Fimbriae of Phytopathogenic and Symbiotic Bacteria

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ABSTRACT

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Fimbriae are proteinaceous, filiform appendages on the bacterial cell surface that are thought to be important in attachment of bacteria to plant and other surfaces. We report here that fimbriae are common in many genera and species of plant pathogenic bacteria. Seventy-seven strains, including all genera and many species of plant pathogenic bacteria, were grown under appropriate cultural conditions and then examined for the presence of fimbriae by electron microscopy. Fimbriae were detected for the first time in *Pseudomonas syringae* pv. *glycinea* and pv. *syringae*, *P. savastanoi* (one strain each), and in *Agrobacterium tumefaciens* (14 strains). The presence of fimbriae was also confirmed for *Rhizobium* (*Bradyrhizobium*) *japonicum* (one strain), *R. trifolii* (three strains), *R. leguminosarum* (five strains), and *P. solanacearum* (five strains). Conditions under which fimbriae were produced most abundantly (shallow, static, aerobic cultures) were similar to those reported as most suitable for the production of fimbriae by mammalian pathogens. A detailed study of the fimbriae of *P. solanacearum* B1, an avirulent derivative of strain K60, was made. B1 cells are strongly fimbriated, whereas K60 cells are almost devoid of fimbriae. A new procedure for purification of B1 fimbriae was developed, consisting of precipitating them from the culture supernatant with 20% ammonium sulfate, followed by repeated sonication and washing with 0.5% sodium deoxycholate and 6 M urea. The fimbrial subunit is composed of a single protein with a molecular weight of about 9,500.

There is increasing evidence that attachment to plant surfaces is important for expression of the virulence or symbiosis of plant-associated bacteria (8,9,14,19,22). Several hypothetical mechanisms have been proposed to explain bacterial attachment to plant surfaces. A popular hypothesis involves the specific interaction of a proteinaceous lectin on the plant surface with a polysaccharide on the bacterial surface (6,26,29). An alternative hypothesis considers that a lectin on the bacterial surface binds to a particular carbohydrate on the plant surface (29). The bacterial fimbriae (pili) are proteinaceous appendages that have long been known to have lectinlike activity (11). Support for the role of fimbriae in attachment of bacteria to plant surfaces was obtained by Young and Sequeira (37), who showed that the fimbriae of *Pseudomonas solanacearum* (Smith) Smith bind to tobacco leaf cell walls. Similarly, Romantschuk and Bamford (27) reported that fimbriae are involved in attachment of *P. syringae* van Hall pv. *phaseolicola* to bean leaves. It seemed important, therefore, to determine whether fimbriae are commonly produced by other plant pathogenic bacteria.

There are a few reports on the production of pili by plant-associated bacteria. Fuerst and Hayward (13) and Christofi et al (4) reported the presence of fimbriaelike appendages in *P. solanacearum* and in three species of *Erwinia* (*E. atroseptica*, *E. carotovora*, and *E. rhapontici*). Cuppels et al (5) reported the presence of fimbriae in *P. syringae* pv. *phaseolicola*. Similarly, there are reports of the production of fimbriae by symbiotic bacteria, including *Rhizobium trifolii* and *R. leguminosarum* (6,7), *R. lupini* (15), *R. japonicum* (33,36), and *R. phaseoli* (35).

These previous reports did not establish with certainty whether the fibers interpreted as fimbriae by electron microscopy were biochemically comparable to the proteinaceous fimbriae of mammalian pathogens. Several plant pathogens and symbionts are known to produce cellulose fibrils or other carbohydrate polymers that form strands on dehydration and these can easily be mistaken for fimbriae. Thus, in addition to a systematic survey of plant pathogenic bacteria for production of fimbriae, we report here on a new method for purification of of the fimbrial subunit of *P. solanacearum*. Two brief accounts of this work have been published (33,34).

