Escherichia coli	
DH5α	*EndA1 gyrSA96 hrdR17(rK- mK-) supE44 recA1*. Used for transformation and propagation of plasmids (Boyer and Roulland-Dussoix 1969).
Fungi	
Fusarium oxysporum f. sp. *radicis-lycopersici* strain ZUM2407	Cause of tomato foot and root rot, IPO-DLO, Wageningen, The Netherlands.
Plasmids	
pRK2013	Helper plasmid for triparental mating (Ditta et al. 1980).
pWTT2081	Plasmid stably maintained in the rhizosphere (van der Bij et al. 1996).
pMP4641	Stable plasmid pME6010 (Heeb et al. 2000) containing the *ecfp* gene.
pMP4655	Stable plasmid pME6010 (Heeb et al. 2000) containing the *egfp* gene.
pMP4658	Stable plasmid pME6010 (Heeb et al. 2000) containing the *eyfp* gene.
pMP4662	Stable plasmid pME6010 (Heeb et al. 2000) containing the *DsRed* gene.
pMP5215	Plasmid pWTT2081 harboring the 5-kb *Hin*dIII-*Hin*dIII *sss*-containing DNA fragment of *P. fluorescens* strain WCS365 which is able to complement the *sss* mutation in mutant PCL1233 (Dekkers et al. 1998b).

crocolonies gradually decreased in the direction of the root tip, on which low numbers of cells were found (Fig. 1). On the upper part of the root system, larger microcolonies were observed. The microcolonies were of both mixed and one cell type (Fig. 1A and B). On the middle part of the root (3 to 7 cm from the top), small microcolonies and single cells were observed. Most microcolonies were of one cell type, although a mixed microcolony occasionally was present (Fig. 1D and F). On the root tip, single cells or small groups of cells (two to four) often were observed. These small microcolonies are predominantly of one cell type (data not shown). Starting seed inoculation with equal numbers of cells of WCS365 and PCL1391, microcolonies consisting of WCS365 cells were found approximately five times more abundantly than microcolonies consisting of PCL1391 cells, especially on the middle part of the root (Fig. 1D). In contrast, root hairs were colonized almost exclusively with PCL1391 cells (Fig. 1E). Equal but very low numbers of WCS365 and PCL1391 cells were found on the root tip (data not shown).

Quorum sensing plays a crucial role in the production of phenazine-1-carboxamide (PCN) by PCL1391 (T. F. C. Chin-A-Woeng, D. van den Broek, G. de Voer, K. M. G. M. van der Drift, J. E. Thomas-Oates, B. J. J. Lugtenberg, and G. V. Bloemberg, *unpublished data*), and PCN is crucial for biocontrol by this strain (Chin-A-Woeng et al. 1998); therefore, we wondered what the effect would be on biocontrol of the observed dilution of PCL1391 cells with WCS365 cells, which do not produce detectable acylhomoserine lactones (T. F. C. Chin-A-Woeng, *unpublished data*). The presence of WCS365 cells also dilutes the quorum sensing signals, especially on the upper parts of the root. The results of four experiments (Table 3) show that inoculation with either PCL1391 or WCS365 was significant in only two out of four experiments. In contrast, in all four experiments of mixed inoculation, significant biocontrol was observed.

Effect of colonization on biocontrol by WCS365.

Recently, it was shown in our laboratory that root colonization is a requirement for biocontrol by *P. chlororaphis* strain PCL1391 (Chin-A-Woeng et al. *in press*), which exerts its biocontrol action through the production of phenazine-1-carboxamide (Chin-A-Woeng et al. 1998). In order to see whether the same applies for biocontrol by *P. fluorescens* strain WCS365, which acts through ISR, biocontrol activity of colonization mutants of the latter strain was compared with that of the parental strain. The tested colonization mutants were mutant PCL1210, mutated in a two-component system (Dekkers et al. 1998a); mutant PCL1233, which lacks a site-specific recombinase (Dekkers et al. 1998b); mutant PCL1209, which lacks the O-antigen of lipopolysaccharide [LPS] (Dekkers et al. 1998c); and the nonmotile mutant PCL1269. In all seven experiments, the wild-type strain WCS365 showed significant biocontrol (Table 4). Of the tested colonization mutants, strain PCL1209 consistently caused biocontrol. The other three mutants caused biocontrol in some, but not all, experiments (Table 4).

