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Genetic Analysis of the *aggA* Locus Involved in Agglutination and Adherence of *Pseudomonas putida*, a Beneficial Fluorescent Pseudomonad

C. R. Buell and A. J. Anderson

Department of Biology and Molecular Biology Program, Utah State University, Logan, UT 84322-5305.
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An isolate of *Pseudomonas putida*, which rapidly adheres to plant roots, is agglutinated by a glycoprotein from root surfaces. Agglutination is prevented and adherence to the root surface is diminished by Tn*5* insertion in mutant 5123. Two cosmid clones from wild type *P. putida* and a 2.7-kbp *Eco*RI-*Hin*dIII subclone present in both cosmid clones restored agglutinability to wild type levels in transconjugants of the nonagglutinable (Agg^-) Tn*5* mutant 5123. These three clones increased agglutinability in transconjugants of the parental Agg^+ isolate. The 2.7-kbp *Eco*RI-*Hin*dIII subclone restored adherence to bean root surfaces of 5123 to wild type levels in a short-term binding assay. Deletion analysis of the 2.7-kbp fragment indicated only 1.45 kbp was necessary for complementation of agglutinability in 5123. This sequence, termed the *aggA* locus, contains an open reading frame of 1,356 nucleotides encoding a predicted 50,509-Da protein. The distribution of the *aggA* locus in plant-associated bacteria, as detected through Southern hybridization, is limited to bacteria that express the agglutination phenotype.

Bacteria belonging to the *Pseudomonas fluorescens-putida* group promote plant growth and suppress certain plant pathogens (Weller 1988). Biochemical and genetic studies have correlated these beneficial traits with production of siderophores, antibiotics, hydrogen cyanide, and/or plant growth hormones (Bakker *et al.* 1986; de Weger *et al.* 1986; Thomashow and Weller 1988; Thomashow *et al.* 1990; Voisard *et al.* 1989; Weller 1988). Agricultural use of these beneficial fluorescent pseudomonads includes coating seeds with a bacterial suspension. As demonstrated by Bull *et al.* (1991), subsequent colonization of root surfaces from a seed inoculum can be critical for effective biocontrol activity.

Root colonization by beneficial bacteria proceeds through several stages including attachment, and different bacterial surface features may be involved. Lipopolysaccharides (LPS) were not found to have a role in attachment but may function in other events of colonization by beneficial pseudomonads (de Weger *et al.* 1989). Pili increase the attachment of *P. fluorescens* (Trevisan) Migula to corn roots (Vesper 1987). Flagella are involved in colonization by *P. putida* (Trevisan) Migula on potato roots (de Weger *et al.* 1987) but are not important for colonization of wheat by *P. fluorescens* (Weller 1988).

One event in colonization of bean roots by a beneficial isolate of *P. putida* involves an interaction between the bacterial cell surface and a plant root surface glycoprotein, an agglutinin (Anderson and Jasalavich 1979; Anderson 1983; Anderson *et al.* 1988; Jasalavich and Anderson 1981; Tari and Anderson 1988). Mutants of *P. putida* lacking in agglutinability were demonstrated to be reduced in their potential to bind within 15 min to roots, colonize roots from a seed inoculum, promote plant growth, and suppress Fusarium wilt (Anderson *et al.* 1988; Tari and Anderson 1988). Additional studies also imply agglutination is important in root colonization. van Peer *et al.* (1990) suggest that agglutination is associated with colonization of the endorhizosphere and Glandorf *et al.* (in press) indicate a possible correlation of agglutination with crop specificity in some rhizosphere isolates.

To further understand the role of agglutinability in adherence, we describe the identification and characterization of a genetic locus responsible for agglutinability in an agglutinable isolate of *P. putida*.

Nucleotide and/or amino acid sequence data have been submitted to GenBank, EMBL, and DDBJ as accession number M64540.

MATERIALS AND METHODS

Chemicals and enzymes. Molecular biology grade enzymes and DNA modifying enzymes were obtained from Stratagene Inc. (La Jolla, CA), Boehringer Mannheim Biochemicals (Indianapolis, IN), Bethesda Research Labs (Gaithersburg, MD), or U.S. Biochemical Inc. (Cleveland, OH). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Table 1. *P. putida* Nal^r (nalidixic acid resistant) Rif^r (rifampin resistant) Agg^+ (Anderson and Guerra 1985) and ethyl methanesulfonate Agg^- mutants (Anderson *et al.* 1988) were stored in 15% glycerol at −80° C and streaked regularly onto King's medium B (KB) (King *et al.* 1954) containing nalidixic acid and rifampin. Mutants deficient in agglutination (Agg^-) generated by Tn*5* mutagenesis (Anderson *et al.* 1988) were streaked from −80° C freezer stocks onto KB medium containing nalidixic acid, rifampin, and neomycin. Overnight cultures of *P. putida* and isolates used in the Southern hybridizations

were grown in rich medium (Anderson and Jasalavich 1979) at 22° C. *Escherichia coli* (Migula) Castellani and Chalmers was grown at 37° C in Luria broth (LB) (Maniatis *et al.* 1982). Antibiotics were used at the following concentrations: 50 μg/ml of ampicillin (Ap), 50 μg/ml of nalidixic acid (Nal), 50 μg/ml of neomycin (Km), 50 μg/ml of rifampin (Rif), 20 μg/ml of tetracycline (Tc) for *E. coli* and 75 μg/ml for *P. putida* cells. $MgSO_4$ was omitted from the medium when selection for tetracycline resistance was employed.

Assays. The agglutination assay was performed using Red Kidney bean root wash as the crude agglutinin source and bacteria grown to late log phase in rich medium as described previously (Anderson and Jasalavich 1979). Bacterial cells used in the adhesion assay were grown to late-log phase in rich medium (Anderson and Jasalavich 1979) without antibiotic selection and washed twice in sterile water. For the adhesion assay, sterile Dark Red Kidney bean plants (Idaho Bean Seed Company, Twin Falls, ID) were grown at 26° C with a 16-hr light/8-hr dark photoperiod. Detached roots of plants were immersed in the cell suspension (3×10^8 cfu/ml) and shaken at 22° C for 15 min. Roots were transferred through three successive 10-ml water washes each with 30 s of vortexing. Samples (0.1 ml) of the washes were plated onto KB medium, and colonies were counted after 2–4 days of growth. One hundred random colonies from the plated washings were selected and replicated onto appropriate KB selection plates to confirm multiple antibiotic resistance. The experiment was repeated twice with four roots per bacterial cell type.

