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Amino Acid Synthesis Is Necessary for Tomato Root Colonization by *Pseudomonas fluorescens* Strain WCS365

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In this work the bio-availability of amino acids for the root-colonizing *Pseudomonas fluorescens* strain WCS365 in the tomato rhizosphere was studied. The amino acid composition of axenically collected tomato root exudate was determined. The results show that aspartic acid, glutamic acid, isoleucine, leucine, and lysine are the major amino acid components. The concentrations of individual amino acids in the rhizosphere of gnotobiotically grown tomato plants were estimated and considered to be too low to support growth of rhizosphere micro-organisms to numbers usually found in the tomato rhizosphere. To test this experimentally, mutants of *P. fluorescens* WCS365 auxotrophic for the amino acids leucine, arginine, histidine, isoleucine plus valine, and tryptophan were isolated after mutagenesis with Tn*5lacZ*. Root tip colonization of these mutants was measured after inoculation of germinated tomato seeds and subsequent growth in a gnotobiotic quartz sand system (M. Simons, A. J. van der Bij, I. Brand, L. A. de Weger, C. A. Wijffelman, and B. J. J. Lugtenberg. 1996. Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting *Pseudomonas* bacteria. Mol. Plant-Microbe Interact. 9:600-607). In contrast to the wild-type strain, none of the five amino acid auxotrophs tested was able to colonize the tomato root tip, neither alone nor after co-inoculation with the wild-type strain. However, addition of the appropriate amino acid to the system restored colonization by the auxotrophic mutants, usually to wild-type levels. Analysis of the root base showed that cells of auxotrophic mutants were still present there. The results show that, although amino acids are present in root exudate, the bio-availability of the tested amino acids is too low to support root tip colonization by auxotrophic mutants of *P. fluorescens* strain WCS365. The genes that are required for amino acid synthesis are therefore necessary for root colonization. Moreover, these compounds apparently play no major role as nutrients in the tomato rhizosphere.

Since the numbers of micro-organisms on plant roots are usually higher than in distant soil (Hiltner 1904), the plant root is thought to be the major source of nutrients for micro-organisms living in the rhizosphere (Lynch and Whipps 1990). Therefore, bacterial growth on compounds present in the rhizosphere is assumed to be essential for efficient colonization of and establishment in the rhizosphere. Despite the importance of bacterial growth in the rhizosphere, hardly anything is known about bacterial nutrition in this complex niche. Identification of the exudate nutrients and their utilization by rhizosphere microbes is therefore a key to unraveling the microbial competition for nutrients in the rhizosphere.

Amino acids, sugars, and organic acids are quantitatively the major components of root exudate, which is supposed to be the major source of nutrients for rhizosphere-colonizing micro-organisms. Knowledge of the bacterial growth conditions in the rhizosphere is important for understanding rhizosphere colonization. This in turn is critical for the application of beneficial micro-organisms as inoculants to support plant growth (Schippers et al. 1987; Weller 1988). A few years ago we initiated a study to elucidate the molecular basis of rhizosphere colonization by *Pseudomonas* bacteria. Our approach was to predict bacterial traits that might be involved in colonization. After mutants defective in such traits were isolated, the hypothesis was tested by analyzing the colonization ability of these mutants in comparison with that of the wild type. In this way it was shown that the presence of flagella (de Weger et al. 1987) and the presence of the O-antigen of lipopolysaccharide (de Weger et al. 1989) are important colonization traits. More recent results indicate that the ability to synthesize vitamin B1 and a high growth rate are also important colonization traits (Simons et al. 1996). In this paper we focused on the bio-availability of amino acids for the efficient tomato root-colonizing *P. fluorescens* strain WCS365, and on their role in colonization. We determined the amino acid composition of axenically collected tomato root exudate and the ability of newly isolated amino acid auxotrophic Tn*5lacZ* mutants to colonize the roots.

RESULTS

Analysis of tomato root exudate.