Effect of extra copies of the *sss*-containing colonization operon on competitive root tip colonization of *Pseudomonas* wild-type strains.

In a second attempt to test a role of colonization genes in the biocontrol ability of strain WCS365, we increased the copy number of the *sss*-containing colonization operon by incorporation of this DNA fragment into the rhizosphere-stable plasmid pWTT2081 (four to eight copies). The increase in copy number had no significant effect on the competitive colonization ability of *P. fluorescens* WCS365 (Table 5). however, the increase in copy number resulted in a statistically significant 8- to 16-fold increase in the colonization ability of *P. fluorescens* F113 (Table 5) and even in a 13- to 40-fold increase for *P. fluorescens* WCS307 (Table 5). Strains WCS307 and F113 contain DNA fragments that hybridize with the *sss*-containing fragment of WCS365. We explain the result by assuming that the majority of the cells of WCS365 are in the rhizosphere-competent state, in contrast to the situation in the other two strains. Multiple copies would result in an equilibrium situation and, therefore, cause poorer colonization of WCS365 and better colonization of the other two strains.

DISCUSSION

Biocontrol experiments performed in an *F. oxysporum* f. sp. *radicis-lycopersici*-tomato system showed that disease suppression by *P. fluorescens* WCS365 was statistically significant to a similar extent as *P. chlororaphis* strain PCL1391 (Table 2). These results show that WCS365 not only is an excellent colonizer but also an excellent biocontrol strain. *P. fluorescens* WCS365 is not antagonistic against phytopathogenic fungi under laboratory conditions; therefore, the mechanism underlying biocontrol is likely to be of another nature. Indeed, it has been reported that *P. fluorescens* strain WCS365 can induce ISR in *A. thaliana* ecotype Columbia, which thereby is protected against the pathogen *P. syringae* pv. *tomato* (Gerrits and Weisbeek 1996). Whether ISR is also responsible for biocontrol in the *F. oxysporum* f. sp. *radicis-lycopersici*-tomato system is not known with certainty.

Table 2. Biocontrol activity of *Pseudomonas fluorescens* WCS365 and *P. chlororaphis* PCL1391 in a *Fusarium oxysporum* f. sp. *radicis-lycopersici*-tomato system[z]