DNA techniques. Large scale plasmid DNA isolations from *E. coli* were by the alkaline lysis method as described by Maniatis *et al.* (1982); plasmid mini-preparations were conducted using the method of Del Sal *et al.* (1989). Chromosomal DNA was isolated from *P. putida* according to Comai *et al.* (1982). Cosmid libraries of *P. putida* wild

Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics[u]	Source/reference
Pseudomonas putida		
Corvallis	Agg$^+$, Nal^r, Rif^r, parental	Anderson and Guerra 1985
1104	Agg$^-$, Corvallis::Tn*5*, Nm^r	Anderson *et al.* 1988
5123	Agg$^-$, Corvallis::Tn*5*, Nm^r	Anderson *et al.* 1988
6000	Agg$^-$, Corvallis::Tn*5*, Nm^r	A. J. Anderson
1202	Agg$^-$, Corvallis, EMS-induced	Anderson *et al.* 1988
1236	Agg$^-$, Corvallis, EMS-induced	Anderson *et al.* 1988
P. putida		
Fort Collins		Scher and Baker 1982
P. fluorescens		
NRRL15132		USDA-ARS patent culture collection[v]
NRRL15133		USDA-ARS patent culture collection[v]
NRRL15134		USDA-ARS patent culture collection[v]
NRRL15135		USDA-ARS patent culture collection[v]
AJA		A. J. Anderson
Pf5		J. Loper[w]
P. syringae pv. *phaseolicola*		Anderson 1984
P. s. pv. *pisi*		
Race 3		Anderson 1984
P. s. pv. *syringae*		Anderson 1984
Agrobacterium tumefaciens		
A208		E. E. Hood[x]
Erwinia herbicola		
Eh318Nr$^+$		S. Thomson[x]
E. coli		
JM109	*recA1 endA1 gyrA6 thi hsdR17supE44 relA1* Δ (*lac-proAB*) {F′ *traD36 proAB lacI*^{q}ZΔM15}	Stratagene Inc.[y]
Plasmids/clones		
pLAFR3	*IncP*, Tc^r, *cos*$^+$, *rlx*$^+$	Staskawicz *et al.* 1987
pRK2013	Km^r, Mob$^+$, Tra$^+$	Figurski and Helinski 1979
puc18	Ap^r	Bethesda Research Labs[z]
pBSKS	Ap^r	Stratagene Inc.
pRK415	Tc^r	Keen *et al.* 1988
pRB4	Tc^r, Nm^r; pLAFR3 AGG::Tn*5* from 5123 genomic DNA	This study
pRB5	Tc^r, Nm^r; pLAFR3 AGG::Tn*5* from 5123 genomic DNA	This study
pRB401	Ap^r; puc18 1.8 kb *Hpa*I-*Eco*RI from 5123 pRB4 cosmid clone	This study
pRB501	Ap^r; puc18 1.2 kb *Hpa*I-*Hin*dIII from 5123 pRB4 cosmid clone	This study
pAGG1	Tc^r; pLAFR3 *aggA* from parent	This study
pAGG2	Tc^r; pLAFR3 *aggA* from parent	This study
pAGG2001	Tc^r; pLAFR3 2.7 kb *Eco*RI-*Hin*dIII *aggA* locus subcloned from pAGG2	This study
pRKAGG201	Tc^r; pRK415 2.7 kbp *Eco*RI-*Hin*dIII *aggA* locus subcloned from pAGG2	This study
pBAGG201	Ap^r; pBSKS 2.7 kb *Eco*RI-*Hin*dIII *aggA* locus subcloned from pAGG2	This study
pBAGG201 Δ*Eco*RI A-N	Ap^r; pBAGG201 deletions at *Eco*RI terminus of *aggA*	This study
pBAGG201 Δ*Hin*dIII A-N	Ap^r; pBAGG201 deletions at *Hin*dIII terminus of *aggA*	This study
pRKAGG201 Δ*Eco*RI A-N	Tc^r; pBAGG201 deletions at *Eco*RI terminus of *aggA*	This study
pRKAGG201 Δ*Hin*dIII A-N	Tc^r; pBAGG201 deletions at *Hin*dIII terminus of *aggA*	This study

[u] Ap = ampicillin, Nal = nalidixic acid, Rif = rifampin, Tc = tetracycline, Nm = neomycin, EMS = ethylmethanesulfonate.
[v] Peoria, IL.
[w] USDA-ARS, Corvallis, OR.
[x] Utah State University, Logan, UT.
[y] La Jolla, CA.
[z] Gaithersburg, MD.

type and Tn*5* Agg$^-$ mutant 5123 were constructed in the wide host range cosmid vector, pLAFR3 (Staskawicz *et al.* 1987). Genomic DNA was subjected to a *Sau*3A partial digest and size-fractionated on a 10–40% sucrose gradient. Fractions enriched in 18- to 30-kbp fragments were pooled and ligated to *Bam*HI-restricted alkaline phosphatase-treated pLAFR3. Ligation products were packaged into phage particles using Gigapack Packaging Extract (Stratagene Inc., San Diego, CA.) and transduced into *E. coli* JM109 as described in the Stratagene protocol. The parental library was plated onto LB Tc, and 995 colonies were stored at −20° C in 15% glycerol. Cosmid clones containing the Tn*5* locus from DNA of mutant 5123 were selected by direct plating onto LB Tc Nm plates.

Hybridizations. Colony hybridizations were performed according to Maniatis *et al.* (1982). Southern hybridizations using Zeta-probe nylon membranes (BioRad, Richmond, CA) were performed following standard protocols recommended by the manufacturer that utilize temperature and sodium dodecyl sulfate (SDS) concentration as stringency determinants. Genomic Southern blots were hybridized for 16 hr at 68° C in 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, and 7% SDS. Low stringency washes for the genomic Southerns were at 68° C in 40 mM sodium phosphate (pH 7.2), 1 mM EDTA, and 5% SDS for 1 hr. High stringency washes followed the low stringency washes and were at 68° C in 40 mM sodium phosphate (pH 7.2), 1 mM EDTA, and 1% SDS. DNA probes prepared from gel-purified inserts were labeled with ^{32}P dCTP using a random primer reaction kit from Boehringer Mannheim Biochemicals. Blots were exposed at −80° C to Kodak X-OMAT AR film using Dupont Cronex Lightening Plus intensifying screens (DuPont, Wilmington, DE).

Matings. Cosmid clones were introduced into *P. putida* by triparental mating using *E. coli* containing the helper plasmid pRK2013 (Figurski and Helinski 1979). Stationary phase cells of *E. coli* (grown in LB) and *P. putida* (grown in rich medium) were washed and suspended in fresh LB. Aliquots (30 μl) of each parent were mixed, plated onto LB, incubated at 28° C for 24 hr, then streaked onto appropriate selection plates to select for transconjugants. Tetracycline was used at 50 or 75 μg/ml for selection of transconjugants, a concentration necessary to restrict growth of nontransconjugant *P. putida*.