Tomato root exudate was collected from 80 sterile 7-day-old tomato plantlets grown in 80 ml of sterile PNS. After

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samples from the exudate were shown to not show microbial growth, amino acids were isolated by trapping them on a Dowex AG50W8 ion exchange column. After derivatization with PITC, the amino acids were analyzed by high-performance liquid chromatography (HPLC), as described in Materials and Methods. Based on the elution profiles of mixtures of standard amino acids and exudate samples, exudate amino acids were identified and quantified after correction for losses during the sample preparation, which were determined individually for each amino acid. Amino acids that were not detected were therefore apparently absent or present below the detection limit of approximately 1 µM in exudate. Detection could not be improved by further sample concentration since this procedure resulted in the appearance of background peaks. Data is shown in Table 1. Aspartic acid, glutamic acid, isoleucine, leucine, and lysine appeared to be the major amino acids. Amino acids not found in exudate are alanine, proline, tyrosine, valine, methionine, and cysteine. The results show that the amounts exuded in 1 week per plant are in the nanomole range.

Based on these data we attempted to estimate the amino acid concentrations in the rhizosphere assuming that (i) the average root length is 80 mm, (ii) the average root diameter is 1 mm, (iii) the average rhizosphere thickness is 1 mm, (iv) the secreted amino acids are not metabolized by the plant, and (v) the exuded amino acids do not inhibit further exudation. This calculation yields an estimated root volume of $80 \cdot \pi \cdot 0.5^2 = 63$ mm^3. The volume of root plus rhizosphere would then be $80 \cdot \pi \cdot 1.5^2 = 567$ mm^3. The volume of rhizosphere of one plant would then be $567 - 63 \approx 500$ mm^3. The 80 plants used would have a total rhizosphere volume of 40,320 mm^3, or about 40 ml. According to this calculation the concentrations of individual amino acids in the rhizosphere would be twice the concentration found in the volume of 80 ml of analyzed exudate, i.e., below 10 µM (Table 1). Considering that the concentration of amino acid required for growth of an auxotroph in minimal medium is usually around 200 µM, this result suggested to us that the amino acid concentration found in exudate is insufficient to contribute substantially to the nutrition of rhizosphere bacteria.

To test this notion experimentally, amino acid auxotrophic mutants were constructed.

Table 1. Amino acid composition of axenically collected tomato root exudate

Amino acid	Determined concentration in root exudate (µM)[y]	Estimated concentration in rhizosphere (µM)[z]
Aspartic acid	3.8	8
Glutamic acid	4.8	9
Serine + asparagine	1.3	3
Glycine + glutamine	1.2	3
Histidine	1.2	3
Arginine + threonine	1.1	2
Isoleucine	2.2	5
Leucine	2.5	5
Phenylalanine	1.2	2
Lysine	2.0	4

[y] Amino acids not detected in exudate: alanine, proline, tyrosine, valine, methionine, and cysteine. Concentrations of these compounds in exudate therefore are below the detection limit of approximately 1 µM.
[z] For assumptions used in these estimates, see Results.

Isolation and characterization of amino acid auxotrophic mutants.

Screening of 10,000 *P. fluorescens* strain WCS365:: Tn*5lacZ* colonies yielded 40 auxotrophic mutants (0.4%) that grew on KB but not on SSM. Analysis of these mutants by growth on SSM supplemented with various mixtures of compounds as described by Holliday (1956) showed that the auxotrophy of 12 mutants could not be identified by this method. These mutants were not used further. Mutants requiring tryptophan (PCL1009), arginine (PCL1033), valine plus isoleucine (PCL1022), leucine (PCL1014), and histidine (PCL1003) were selected for further use in colonization experiments.

Tomato rhizosphere colonization of amino acid auxotrophic mutants.

The ability of each auxotrophic mutant to reach the tip of the root system after inoculation of the seedling was determined in the absence (Table 2) and presence (Table 3) of the

Table 2. Colonization of tomato root tip by *Pseudomonas fluorescens* strain WCS365 and selected amino acid auxotrophic Tn*5lacZ* mutants inoculated as single strains in gnotobiotic system, in absence and presence of exogenously added amino acids[y]

	Colonization [$\log_{10}$(CFU + 1)/cm root tip] by strain					
Addition to PNS[z]	WCS365	PCL1014 (*leu*)	PCL1033 (*arg*)	PCL1003 (*his*)	PCL1022 (*ilv*)	PCL1009 (*trp*)
None	2.8 a	0 c	0 c	0 c	0 c	0 c
Leucine	2.1 a	2.7 a	. . .	. . .	. . .	. . .
Arginine	4.3 b	. . .	2.8 a	. . .	. . .	. . .
Histidine	4.4 b	. . .	. . .	4.0 b	. . .	. . .
Valine + isoleucine	2.4 a	. . .	. . .	. . .	2.9 a	. . .
Tryptophan	5.1 b	. . .	. . .	. . .	. . .	5.8 b