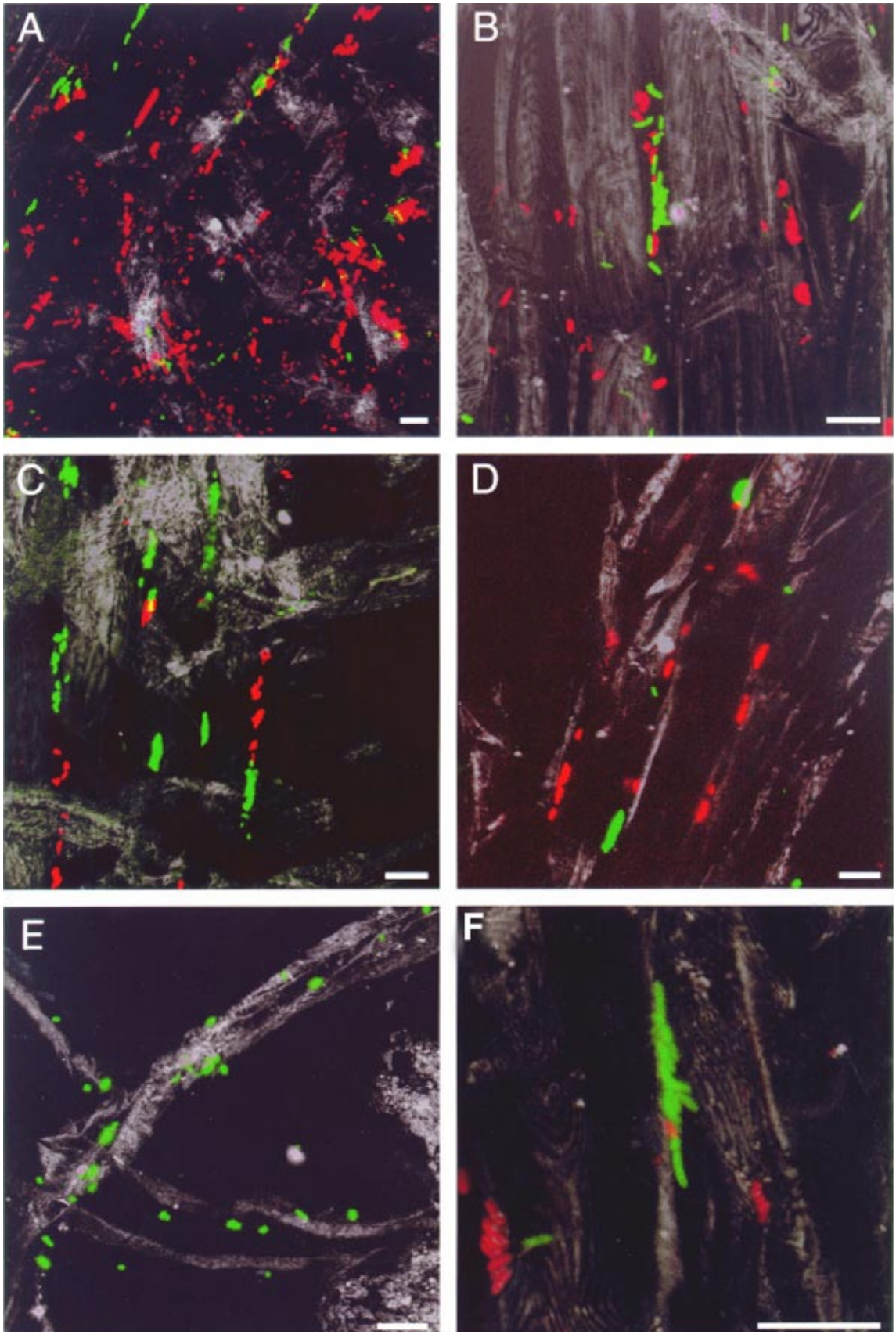
	Diseased plants (%)		
Treatment	**Exp. 1**	**Exp. 2**	**Exp. 3**
Untreated control	45 a	43 a	72 a
WCS365	19 b	25 b	39 b
PCL1391	21 b	27 b	38 b

[z] Data are the result of eight replicates of 12 plants each. Data were analyzed for significance using analysis of variance followed by Fischer's least significant difference test ($\alpha = 0.05$), using SPSS software (SPSS Inc., Chicago). Values followed by different letters indicate a statistically significant difference.

Table 3. Comparison of mixed and single inoculation of tomato with *Pseudomonas fluorescens* WCS365 and *P. chlororaphis* PCL1391 on tomato foot and root rot[z]

	Diseased plants (%)			
Treatment	**Exp. 1**	**Exp. 2**	**Exp. 3**	**Exp. 4**
Untreated control	69 a	60 a	61 a	50 a
PCL1391	52 ab	41 b	47 bc	47 a
WCS365	58 ab	35 b	35 cd	54 a
PCL1391/WCS365	45 b	45 b	25 d	33 b

[z] Data are the result of eight replicates of 12 plants each. Data were analyzed for significance using analysis of variance followed by Fischer's least significant difference test ($\alpha = 0.05$), using SPSS software (SPSS Inc., Chicago). Values followed by different letters indications indicate a statistically significant difference.


A
B
C
D
E
F

To test how two *Pseudomonas* biocontrol strains, which use different mechanisms of action, behave on the root upon inoculation of the seedlings with a 1:1 mixture of cells of these strains, the two cell types were labeled with different fluorescent markers. The results (Fig. 1) show that, although the strains use similar overall colonization strategies (Chin-A-Woeng et al. 1997), their precise behavior differs. Cells of strain WCS365 were more abundant at the middle part of the root (Fig. 1D), whereas cells of PCL1391 were more abundant on root hairs (Fig. 1E). Another striking observation was the frequent occurrence of microcolonies of a single cell type, especially at the lower part of the root system. This suggests to us that initial colonization of new root parts occurs predominantly by single cells.