MATERIAL AND METHODS

Cultures and media. Most of the bacterial strains that were tested for the presence of fimbriae were from the collection in the Department of Plant Pathology, University of Wisconsin, Madison (Table 1). Stocks were maintained in stab culture, as suspensions in sterile distilled water, or lyophilized.

Fimbriae were observed when bacteria were grown in one or more of the following media:

CPG: 10 g of Bacto-peptone (Difco), 1 g of casamino acids (Difco), 5 g of glucose, 18 g of agar, and 1 L of distilled water. Modified TZC medium (17).

AT: 500 ml of AT×2 salts, 20 ml of 10% mannitol, 20 ml of 10% ammonium sulfate, 20 g of agar, 460 ml of distilled water. AT×2 salts: 21.8 g/L of KH_2PO_4, 320 mg/L of $MgSO_4 \cdot 7H_2O$, 10 mg/L of $FeSO_4 \cdot 7H_2O$, 22 mg of $CaCl_2 \cdot 2H_2O$, 4 mg of $MnCl_2 \cdot 4H_2O$, pH 7.0.

BM: Per liter, 0.23 g of K_2HPO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 10 g of mannitol, 0.6 g of KNO_3, 1 ml of trace elements, 1 ml of vitamins. Trace elements stock, per liter: 5 g of $CaCl_2$, 145 mg of H_3BO_3, 125 mg of $FeSO_4 \cdot 7H_2O$, 70 mg of $CaSO_4 \cdot 7H_2O$, 5 mg of $CuSO_4 \cdot 7H_2O$, 4.3 mg of $MnCl_2 \cdot 4H_2O$, 108 mg of $ZnSO_4 \cdot 7H_2O$, 125 mg of Na_2MoO_4, 7 mg of nitrilotriacetate. Vitamins stock, per liter: 20 mg of riboflavin, 20 mg of para-aminobenzoic acid, 20 mg of nicotinic acid, 20 mg of biotin, 20 mg of thiamine HCl, 20 mg of pyridoxine HCl, 20 mg of Ca panthothenate, 120 mg of inositol.

HK2: Modified Husain & Kelman medium (16), per liter: 0.66 g of $(NH_4)_2SO_4$, 4 mg of $MgSO_4 \cdot 7H_2O$, 4 mg of $ZnSO_4 \cdot 7H_2O$, 1.2 mg of $FeCl_3$, 0.9 mg of $MnSO_4 \cdot H_2O$, 0.52 g of glutamic acid, 1 g of sucrose, 5 ml of 44.1 g/L of KH_2PO_4, 8.5 ml of 117.4 g/L of K_2HPO_4, pH 7.0.

SH: Sherwood synthetic broth (31).

LB: Luria broth (21).

Preparation of root exudates. Sunflower (*Helianthus annuus* L. 'Vanity') seeds were germinated in Jiffy mix and, after 1 wk, 10 seedlings were carefully removed, gently shaken to remove most of the Jiffy mix particles from the roots, then placed in a flask containing 20 ml of Hoagland's solution, and grown under Gro-lux fluorescent lights at 26 C for 28 hr. Then the seedlings were discarded and the nutrient solution was filter sterilized. This root exudate solution was added to AT culture medium (1:1, v/v) in certain experiments.

Detection of fimbriae. To prepare fimbriae for electron microscopy (EM), bacteria were grown statically in 0.7-ml volumes of broth in shallow wells (2 cm diameter) in glass plates. Cultures were transferred two to five times, but only the pellicle was used as inoculum. After 10–48 hr of growth (depending on the strain), when the pellicle first became visible, a Formvar-coated, 200-mesh copper grid was placed on the surface of the culture. Grids were briefly floated on distilled, sterilized water to remove debris, then floated on a drop of 1% (w/v) phosphotungstic acid (pH 7.0) for 2 min. The stain had been stored frozen and was filtered through a Millipore membrane (0.2 μm pore) immediately before use. The grid was blotted dry and then dried thoroughly under an infrared lamp. In some instances, grids were shadowcast with platinum-carbon in a vacuum evaporator. Grids were examined at 75 kV in a JEOL JEM-7 electron microscope at a magnification of approximately 20,000.