[y] Sterile, germinated tomato seeds were dipped in a 10^7 CFU/ml suspension of cells of WCS365 or an auxotrophic WCS365::Tn*5lacZ* mutants. After 7 days of plant growth, root tip was isolated. Bacteria were isolated from root tip and plated on King's medium B, supplemented with X-gal, in order to differentiate wild-type (white) and mutant (blue) colonies on a single plate. From these numbers, the $\log_{10}$(CFU + 1)/cm value was calculated. Values represent averages of 10 plants. Detection limit for each determination is 2.4 log CFU/cm root tip or 0.24 log CFU/ml for 10 plants. Values with same letter are not significantly different ($P = 0.05$) according to Wilcoxon-Mann-Whitney test.
[z] Plant nutrient solution.

Table 3. Colonization of tomato root tip by auxotrophic Tn*5lacZ* mutants of *Pseudomonas fluorescens* strain WCS365 after co-inoculation with wild-type cells in gnotobiotic system, in absence and presence of exogenously added amino acids[y]

	Colonization [$\log_{10}$(CFU + 1)/cm root tip] by strain					
Addition to PNS[z]	WCS365	PCL1014 (*leu*)	PCL1033 (*arg*)	PCL1003 (*his*)	PCL1022 (*ilv*)	PCL1009 (*trp*)
None	3.3 a	0 c	0 c	0 c	0 c	0 c
Leucine	5.0 b	5.2 b	. . .	. . .	. . .	. . .
Arginine	4.4 b	. . .	1.4 d	. . .	. . .	. . .
Histidine	3.6 a	. . .	. . .	2.9 a	. . .	. . .
Valine + isoleucine	2.2 a	. . .	. . .	. . .	3.4 a	. . .
Tryptophan	5.1 b	. . .	. . .	. . .	. . .	4.9 b

[y] For description of colonization assay, see Table 2 footnote. In the experiments described here, seedlings were co-inoculated a 1:1 mixture of 10^7 CFU/ml of *P. fluorescens* strain WCS365 and an auxotrophic WCS365::Tn*5lacZ* mutant. Values with same letter are not significantly different ($P = 0.05$) according to Wilcoxon-Mann-Whitney test.
[z] Plant nutrient solution.

wild-type strain and in the absence and presence of the required amino acid. When mutant and wild type were tested separately, sterile tomato seedlings were inoculated in a suspension of approximately 10^7 CFU/ml of the single strain. In the case of mixtures, seedlings were inoculated in a 1:1 mixture of mutant and wild type containing a total of 10^7 CFU/ml. With this method, tomato seedlings contained approximately 5×10^4 CFU directly after inoculation. After 7 days of plant growth, the root tip was isolated and the number of CFU was determined for each strain on KB supplemented with X-gal. The results show that none of the auxotrophic mutants colonized the root tips after inoculation, neither alone (Table 2) nor in competition with the wild-type strain (Table 3).

Addition of the required amino acid restored colonization by the mutants when tested alone (Table 2), as well as after co-inoculation with the wild-type strain (Table 3). Addition of the individual amino acids often appeared to affect the level of colonization by the wild-type strain (Tables 2 and 3). This trend was usually followed by the mutants except for the arginine-requiring mutant PCL1033, which never reached the corresponding wild-type level (Tables 2 and 3). Inspection of the plants showed that the addition of tryptophan to the plant growth medium caused shorter and somewhat thicker plant roots.

Analysis of other parts of the root showed that auxotrophic mutants were rarely present on root parts below the first centimeter of the root base. On 1-cm segments below the root base, the wild-type strain was present in numbers that were also found in previous studies, approximately 10^6 CFU/cm (Simons et al. 1996). On the root base (i.e., the part that was inoculated), the number of cells of both wild type and mutant were approximately 10^6 to 5×10^6 CFU/cm after 7 days of plant growth. The total number of wild-type bacteria on the root was about 5×10^6.