Deletions of the *aggA* locus. Nested deletions of the *aggA* locus were made in pBAGG201 from the *Eco*RI restriction site (pBAGG201Δ*Eco*RI A–N) and *Hin*dIII restriction site (pBAGG201Δ*Hin*dIII A–N) using an *Exo*III/Mung Bean deletion kit from Stratagene Inc. After agarose gel size fractionation of deleted inserts, a set of 10 deletion clones, each differing in length by 200–300 bp (pBAGG201Δ*Eco*RI A, C, E, E2, F, and pBAGG201Δ*Hin*dIII A, B, C, E, G), were ligated separately into the broad host range plasmid, pRK415 (Keen *et al.* 1988), to yield pRKAGG201Δ*Eco*RI A, C, E, E2, F, G, and pRKAGG201Δ*Hin*dIII A, B, C, E, G, respectively.

Sequence analysis of the *aggA* locus. DNA sequence analysis of the *aggA* locus was performed on the pBAGG201 nested deletions with the Sequenase 2.0 kit from U.S. Biochemical Inc. Template DNA was prepared using either the alkali denaturation procedure described in the Sequenase protocol or as described by Del Sal *et al.* (1989). Reactions were performed according to the manufacturer's instructions and run on a 5.7% acrylamide, 0.3% bis acrylamide, 50% (w/v) urea, and 1× TBE (89 mM Tris-HCl, 89 mM boric acid, 2.0 mM EDTA, pH 8.0) gel (Maniatis *et al.* 1982). Gels were fixed in 10% acetic acid and 12% methanol before drying and exposure. The nucleotide sequence was analyzed using PC GENE (Intelligenetics, Mountainview, CA) and the University of Wisconsin Genetics Computer Group Package (Madison, WI) programs.

RESULTS

Cloning the *aggA* locus. Two separate cosmid libraries were made in the wide host range vector pLAFR3 (Staskawicz *et al.* 1987) using DNA from the wild type parental strain and the Tn*5* Agg$^-$ mutant 5123. Selection of the 5123 Agg$^-$ Tn*5* cosmid library directly on LB Tc Nm plates yielded two Tc^r Nm^r clones, pRB4 and pRB5 (Table 1). Restriction enzyme mapping and Southern hybridizations with the Tn*5* suicide vector, pSUP2021 (Simon *et al.* 1983), indicated the presence of Tn*5* in these clones. Fragments adjacent to the Tn*5* insertion site were subcloned into pUC18, yielding pRB401 and pRB501, and were subjected to further restriction mapping. Restriction enzyme fragments at least 0.5 kbp from the Tn*5* insertion were used to probe the parental library. Hybridization of pRB401 and pRB501 inserts to the parental library yielded two clones, pAGG1 and pAGG2. A 2.7-kbp *Eco*RI-*Hin*dIII fragment present in both pAGG1 and pAGG2 was subcloned from pAGG2 into pLAFR3, pRK415, and pBSKS, yielding pAGG2001, pRKAGG201, pBAGG201, respectively (Table 1).

Complementation of agglutinability. The pAGG1, pAGG2, and pAGG2001 cosmid clones were introduced into the parental *P. putida* and five Tn*5* and ethyl methanesulfonate Agg$^-$ mutants (1104, 1202, 1236, 5123, and 6000) through triparental matings. pRKAGG201 was introduced by triparental mating into the parent and 5123. No complementation of agglutination was seen with

Table 2. Complementation of agglutination in *Pseudomonas putida* and *P. fluorescens*

	Agglutinability with introduced plasmid[z]				
Bacterium	**None**	**pLAFR3**	**pAGG1**	**pAGG2**	**pAGG2001**
P. putida					
Parental	3+	3+	4+	4+	4+
1104	−	−	−	−	−
1202	−	−	−	−	−
1236	−	−	−	−	−
5123	−	−	4+	4+	4+
6000	−	−	−	−	−
P. fluorescens					
NRRL15132	−	−	−	−	−

[z] Plasmids were introduced into various recipients by triparental mating as discussed in the text. Agglutination assays were performed on stationary phase cells grown in rich medium. Transconjugant bacteria were grown in rich medium containing 50 μg/ml of tetracycline and no magnesium sulfate. Cells were washed twice in sterile water and then assayed with crude bean agglutinin. Agglutination was rated on a scale of − for no agglutination to 4+ for maximal agglutination. The agglutinability of the cells exposed to 100 units of bean root surface agglutinin is reported (Anderson and Jasalavich 1979).

pAGG1, pAGG2, or pAGG2001 in the Agg$^-$ mutants 1104, 1202, 1236, and 6000 (Table 2). Complementation of agglutination was observed in mutant 5123 with pAGG1, pAGG2, pAGG2001 (Table 2), and pRKAGG201 (data not shown). The 2.7-kbp *Eco*RI-*Hin*dIII fragment present in pAGG2001 and pRKAGG201, which was able to complement agglutination in 5123, was termed the *aggA* locus. Complementation of agglutination in 5123 was independent of the orientation of the 2.7-kbp *Eco*RI-*Hin*dIII fragment in the vector pRK415 (Keen *et al.* 1988). pAGG1, pAGG2, and pAGG2001 also increased the agglutinability of the parent (Table 2). pRKAGG201 did not increase parental agglutinability (data not shown). Select nested deletions of the 2.7-kbp *aggA* locus were ligated into the broad host range vector pRK415. Deletion of more than 270 bp from the *Hin*dIII terminus of the locus resulted in loss of complementation capabilities (Fig. 1). Deletion of more than 924 bp from the *Eco*RI terminus also resulted in loss of complementation ability (Fig. 1). Thus, complementation of agglutination appears to reside within a 1.45-kbp fragment of the *aggA* locus.

Complementation of adhesion capabilities. Root adhesion capabilities of parental (Agg$^+$), 5123 (Agg$^-$), 5123 (pRK415) (Agg$^-$), and 5123 (pRKAGG201) (Agg$^+$) cells to Dark Red Kidney bean roots was measured through dilution plating of root washings onto semisolid media. Cell viability problems were observed when a magnesium-free medium required for selection of tetracycline resistance was used in initial adherence assays. Parental *P. putida* cells, when plated onto KB medium without magnesium sulfate after growth to log phase in rich medium, were reduced by over 50% in the proportion of recovered cells