Mixed inoculation with the two cell types tended to give slightly better protection against the disease than single inoculation with the same number of cells (Table 3). Production of PCN by strain PCL1391 is subject to quorum sensing (T. F. C. Chin-A-Woeng, D. van den Broek, G. de Voer, K. M. G. M. van der Drift, J. E. Thomas-Oates, B. J. J. Lugtenberg, and G. V. Bloemberg, *unpublished data*). Although it is not known how many cells are required to allow PCN production, the fact that most microcolonies on the lower root parts consist of one cell type (Fig. 1) may contribute to the slight synergistic effect of the two strains in plant protection (Table 3).

Although an important role for colonization in biocontrol was suggested previously (Bull et al. 1991; Schippers et al. 1987), this notion is not general (Roberts et al. 1994). Now that well-characterized colonization-impaired mutants are available (Dekkers et al. 1998a, 1998b, 1998c; Lugtenberg and Dekkers 1999; Lugtenberg et al. 1996; Simons et al. 1996, 1997; Weller 1988) it is possible to test the influence of colonization on biocontrol experimentally.

Chin-A-Woeng et al. (Chin-A-Woeng et al. *in press*) have constructed colonization mutants in *P. chlororaphis* strain PCL1391 whose PCN production is required for biocontrol of tomato foot and root rot. Individual derivatives of this strain, impaired in *sss/xerC*, motility, and the synthesis of the amino acid phenylalanine, appeared not to be able to control disease in an *F. oxysporum* f. sp. *radicis-lycopersici*-tomato biocontrol system. Controls show that the mutants were still able to inhibit growth of *F. oxysporum* f. sp. *radicis-lycopersici* on plates. Their results clearly showed, for the first time, the crucial role of colonization in biocontrol (Chin-A-Woeng et al. *in press*).

Colonization mutants of strain WCS365 show a less clear effect (Table 4), because some mutant strains (e.g., PCL1269, PCL1233, and PCL1210) show efficient biocontrol in some, but not all, experiments, whereas strain PCL1209 shows biocontrol in all three experiments. We conclude that colonization plays a less important role in biocontrol when strain WCS365 is used than when strain PCL1391 is used. The most likely explanation is the difference in biocontrol mechanisms used by the two strains. In the case of PCL1391, production and secretion of the antifungal metabolite PCN on a substantial part of the root during the whole plant growth period is likely to be necessary to protect the root against pathogens from the soil. In the case of WCS365, which is supposed to act through ISR, it is conceivable that colonization of seedlings for a brief period is sufficient to induce ISR in the whole plant.

Colonization is often the limiting step in biocontrol; therefore, the importance of the *sss* gene in colonization (Dekkers et al. 1998b) raised the question of whether this operon can be used to improve colonization in other *Pseudomonas* spp. Results (Table 5) show that the presence of multiple copies of an *sss*-containing fragment in the wild-type strain WCS365 has no positive effect on root tip colonization. However, the presence of the same fragment in two *P. fluorescens* strains, F113 and WCS307, improved root tip colonization enormously. These experiments show that improvement of colonization through genetic engineering is a realistic goal. This suggests that, in cases in which colonization is limiting for biocontrol, the use of *col* genes can improve disease control.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions.