Table 4. Bacterial strains and relevant characteristics

Strain	Relevant characteristics	Reference or source
Escherichia coli		
S17-1	MM294, RP4-2 Tc::Mu-Km::Tn*7* chromosomally integrated	Simon et al. 1983
Pseudomonas		
WCS365	Wild-type *P. fluorescens*, isolated from potato roots	Geels and Schippers 1983
	Efficient colonizer of the potato rhizosphere	Brand et al. 1990
	Efficient colonizer of the tomato, radish, and wheat rhizosphere	Simons et al. 1996
	Biocontrol strain in hydroponic cucumber system	J. Postma, unpublished
PCL1003	WCS365::Tn*5lacZ*, requiring histidine	This work
PCL1009	WCS365::Tn*5lacZ*, requiring tryptophan	This work
PCL1014	WCS365::Tn*5lacZ*, requiring leucine	This work
PCL1022	WCS365::Tn*5lacZ*, requiring isoleucine and valine	This work
PCL1033	WCS365::Tn*5lacZ*, requiring arginine	This work
Plasmid		
pCIB100	pLRKΔ211 (ColE1) with pSUP5011 mobilization segment. Carries Tn*5lacZ* (Km^r). The *lacZ* is constitutively expressed in *Pseudomonas.*	Lam et al. 1990

DISCUSSION

The amino acid analysis of tomato root exudate (Table 1) shows that many amino acids are present. All major peaks in the chromatograms could be identified as known amino acids.

The amounts we found correspond, with respect to order of magnitude, to the work of others on root exudate amino acids (van Egeraat 1972; Klein et al. 1988; Gamliel and Katan 1992a). We found amino acid concentrations in the micromolar range, in 7-day-old sterile exudate and with 0.5 ml of PNS per tomato plant. We considered it unlikely that the low amounts of amino acids we found can function as major nutrients for rhizosphere-colonizing micro-organisms (see estimate in Results section). This notion was tested by an in situ assay in the rhizosphere, i.e. colonization, based on previous work on tomato rhizosphere colonization (Simons et al. 1996). This work indicated that the most sensitive assay for putative colonization mutants is the analysis of the lower root parts, i.e., the root tip.

Our results on tomato root tip colonization by five amino acid auxotrophic mutants show that the mutants do not colonize the root tip unless the required amino acid is added (Tables 2 and 3). We do not understand the effects usually found, after the addition of amino acids, on bacterial numbers of both the wild-type strain and mutants. Since colonized roots generally show a decreasing number of bacteria from root base to root tip (Simons et al. 1996), the increased colonization observed in the presence of tryptophan may be explained by the shorter roots.

Restoration of colonization by the addition of the required amino acid, as observed in most experiments (Tables 2 and 3), shows that the colonization defect is due to the lack of the amino acid. The colonization results (Tables 2 and 3) therefore confirm our notion (Table 1 and text) that the amount of amino acids in exudate is too low to contribute substantially to supporting growth of strain WCS365 in the rhizosphere. Inoculant bacteria may use a survival strategy in the rhizosphere that requires the expression of specific genes (Matin 1992). The possibility was considered that insufficient amino acid is available for the auxotrophic mutants to produce these proteins and that they therefore cannot survive, let alone grow. However, considering the number of cells actually present on a freshly inoculated seedling, approximately 5×10^4, the 100-fold higher number we isolated from the root base after 1 week indicates that substantial growth of the bacteria occurs in the rhizosphere. The number of cells of auxotrophic mutants on the root base after 7 days is usually equal to or not more than five-fold lower than the numbers of wild-type cells, which indicates that these cells must have grown. Usually, no auxotrophic cells can be detected at the lower root parts after 7 days of plant growth. Apparently, the mutants cannot take part in the actual colonization process.