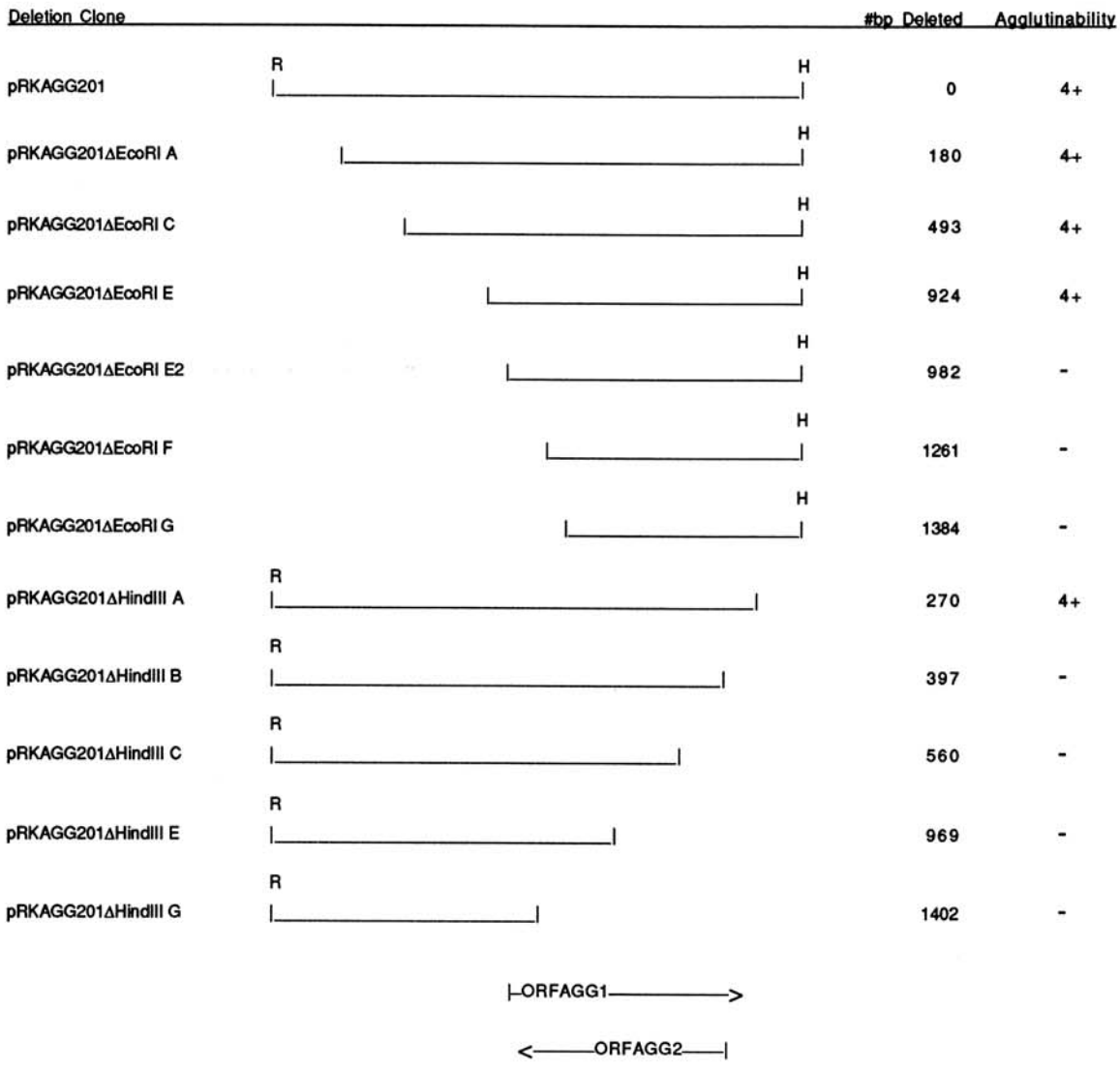

Fig. 1. Deletion analysis of the *aggA* locus. Plasmids were introduced into various recipients by triparental matings as discussed in the text. Agglutination assays were performed on transconjugant bacteria grown in rich media containing 50 μg/ml of tetracycline and no magnesium sulfate. Cells were washed twice in sterile water and then assayed with bean agglutinin. Agglutination was rated on a scale of −, for no agglutination and 4+, for maximal agglutination with the agglutinability reported for 100 units of agglutinin. The positions for ORFAGG1 and ORFAGG2 as predicted by sequence analysis are noted at the bottom. R = *Eco*RI and H = *Hin*dIII.

(data not shown). Thus, in the adherence assay, magnesium was included in the KB medium without the addition of antibiotics to remove bias in cell recovery. Replica plating of 5123 (pRKAGG201) and 5123 (pRK415) transconjugant cells initially recovered on KB medium to tetracycline selection media indicated 84.9 and 62.2% of the colonies, respectively, were tetracycline resistant.

Adherence assays indicated the *aggA* locus increased the ability of mutant 5123 to adhere to bean root surfaces (Table 3). The root adherence capability of 5123 was increased in transconjugants with pRKAGG201 but not the vector pRK415 at wash 1 (Table 3). In addition, recovery in wash 2 and wash 3 of 5123(pRKAGG201) transconjugant cells was similar to parental levels and higher than both 5123 and 5123(pRK415).

Distribution of the *aggA* locus in plant-associated bacteria. Hybridization of the insert of pBAGG201Δ*Eco*RI E to Southern blots of *Eco*RI restricted total DNA from an array of plant-associated bacteria occurred only in organisms agglutinable with agglutinin from bean root surfaces (Fig. 2). *P. fluorescens* NRRL15133, *P. fluorescens* NRRL15135, *P. putida* Fort Collins, and *P. s. tabaci* showed hybridization to the pBAGG201Δ*Eco*RI sequence under both low and high stringency wash conditions. Each of these isolates was agglutinable by crude bean root surface agglutinin preparations. Other isolates of saprophytic pseudomonads and pathogenic bacteria, which were not agglutinable with bean root surface agglutinin, failed to hybridize to the pBAGG201Δ*Eco*RI sequence under low or high stringency conditions.

Sequence analysis of the *aggA* locus. Potential open reading frames (ORFs) in the 1.45 kbp of the *aggA* locus that complemented agglutination in 5123 (Figs. 3,4) were determined using the method of Kolaskar and Reddy (1985). An ORF of 1,356 bp was detected on one strand and termed ORFAGG1. The nucleotide sequence of ORFAGG1 is predicted to encode a protein with molecular weight of 50,509 Da and a pI of 5.24. A consensus sequence for a ribosome-binding site (AGGGA) (Shine and Dalgarno 1974) was located at nucleotide 216, eight bases upstream from the ATG translational start site of ORFAGG1. Various potential promoter consensus sequences (Deretic *et al.* 1989) were located upstream from the putative translational start site. The sequence TGCA that resembles the −12 canonical sequence (TTGCA) recognized by σ^{54} RNA polymerase holoenzyme was present beginning at nucleotide 150. A second sequence beginning at nucleotide 166 (ATAA) resembles the −10 canonical sequence recognized by σ^{70} polymerase (TATAAT). Other potential consensus sequences corresponding to canonical −24 (CTGGNA) and −35 (TTGACA) sequences were present further upstream, beginning at nucleotide 81 (TGGCA) and nucleotide 88 (TTGATA), respectively. These sequences were present in regions not required for complementation of agglutination, and they displayed different spacing than usual for σ^{54} and σ^{70} polymerase consensus sequences (Deretic *et al.* 1989). The predicted amino acid sequence of ORFAGG1 contained one transmembrane helix extending from residues 2–17, as predicted by the method of Rao and Argos (1986). A prokaryotic signal cleavage site located between residues 22 and 23 was predicted by the method of von Heijne (1986). Secondary structure of ORFAGG1 as predicted by the Novotny algorithm indicated alternating alpha helix and beta sheet structures.