All *Pseudomonas* strains (Table 1) were grown overnight at 28°C on solidified King's medium B (King et al. 1954) or in

Fig. 1. Tomato root colonization by *Pseudomonas fluorescens* WCS365 and *P. chlororaphis* PCL1391. Germinated seedlings were inoculated with a 1:1 mixture of *P. fluorescens* WCS365 harboring pMP4662 (*DsRed*) or pMP4641 (*ecfp*) and *P. chlororaphis* PCL1391 harboring pMP4655 (*egfp*) or pMP4658 (*eyfp*). Root samples (10 cm long) were inspected after 7 days of growth in the gnotobiotic system (Simons et al. 1996). **A and B,** Examples of the upper part of the tomato root system or root base (first 1 to 3 cm). **C and D,** Examples of the middle part of the root system (3 to 7 cm). **E,** Root hairs and **F,** close up of two microcolonies on the middle part (3.5 cm) of the tomato root system. **A, D, and E,** A combination of DsRed-marked WCS365 and EGFP-marked PCL1391 cells. **B, C, and F,** A combination of ECFP-marked WCS365 and EYFP-marked PCL1391 cells. **A–F,** WCS365 cells are depicted in red and PCL1391 cells are depicted in green. **A–F,** The size bars represent 10 μm.

Table 4. Biocontrol activity of *Pseudomonas fluorescens* WCS365 and its colonization mutants[z]

	Diseased plants (%)						
Inoculated strain	**Exp. 1**	**Exp. 2**	**Exp. 3**	**Exp. 4**	**Exp. 5**	**Exp. 6**	**Exp. 7**
None	42 a	37 a	63 a	46 a	45 a	43 a	72 a
WCS365	26 c	21 b	44 b	36 b	19 b	25 b	39 bc
PCL1269	…	…	…	…	…	36 a	42 bc
PCL1209	…	…	…	…	16 b	26 b	51 b
PCL1233	35 abc	23 b	58 a	47 a	16 b	30 a	47 bc
PCL1210	33 ab	…	…	45 ab	20 b	27 b	37 c

[z] Data are the result of eight replicates of 12 plants each. It should be noted that the methods used for seed inoculation in experiments 1 through 4 and 5 through 7 were different. Data were analyzed for significance using analysis of variance followed by Fischer's least significant difference test (α = 0.05), using SPSS software (SPSS Inc., Chicago). Values with different letter indications indicate a statistically significant difference.

liquid King B under vigorous aeration. For the transfer of plasmids from *Escherichia coli* to *Pseudomonas* spp., triparental mating was performed using pRK2013 as the helper plasmid (Ditta et al. 1980). Selection for plasmid pWTT2081 and its derivatives (van der Bij et al. 1996) was performed on medium supplemented with nalidixic acid and tetracycline to final concentrations of 15 and 80 μg/ml, respectively. *E. coli* cells used for propagation of plasmids were grown overnight in liquid or solidified Luria-Bertani medium (LB) (Sambrook et al. 1989) supplemented with tetracycline (40 μg/ml). If appropriate, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was added to the medium to a final concentration of 40 μg/ml as an indicator for β-galactosidase activity.

Colonization experiments on tomato plants.

The ability of various *Pseudomonas* strains to colonize tomato root tips was studied using the gnotobiotic system described by Simons et al. (1996). Briefly, sterile germinated tomato seeds were inoculated with a 1:1 mixture of cells of two strains, one of which was marked with Tn*5lacZ*. After growth for 7 days, bacteria were isolated from the root tip (1 to 2 cm). When plasmid-containing strains had to be tested, pWTT2081, a rhizosphere-stable plasmid (van der Bij et al. 1996), was used as the vector. This plasmid acts as a genetic or physiological burden (van der Bij et al. 1996); therefore, it also was present in the control cells. The ratio of cells harboring plasmid pMP5215 (yellow/white colonies) and cells marked with Tn*5lacZ* and additionally harboring plasmid pWTT2081 (blue colonies) was determined by plating on King B supplemented with X-gal.

Biocontrol experiments.