The observation of Glandorf (1992), using mutants provided by our group, that amino acid auxotrophs of strain WCS365 are also impaired in potato root colonization tested in field soil, indicates that our results can also be valid in

other systems. Our results add one important trait to the known bacterial traits required for efficient rhizosphere colonization by fluorescent pseudomonads. To these traits, namely, motility (de Weger et al. 1987), chemotaxis (Gamliel and Katan 1992b), the production of the O-antigenic side chain of lipopolysaccharide (de Weger et al. 1989), the ability to synthesize vitamin B1, and a high bacterial growth rate (Simons et al. 1996), we can now add amino acid prototrophy.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains used in this study are shown in Table 4. *P. fluorescens* WCS365 was grown at 28°C on King's medium B (KB; King et al. 1954) solidified with 1.8 % agar (Bacto Agar, Difco Inc., Detroit, MI). A derivative of *Escherichia coli* S17-1 harboring plasmid pCIB100, which is a suicide plasmid in *Pseudomonas*, was grown at 37°C on solidified LC medium (Maniatis et al. 1982) supplemented with 20 µg of kanamycin (Sigma, Bornhem, Belgium) per ml. Stock cultures of all strains were kept in 35% glycerol at –80°C. Bacteria on agar were kept at 4°C until use.

P. fluorescens WCS365::Tn*5lacZ* transconjugants were generated by a two-parental mating of strains *P. fluorescens* WCS365 and *E. coli* S17-1 harboring plasmid pCIB100 (Lam et al. 1990) as described previously (Simons et al. 1996). Auxotrophic transconjugants were isolated after screening of WCS365::Tn*5* colonies on standard succinate medium (SSM; Meyer and Abdallah 1978) and on KB. The auxotrophies of mutants that grew on KB but not on SSM were further characterized by the method of Holliday (Holliday 1956). Briefly, colonies were transferred to SSM plates containing specific amino acid mixtures. Mutants that grew on two of these plates require a single compound for growth, which was confirmed by determination of growth on SSM supplemented with the appropriate compound. Mutants that grew on only one plate containing a mixture of amino acids apparently need a combination of compounds. The latter mutants were further characterized by determination of growth on SSM supplemented with various mixtures of compounds that were present in the original mixture as described by Holliday (1956).

Collection of root exudate.

Tomato (*Lycopersicon esculentum* Mill. 'Carmello') seeds (S&G Seeds B.V., Enkhuizen, The Netherlands) were sterilized by gentle shaking for 3 min in 5% household sodium hypochlorite. The sterilized seeds were soaked six times for 30 min in sterile, demineralized water. To synchronize the germination process, seeds were placed on petri plates containing PNS (plant nutrient solution, Hoffland 1992), consisting of 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM $MgSO_4$, 1 mM KH_2PO_4, and micronutrients, solidified with 1.5% Pronarose D1 (Hispanagar, Burgos, Spain). The plates were incubated overnight upside down in the dark at 4°C, followed by incubation at 28°C for 2 days. Root exudate was collected in Magenta vessels (Sigma, Bornhem, Belgium), equipped with a stainless steel, perforated tray and filled with a volume of 80 ml of PNS up to the tray. Eighty germinated seedlings were placed on the tray with their roots into the solution. After 1 week of growth at 18°C, exudate was collected by filtration over Whatman No. 3 filter paper (Whatman International Ltd., Maidstone, England) to remove solid plant material, and frozen immediately with liquid nitrogen. A sample of exudate was taken directly from the Magenta vessels and tested for sterility on solidified KB. The exudate was then lyophilized and the solid material was redissolved in 2.0 ml of demineralized water. This 40-fold concentrated exudate was filtered through a 0.45-µm disposable cellulose-nitrate filter (Sartorius GmbH, Göttingen, Germany) to remove undissolved particles, and stored at –20°C until use. Only material from exudates in which no microbial growth could be detected was used.

Amino acid isolation and analysis.