Table 3. Complementation of adhesion capabilities

Bacterium[z]	Number of cfu × 10^4/g fresh weight root[x,y]		
	Wash 1	Wash 2	Wash 3
Parent (Agg$^+$)	75,329 a	5,857 a	743 a
5123 (Agg$^-$)	8,318 c	669 b	79 b
5123 (pRKAGG201) (Agg$^+$)	29,888 b	5,384 a	592 a
5123 (pRK415) (Agg$^-$)	4,289 c	538 b	55 b

[x] Adhesion assays were performed as described in the text. Data are averages of eight roots per treatment.

[y] Letters in the same column followed by the same letter are not significantly different ($P < 0.05$) as determined by the Duncan's multiple range test (Lyman, 1988).

[z] All cells were grown overnight in rich medium without antibiotic selection as described in the text. 5123(pRKAGG201) cells were agglutinable, and 84.9% of the colonies were recoverable on tetracycline-containing media. 5123(pRK415) cells were nonagglutinable, and 62.2% of the colonies were recoverable on tetracycline-containing media.

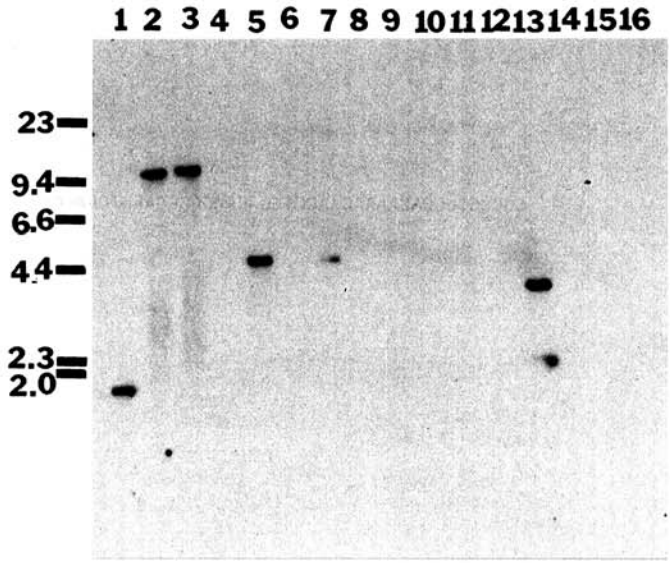

Fig. 2. Southern analysis of the distribution of the *aggA* locus in plant-associated bacteria. Total DNA was isolated from cells grown to stationary phase in rich medium as described in the text. The DNA (1 μg) was restricted with *Eco*RI, fractionated on a 0.8% agarose gel, and transfered to a nylon membrane. The blot was hybridized to a ^{32}P-labeled pBAGG201Δ*Eco*RI E insert and washed under high stringency wash conditions as described in the text. pBAGG201Δ*Eco*RI E is a 924-bp deletion from the *Eco*RI terminus of pBAGG201. The agglutinability of plant-associated bacteria was tested on cells grown to late log to stationary phase in rich medium, washed twice in water, and then assayed with crude bean agglutinin preparations. The agglutinability of the isolates with 100 units of bean root surface agglutinin is listed in parentheses. Lane 1: pBAGG201Δ*Eco*RI E insert; lane 2: *Pseudomonas putida* Corvallis (4+); lane 3: *P. putida* Fort Collins (4+); lane 4: *P. fluorescens* NRRL15132 (−); lane 5: *P. fluorescens* NRRL15133 (3+); lane 6: *P. fluorescens* NRRL15134 (−); lane 7: *P. fluorescens* NRRL15135 (+/−); lane 8: *P. fluorescens* AJA (−); lane 9: *P. fluorescens* Pf5 (−); lane 10: *Pseudomonas syringae* pv. *syringae* (−); lane 11: *P. s. pisi* (−); lane 12: *P. s. phaseolicola* (−); lane 13: *P. s. tabaci* (3+); lane 14: *Erwinia herbicola* (−); lane 15: *Agrobacterium tumefaciens* (−); lane 16: *Escherichia coli* (−).

The codon bias and nucleotide preference in ORFAGG1 were similar to those of other *P. putida* ORFs (Essar *et al.* 1990). ORFAGG1 was 63.4% G + C at position 1, 41.5% G + C at position 2, and 81.1% G + C at position 3 of each codon, with 62.0% G + C used throughout ORFAGG1.

A second potential ORF of 1,254 nucleotides, ORFAGG2, was present on the opposing strand of the 1.45-kbp region that complemented mutant 5123 (Figs. 1,3,4), ORFAGG2 has a predicted GTG translational start codon at nucleotide 1,515 (relative to ORFAGG1) and has a translation stop codon, TAA, at nucleotide 261 (relative to ORFAGG1). However, ORFAGG2 is a weaker candidate for the functional protein coding sequence because it lacks a ribosome consensus binding site, promoter consensus sequences, and contains a GTG translational initia-

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1    CAGACACTGGCTTTGTAAGCCTTACGCGCATTCGCCATCAGCACTTATTGGTGTCTTTTTTAGATAATAAAGATCTTAAATGGCAATTTGATATGAATAA

                                                  |
101  ATCTTCTTACATATTGCGTTGAATGTACTTAGCTGTGTTGTTAAAAACCTGCAACACTATACGCGATAACATTATCGTCATTTTTTTGGCGAGCAGCAGG
                                                                                                  *
        +                          M  R  V  L  N  P  I  T  S  A  L  L  L  A  L  A  S  P  N  V  Q  A  M  S
201  ATCAATTCCAGGATCAGGGAGATGTACCATGCGCGTTTTGAACCCCATCACCAGTGCAttaCTGTTGGCCCTGGCGAGTCCCAACGTACAGGCGATGTCG

     I  T  E  A  V  Q  S  A  V  D  Y  H  P  Q  V  S  S  N  R  N  S  K  L  S  A  D  E  D  V  K  F  A  R  G
301  ATCACCGAGGCCGTCCAGAGCGCCGTGGACTACCACCCACAAGTCAGCTCCAACCGCAACAGCAAGCTGTCGGCCGATGAGGACGTGAAATTTGCGCGTG

        G  Y  Y  P  S  V  D  L  V  A  G  Y  G  R  Q  R  S  D  N  A  T  T  R  A  E  G  N  H  N  K  E  T  L
401  GTGGCTACTACCCTTCCGTGGATTTGGTCGCTGGCTATGGCCGCCAGCGTTCGGACAACGCCACTACCCGTGCCGAGGGTAACCACAACAAGGAAACCCT

        N  Y  T  Q  S  E  L  R  L  R  Q  M  I  F  D  G  F  N  T  S  N  E  V  G  R  T  E  A  V  S  T  S  R
501  CAACTACACCCAGTCCGAGCTGCGCCTGCGGCAGATGATCTTCGACGGCTTCAACACCTCGAATGAAGTGGGCCGTACCGAGGCAGTCTCCACCTCCCGT