For experiments 1 through 4 (Table 4), suspensions of approximately 10^{12} CFU/ml were made by scraping *Pseudomonas* bacteria from King B plates and suspending them into 6 ml of sterile 10 mM $MgSO_4$. These suspensions were used to coat seeds of tomato (*Lycopersicon esculentum* Mill. cv. Carmello) in a small-scale industrial fluidized bed coater. For all other seed coatings performed for biocontrol experiments, overnight King B cultures were washed and adjusted to an optical density at 620 nm (OD_{620}) of 0.7. For coating with a mixture of both WCS365 and PCL1391, cultures of OD_{620} of 0.7 were mixed in a ratio of 1:1. All suspensions were mixed with an equal volume of 2% (wt/vol) methylcellulose. Tomato seeds were inoculated by dipping in the resulting suspensions for 10 min and air-dried. Biocontrol by various *Pseudomonas* spp. against *F. oxysporum* f. sp. *radicis-lycopersici* on tomato was performed as described by Chin-A-Woeng et al. (1998). Coated tomato seeds were sown in *F. oxysporum*-infested soil in multicell trays. For each coating, at least eight replications containing 12 plants each were used. After incubation for 16 days in a climate-controlled growth chamber at 20°C and 85% relative humidity, the number of diseased root systems was assessed. Data were analyzed for significance using analysis of variance followed by Fischer's least significant difference test ($\alpha = 0.05$), using SPSS software (SPSS Inc., Chicago).

Table 5. Effect of the introduction of the *sss*-containing plasmid pMP5215 on the competitive tomato root tip-colonizing ability of various *Pseudomonas fluorescens* wild-type strains[y]

	Colonizing ability[z]	
Strain	**Wild-type**	**Wild-type harboring plasmid pMP5215**
WCS365	4.4 ± 0.4 a	3.8 ± 0.5 a
F113	3.3 ± 0.8 a	4.5 ± 0.3 b
	3.7 ± 0.3 a	4.6 ± 0.4 b
WCS307	3.0 ± 0.9 a	4.6 ± 0.2 b
	3.3 ± 0.8 a	4.4 ± 0.3 b

[y] In these experiments, *lacZ*-marked wild-type strains containing the empty vector pWTT2081 were compared with the wild-type strain containing pMP5215 (i.e., pWTT2081), in which an *sss*-containing fragment (Dekkers et al. 1998b) has been cloned. It was shown previously (Simons et al. 1996; van der Bij et al. 1996) that the presence of this *lacZ* marker has no influence on the colonizing behavior of WCS365.

[z] Mean $\log_{10}$ (CFU + 1)/cm of root tip. Different letters following colonization values within one experiment indicate that the results are significantly different at $P = 0.05$ according to the Wilcoxon Mann-Whitney test (Sokal and Rohlf 1981).

Microscopy.

Germinated tomato seedlings were inoculated with a 1:1 mixture of *P. fluorescens* WCS365, harboring the *DsRed*- or *ecfp*-containing plasmid, and *P. chlororaphis* PCL1391, harboring *egfp*- or *eyfp*-containing plasmid, and planted in the gnotobiotic system (Simons et al. 1996). After 1 week, the tomato roots were isolated and rinsed in phosphate-buffered saline solution (PBS; 0.9% NaCl buffered with 10 mM sodium phosphate, pH 7.2). Tomato roots were mounted on a microscope slide for observation. Roots were examined with an inverted fluorescence microscope (Leica DMIRBE; Leica, Bensheim, Germany) equipped with filter blocks with spectral properties matching those of CFP (440/21-nm excitation, 480/36-nm emission, XF114; Chroma, Brattleboro, VT, U.S.A.) or EGFP and EYFP (470/20-nm excitation, 515-nm long pass emission, I3; Leica) or DsRed (538/22-nm excitation, 590-nm long pass emission, N2.1; Leica). A Leica SP confocal scanhead was attached to this microscope. Dual color images were obtained by sequentially scanning with settings optimal for CFP (excitation with the 457-nm argon laser line, detection of emitted light between 470 and 490 nm), EGFP (excitation with a 488-nm argon laser line, detection of emitted light between 500 and 520 nm), EYFP (excitation with the 488-nm argon laser line, detection of emitted light between 530 and 550 nm), or DsRed (excitation with the 568-nm krypton laser line, detection of the emitted light between 580 and 620 nm.) Cross talk between the channels in this set-up was monitored and, in all cases, was negligible. Pictures obtained using different channels were merged using Photoshop 5.0 (Adobe, San Jose, CA, U.S.A.) to facilitate projection.

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