Root exudate amino acids were trapped on a Dowex AG50W-X8 cation exchanger (Biorad, Veenendaal, The Netherlands), according to the method of Klein et al. (1988). Before use, the resin was washed and equilibrated by subsequent washings with 0.5 M ammonium sulfate for 2 h and 7% formic acid for 8 h. The resin was finally rinsed with and stored in 1.0% formic acid. A disposable column (EconoPac, Biorad, Veenendaal, The Netherlands) was filled with 20 ml of cation exchanger and washed with 80 ml of 1.0% formic acid (Sigma), followed by demineralized water until the eluent was neutral. The exudate sample was loaded on this column, and washed with 2.0 ml of 1 mM HCl. The neutral and negatively charged compounds were washed off with four column volumes of 50 mM HCl. Subsequently, the trapped amino acids were eluted with four column volumes of 4 M ammonium hydroxide. The resulting solution was freed from ammonia overnight in vacuo with a cold trap and subsequently lyophilized. Prior to HPLC analysis the samples were derivatized with PITC (phenylisothiocyanate; Pierce, Rockford, IL) (Janssen et al. 1986). Briefly, lyophilized samples from the cation exchanger were dissolved in the redrying mix EMT (ethanol–Milly Q water–triethylamine, 2:2:1, vol/vol/vol). Dissolved samples were dried in a Speedvac concentrator for 30 min. This procedure was conducted two times. Subsequently, 20 µl of freshly prepared PEMT (PITC-EMT, 1:7:1:1 vol/vol/vol/vol) derivatization mixture was added. After the sample was carefully dissolved, the mixture was allowed to react at ambient temperature for 20 min. After drying in a Speedvac concentrator, the sample was dissolved in 400 µl of buffer A (0.14 M sodium acetate, 0.05% TEA [triethylamine; Sigma, Bornhem, Belgium], 4% acetonitrile in Milly Q water; adjusted to pH 6.40 with acetic acid) prior to injection on the column. The HPLC system consisted of a C_{18} reversed-phase column (Shandon hypersil ODS 250 · 4 mm, Shandon BV, Zeist, The Netherlands) at 50°C in a column oven and gradient elution with buffer A and buffer B (60% acetonitrile in water). Even with an optimized gradient, amino acids eluted in clusters, as has been observed by others (Findlay and Geishow 1986). Ornithine was used as an internal standard. A standard mixture of amino acids (Pierce amino acid standards mixH nr.20088) was used as an external standard. This mixture contains alanine, arginine, aspartic acid, cysteic acid, glutamic acid, glycine, histidine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine. With known concentrations of the standard amino acids, exudate amino acid concentrations were corrected for losses during the isolation and derivatization procedure. For peak detection the variable wavelength monitor (Pharmacia LKB, Uppsala, Sweden) was set to 254 nm. Nelson 2600 /

HPLC Manager software (Pharmacia) was used for peak integration and identification.

Amino acids used for peak identification (amino acid standard kit 22, Pierce nr. 20065) were derivatized individually and analyzed in couples to determine elution volumes. A mixture of external standards was used between runs to determine elution volume of appropriate peaks. Peaks were identified on the basis of elution volumes and by mixing samples with known amino acids.

Gnotobiotic colonization assay.

For studying the colonization of the rhizosphere a recently developed gnotobiotic tube system for tomato was used (Simons et al. 1996). Briefly, quartz sand (Wessem filterzand, Wessem, The Netherlands) was mixed with PNS (10% vol/wt), pH 5.8. If required, 1 mM amino acid (Pierce amino acid standard kit 22 nr. 20065) was added to the PNS. The complete tube was sterilized by autoclaving at 110°C for 40 min. After autoclaving, the tubes were stored at room temperature for at least 48 h to equilibrate. Inoculation of tomato seeds was performed by dipping germinated seeds for 5 min in a bacterial suspension of 10^7 CFU/ml. After inoculation, the seeds were placed under the surface of the quartz sand with sterile forceps. The plant growth tubes were kept in a climate-controlled growth chamber (18°C, 70% relative humidity, 16 h daylight) to allow the tomato plantlets to grow. After 7 days, in which the root systems grew to an average length of 8 cm, a 1-cm fragment was cut off the root tip and shaken vigorously in 1.0 ml of PNS on an Eppendorf shaker for 15 min to remove the bacteria. The bacterial suspension was diluted 10-fold and 37 µl was plated on KB plates supplemented with 40 µg of X-gal per ml, with an spiral plater (Spiral Systems Inc., Cincinnati, OH). After 2 days of incubation at 28°C the numbers of yellow/white (wild-type) and blue (Tn*5lacZ* auxotrophic mutants) colonies were counted. The CFU/cm was calculated (Davies and Whitbread 1989), $\log_{10}$(CFU + 1)/cm transformed (Loper and Schroth 1984) to avoid the nonexistent log(0) situation. Estimates of mean and standard deviation were calculated, to be used as point estimates. All colonization experiments were carried out in 10-fold, and repeated at least once. For statistical comparison of strains in mixed inocula the nonparametric Wilcoxon-Mann-Whitney test was used (Siegel 1956; Sokal and Rohlf 1981).

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