     A  Y  Y  T  Q  A  V  A  Q  D  V  A  L  R  A  V  E  V  Y  L  E  V  L  K  R  R  E  L  V  T  L  A  K  N
601  GCCTACTACACCCAGGCCGTTGCCCAGGATGTCGCCTTGCGTGCCGTGGAGGTGTACCTGGAAGTGCTCAAGCGCCGCGAGCTGGTAACCCTGGCCAAGA

        N  L  Q  A  H  L  R  V  N  D  Q  I  G  L  R  N  E  R  G  V  G  S  T  A  D  L  D  Q  S  R  A  R  R
701  ACAACCTGCAAGCTCACCTGCGCGTCAACGACCAGATCGGCCTGCGTAACGAGCGCGGCGTTGGCAGCACCGCCGACCTCGACCAGTCCCGCGCACGTCG

     A  L  A  E  N  N  L  D  T  A  E  V  D  L  A  D  A  E  A  N  F  F  S  V  I  G  R  M  P  D  E  L  E
801  CGCCCTGGCAGAAAACAACCTGGACACCGCCGAAGTCGACCTGGCCGATGCCGAGGCCAACTTCTTCAGCGTGATCGGCCGCATGCCGGACGAACTGGAA

     S  P  Q  T  I  K  A  E  V  P  D  T  L  D  G  A  R  D  S  M  R  Q  N  N  P  Y  I  K  S  A  Q  A  D  V
901  AGCCCGCAGACCATCAAGGCCGAGGTGCCTGACACCCTCGACGGCGCGCGCGACAGCATGCGTCAGAACAACCCCTACATCAAATCTGCCCAGGCTGACG

        N  A  A  E  Q  Q  Y  E  V  G  K  S  T  F  Y  P  R  F  D  A  I  L  A  T  G  A  N  N  N  T  G  G  E
1001 TCAACGCGGCCGAGCAGCAGTACGAAGTGGGCAAGTCGACTTTCTACCCGCGCTTTGACGCCATTCTGGCAACCGGTGCCAACAACAACACCGGCGGCGA

     K  G  H  N  N  N  D  W  Q  A  G  V  E  M  N  Y  N  L  F  R  G  G  S  D  K  A  R  L  Q  S  D  A  H
1101 GAAAGGCCACAACAACAACGACTGGCAGGCCGGTGTAGAGATGAACTACAACCTGTTCCGCGGTGGCAGTGACAAGGCCCGCCTGCAGTCTGACGCGCAC

     K  I  N  Q  A  L  D  I  R  N  N  A  L  R  E  L  T  E  N  L  S  L  A  W  N  A  M  N  N  A  S  K  Q  L
1201 AAGATCAACCAGGCCCTGGACATCCGCAACAACGCCCTGCGTGAGCTGACCGAAAACCTGAGCCTGGCATGGAACGCCATGAACAACGCCAGCAAGCAGC

        P  T  A  R  E  Y  A  E  T  T  K  R  V  R  A  A  Y  Q  D  Q  F  G  L  G  Q  R  T  L  L  D  V  L  D
1301 TGCCGACCGCGCGTGAATATGCCGAGACCACCAAGCGCGTGCGTGCTGCCTACCAGGACCAGTTCGGCCTGGGCCAGCGTACCCTGCTGGACGTGCTGGA

     S  E  N  E  L  Y  N  A  D  R  R  Y  T  E  V  R  Y  T  E  E  F  S  R  Y  R  V  L  A  T  M  G  E  L
1401 CAGTGAAAACGAACTGTACAACGCCGACCGCCGCTACACCGAAGTGCGTTATACCGAGGAGTTCTCGCGCTACCGCGTGCTGGCGACCATGGGTGAGTTG

     L  S  K  Q  H  I  S  L  P  P  E  A  L  A  T  T  E  V  R  T  E  A  R  L  P  E  M  R  -              ^
1501 CTGAGCAAGCAAcacATCTCGCTGCCGCCAGAGGCACTGGCTACCACTGAAGTACGCACCGAGGCGCGCCTGCCCGAGATGCGTTGATCGGGTTTGAGAT

1601 TCGTTTGCGGCCCCCAGCCCTCACTCGGTGCCATGGTTACGCTCAAACGTCTCCATCCCCATCGCCTTGATCTGCCCCTCGATCAAGCCTCGAACGGCGC

1701 CAGCAGTGCATCGAAGCAGGCCGCTGCTCCAGCGTATTCAATGCCCCCA
```

Fig. 3. Nucleotide and amino acid sequence of 1,749 nucleotides of the *aggA* locus encoding ORFAGG1. The predicted ORFAGG1 is 1,356 bases in length. One letter amino acid abbreviations are listed above each codon. The putative ribosome binding site of ORFAGG1 beginning at nucleotide 216 is double underlined. Putative promoter consensus sequences are underlined once. The translation start ATG codon of ORFAGG1 at nucleotide 229 is in bold. The terminus of pRKAGG201Δ*Eco*RI E at nucleotide 149 is noted with |. The terminus of pRKAGG201Δ*Eco*RI E2 at nucleotide 206 is noted with a +. The putative signal cleavage site of the ORFAGG1 protein is noted with a *. The terminus of pRKAGG201Δ*Hin*dIII A is noted with a ^. Also, noted in lowercase letters are the positions complementary to the translation start codon of ORFAGG2 at nucleotide 1,515 and the translation termination codon of ORFAGG2 at nucleotide 261.

tion signal. Also, there are no inframe translational stop codons in the 86 bases 5′ of the ORFAGG2 GTG translational start site that are necessary for complementation, suggesting the GTG codon may be an internal codon of a longer protein.

The predicted amino acid sequences for ORFAGG1 and ORFAGG2 were used to search the NBRF and GenEMBL databases using the TFASTA program (Lipman and Pearson 1988) of the University of Wisconsin Genetics computer Group Package. The database search did not reveal any sequences of significant similarity.

DISCUSSION

A genetic locus, *aggA*, involved in recognition between a beneficial fluorescent pseudomonad, *P. putida* isolate Corvallis, and a root surface glycoprotein, termed an agglutinin (Anderson and Jasalavich 1979), has been characterized. This locus was identified because insertion of transposon Tn*5* in mutant 5123 resulted in loss of agglutinability and reduction in adherence to bean roots (Anderson *et al.* 1988). A 2.7-kbp *Eco*RI-*Hin*dIII fragment of Agg^+ *P. putida* Corvallis DNA bearing the wild type *aggA* locus restored agglutinability in the Agg^- mutant 5123 to wild type levels. Neither the *aggA* locus nor larger fragments containing the *aggA* locus were able to complement agglutination in four other Agg^- mutants or convert the nonagglutinable *P. fluorescens* NRRL15132 (2-79) isolate to agglutinability. These data suggest that multiple genetic loci are involved in the manifestation of the agglutination phenotype. The increased agglutinability of the parental isolate when containing the additional copies of the *aggA* locus suggests this locus may be a key component in generation of the phenotype.

Adherence of 5123 to bean root surfaces was also restored in the presence of a wild type *aggA* locus. The ability of the *aggA* locus to complement 5123 to wild type levels of attachment as well as agglutinability further strengthens the proposed role of agglutination in binding of *P. putida* to roots (Anderson *et al.* 1988; Tari and Anderson 1988).

Agglutination is a phenotype present in several beneficial saprophytic, root-colonizing fluorescent pseudomonads and in *P. s.* pv. *tabaci* (Anderson 1983; Chao *et al.* 1988; Glandorf, in press). Southern analysis with a 1.75-kbp fragment of the *aggA* locus revealed hybridization was restricted to DNA from isolates that expressed the agglutination phenotype. This hybridization pattern suggests a common component may be involved in agglutination of these isolates.

Deletion analysis of the 2.7-kbp fragment containing the *aggA* locus revealed 924 bp from the *Eco*RI terminus and 270 bp from the *Hin*dIII terminus were not necessary for complementation of agglutinability in 5123. Sequence analysis of 1.75 kbp of the fragment indicated the presence of two potential ORFs, ORFAGG1 and ORFAGG2, on opposing strands. Sequences at the 5′ side of the predicted ORFAGG1 ATG translational start site revealed a consensus ribosome binding site (Shine and Dalgarno 1974) and promoter consensus sequences (Deretic *et al.* 1989), although both components of the −35/−10 and −24/−12 consensus sequences were not present within 5′ noncoding regions of ORFAGG1 required for complementation. This resembles other *Pseudomonas* genes that lack complete canonical consensus sequences 5′ of the translational start site (Deretic *et al.* 1989). Complementation of agglutination in 5123 was independent of the orientation of the 2.7-kbp *aggA* locus in the vector pRK415 (Keen *et al.* 1988), suggesting sequences necessary for transcription initiation are present in the 2.7-kbp *aggA* locus. The functionality of sequences within the *aggA* promoter is being examined currently in reporter gene fusions.

ORFAGG1 is predicted to encode a 50,509-Da protein

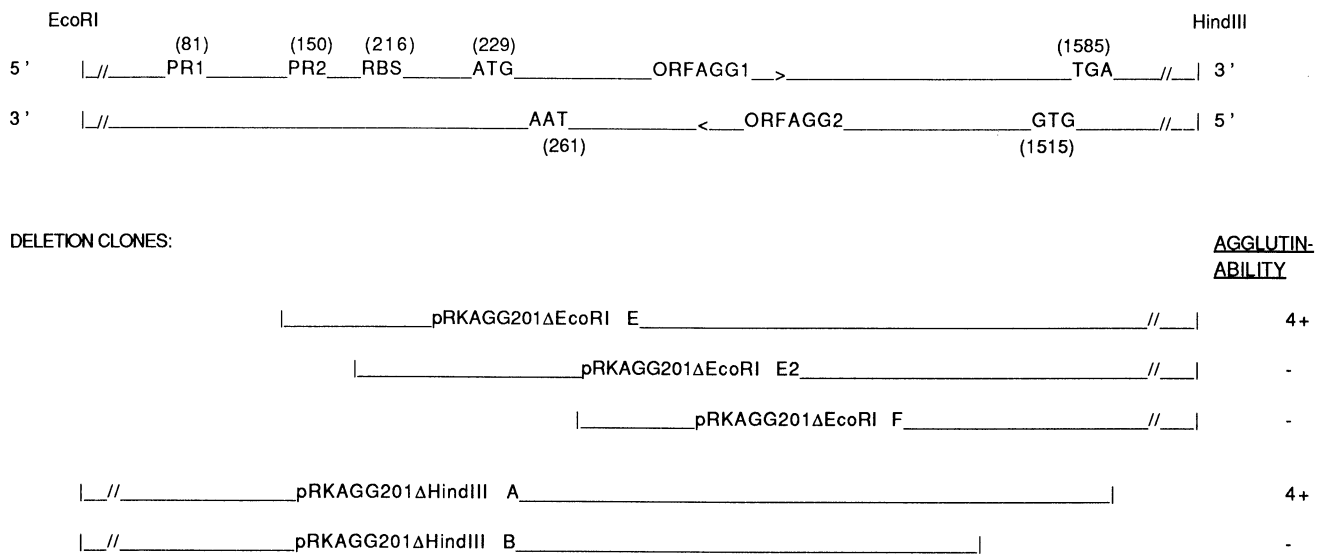

Fig. 4. Deletion and sequence analysis of ORFAGG1 and ORFAGG2. The *aggA* locus with key sequences of ORFAGG1 and ORFAGG2 is drawn. PR1 = −24 and −35 promoter consensus sequences; PR2 = −12 and −10 promoter consensus sequences; RBS = ribosome-binding site; ATG and GTG = translational start codons; TGA and TAA = translational stop codons. The nucleotide positions, as taken from Figure 3 and relative to ORFAGG1, of each key sequence is noted in parentheses above (ORFAGG1) or below (ORFAGG2) the sequence. The site of the altered terminus of each deletion clone is marked. The ability of these deletion clones to complement agglutinability is also noted. The figure is not drawn to scale.

with a pI of 5.24. The prediction of a transmembrane helix at the N-terminus of ORFAGG1 and signal peptide cleavage signal is consistent with export of the putative protein out of the cytoplasm (Model and Russel 1990). A survey of several databases revealed no significant sequence similarities for ORFAGG1.

Sequence analysis also revealed the presence of a second ORF, ORFAGG2, on the opposing strand. ORFAGG2 contains a putative GTG translational start site. Sequences 5′ of this GTG revealed no ribosome binding site or promoter consensus sequences, and inframe translational stop codons in ORFAGG2 5′ noncoding sequences are also absent. Sequence and deletion analysis with deletion clones pRKAGG201Δ*Eco*RI, which complements 5123, and pRKAGG201Δ*Eco*RI E2, which does not complement 5123, further supports the idea that ORFAGG1 rather than ORFAGG2 is the ORF in *aggA* responsible for complementation of agglutination. Complementation is lost when 982 bp (pRKAGG201Δ*Eco*RI 2) but not 924 bp (pRKAGG201Δ*Eco*RI E) are deleted from the *Eco*RI terminus of *aggA*. Noncoding regions but not coding regions are deleted in both ORFAGG1 (5′) and ORFAGG2 (3′) within this additional 58-bp deletion. In pRKAGG201Δ*Eco*RI E2, ORFAGG1 retains only 10 bp 5′ of the putatative ribosome binding site, whereas ORFAGG2 retains all 5′ regions and 54 bp 3′ of the translational stop codon. These data suggest ORFAGG2 may be a partial ORF and therefore unlikely to be involved in generating a functional product concerned with agglutinability.

The data presented in this paper provide evidence for a genetic link between agglutinability and events at the plant-bacterium interface. They further suggest that the agglutination phenotype is beneficial in the adherence between the plant root and *P. putida*.

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LITERATURE CITED

Anderson, A. J., and Jasalavich, C. 1979. Agglutination of pseudomonad cells by plant products. Physiol. Plant Pathol. 15:149-159.

Anderson, A. J. 1983. Isolation from root and shoot surfaces of agglutinins that show specificity for saprophytic pseudomonads. Can. J. Bot. 61:2438-3443.

Anderson, A. J. 1984. Differences between lipopolysaccharide compositions of plant pathogenic and saprophytic *Pseudomonas* spp. Appl. Environ. Microbiol. 48:31-35.

Anderson, A. J., and Guerra, D. 1985. Responses of bean to root colonization with *Pseudomonas putida* in a hydroponic system. Phytopathology 75:992-995.

Anderson, A. J., Habibzadegah-Tari, P., and Tepper, C. S. 1988. Molecular studies on the role of a root surface agglutinin in adherence and colonization by *Pseudomonas putida*. Appl. Environ. Microbiol. 54:375-380.

Bakker, P. A. H. M., Lamers, J. G., Bakker, A. W., Marugg, J. D., Weisbeek, P. J., and Schippers, B. 1986. The role of siderophores in potato yield increase by *Pseudomonas putida* in a short rotation of potato. Neth. J. Plant Pathol. 92:249-256.

Bull, C. T., Weller D. M., and Thomashow, L. S. 1991. Relationship between root colonization and suppression of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* strain 2-79. Phytopathology 81:954-959.

Chao, W., Li, R., and Chang, W. 1988. Effect of root agglutinin on microbial activities in the rhizosphere. Appl. Environ. Microbiol. 54:1838-1841.

Comai, L. A., and Kosuge, T. 1982. Transposable element that causes mutations in a plant pathogenic *Pseudomonas* sp. J. Bacteriol. 154:1162-1167.

Del Sal, G., Manfioletti, G., and Schneider, C. 1989. The CTAB-DNA precipitation method: A common mini-scale preparation of template DNA from phagemids, phages, or plasmids suitable for sequencing. BioTechniques 7:514-519.

Deretic, V., Konyecni, W. M., Mohr, C. D., Martin, D. W., and Hibler, N. S. 1989. Common denominators of promoter control in *Pseudomonas* and other bacteria. Bio/Technol. 7:1249-1254.

de Weger, L. A., van Boxtel, R., van der Burg, B., Gruters, R. A., Geels, F. P., Schippers, B., and Lugtenberg, B. 1986. Siderophores and outer membrane proteins of antagonistic, plant-growth-stimulating, root-colonizing *Pseudomonas* spp. J. Bacteriol. 165:585-594.

de Weger, L. A., van der Vlugt, C. I. M., Wijfjes, A. H. M., Bakker, P. A. H. M., Schippers, B., and Lugtenberg, B. 1987. Flagella of a plant-growth-stimulating *Pseudomonas fluorescens* strain are required for colonization of potato roots. J. Bacteriol. 169:2769-2773.

de Weger, L. A., van Loosdrecht, M. C. M., Klassen, H. E., and Lugtenberg, B. 1989. Mutational changes in physiochemical cell surface properties of plant-growth-stimulating *Pseudomonas* spp. do not influence the attachment properties of cells. J. Bacteriol. 171:2756-2761.

Essar, D. W., Eberly, L., and Crawford, I. R. 1990. Evolutionary differences in chromosomal locations of four early genes of the tryptophan pathway in fluorescent pseudomonads: DNA sequences and characterization of *Pseudomonas putida trpE* and *trpGDC*. J. Bacteriol. 172:867-883.

Figurski, D. H., and Helinski, D. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA 76:1648-1652.

Glandorf, D. C. M., Bakker, P. A. H. M., and Schippers, B. Crop specificity of fluorescent pseudomonads and the involvement of root agglutinins. In: Biotic Interactions and Soil-borne Diseases, in press.

Jasalavich, C. A., and Anderson, A. J. 1981. Isolation from legume tissues of an agglutinin of saprophytic pseudomonads. Can. J. Bot. 59:264-271.

Keen, N. T., Tamaki, S., Kobayashi, D., and Trollinger, D. 1988. Improved broad-host range plasmids for DNA cloning in Gram-negative bacteria. Gene 70:191-197.

King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.

Kolaskar, A. S., and Reddy, B. V. B. 1985. A new method to locate protein coding sequences in DNA of prokaryotic systems. Nucleic Acids Res. 13:185-194.

Lipman, D. J., and Pearson, W. R. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA 85:2444-2448.

Lyman, O. H. 1988. An introduction to statistical methods and data analysis, 3rd ed. PWS-Kent Publishing Co, Boston, MA.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Model, P., and Russel, M. 1990. Prokaryotic secretion. Cell 61:739-741.

Rao, M. J. K., and Argos, P. 1986. A conformational preference parameter to predict helices in integral membrane proteins. Biochim. Biophys. Acta. 869: 197-214.

Scher, F. M., and Baker, R. 1982. Effect of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to Fusarium wilt pathogens. Phytopathology 72:1567-1573.

Shine, J., and Dalgarno, L. 1974. The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: Complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA 71:1341-1346.

Simon, R. U., Priefer, U., and Puhler, A. 1983. A broad host range mobilization system for in vivo genetic engineering: Transposon mutagenesis in gram negative bacteria. Bio/Technol. 9:784-791.

Staskawicz, B., Dahlbeck, D., Keen, N., and Napoli, C. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 169:5789-5794.

Tari, P. H., and Anderson, A. J. 1988. Fusarium wilt suppression and agglutinability of *Pseudomonas putida*. Appl. Environ. Microbiol.

54:2037-2041.
Thomashow, L. S., and Weller, D. M. 1988. Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. J. Bacteriol. 170:3499-3508.
Thomashow, L. S., Weller, D. M., Bonsall, R. F., and Pierson, L. S. 1990. Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. Appl. Environ. Microbiol. 56:908-912.
van Peer, R., Punte, H. L. M., de Weger, L. A., and Schippers, B. 1990. Characterization of root surface and endorhizosphere pseudomonads in relation to their colonization potential. Appl. Environ. Microbiol. 56:2462-2470.
Vesper, S. J. 1987. Production of pili (fimbriae) by *Pseudomonas fluorescens* and correlation with attachment to corn roots. Appl. Environ. Microbiol. 53:1397-1405.
Voisard, C., Keel, C., Hans, D., and Defago, G. 1989. Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. EMBO J 8:351-358.
von Heijne, G. 1986. A new method for predicting signal sequence cleavage signals. Nucleic Acids Res. 11:4683-4690.
Weller, D. M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. 26:279-407.