Purification of B1 fimbriae. Strain B1 of *P. solanacearum* was grown for about 24 hr three consecutive times in shallow, static culture, as described previously. Then, the pellicle of the last subculture was transferred into 25 ml of low-glucose (2 g/L) CPG broth. Aliquots (100 μl per plate) of this suspension were spread on low glucose CPG plates; cultures were grown for 3 or 4 days at 28 C. For preparative work, up to 900 plate cultures were used. The growth from these plates was suspended in double-distilled water (10 ml per plate) and stirred vigorously for 3 hr at 4 C. Then cells and debris were removed by centrifuging the suspension at 12,000 *g* for 15 min at 4 C and recentrifuging the supernatant fluid twice. Ammonium sulfate was added to 20% saturation, and the mixture was allowed to settle for about 1 hr at 4 C. The precipitate was resuspended in distilled water, sonicated for 30 sec at 60 W, and centrifuged at 12,000 *g* for 10 min at 4 C. This washing procedure was repeated once in distilled water, six to eight times in 0.5% Na deoxycholate solution in 50 mM Tris-Cl, pH 7.5, three times in 100 mM citrate buffer, pH. 2.6, three times in 100 mM Tris-Cl, pH 9.0, twice in distilled water, twice in 6 M urea, and once in distilled water. Then the protein content of the precipitate was determined and purity of the sample was tested by electrophoresis.

Because the fimbrial protein did not stain properly with either Coomassie blue or silver, further purification was monitored by means of labeling with ^{125}I. For this purpose, a modification of the procedure of Salacinski et al (28) was used. Glass scintillation vials were coated by adding 2 ml of Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril; Pierce Chemical Co., Rockford, IL) in chloroform (1.5 μg/ml) and evaporating the solvent under a stream of nitrogen in the dark. Purified fimbriae (1,100 μg of protein) were suspended in 100 mM Tris-Cl, pH 7.4, and 1-2 mCi ^{125}I was added to a final volume of 1 ml. The mixture was stirred in the dark at room temperature for about 1 hr. Then, free iodine was removed by exhaustive dialysis against the same buffer. The iodinated fimbriae were recovered by centrifugation, and radioactivity was determined with a Packard Prias Tri-Carb scintillation counter. A specific activity of about 3×10^5 cpm/μg of protein was obtained.

The iodinated fimbriae were extracted three times with 0.5% deoxycholate in 50 mM Tris-Cl, pH 7.5, and were sonicated at each extraction step, as indicated previously.

Electrophoresis. Polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) was performed in minislab gels containing 6 M urea to improve separation of low molecular weight proteins (1). Gels were stained for proteins with Coomassie Brilliant Blue or with silver (24).

Protein determination. Protein content was estimated according to the procedures of Lowry et al (20) or Bradford (2), with bovine serum albumin as the standard. Because of the low content of arginine and lysine in the fimbriae of *P. solanacearum* B1, the amount of protein was consistently underestimated when Coomassie dye-binding assays (e.g., Bradford's) were used.

Amino acid analysis. Purified fimbriae were hydrolyzed with 6 N HCl and 0.2% (v/v) phenol for 20 hr at room temperature (23). Concentrations of amino acids in the hydrolysate were determined with a Durrum amino acid analyzer, model D-500.

RESULTS

Screening bacterial strains for fimbriae. Production of fimbriae by the bacterial strains listed in Table 1 was evaluated under a wide variety of cultural conditions. Major variations in conditions were: solid vs. liquid media, static vs. shaken liquid culture, addition of plant extracts, etc. In general, fimbriae were most numerous when bacterial strains were grown under static, aerobic conditions in 0.7-ml volumes of broth for 10–18 hr at 28 C.

For determination of fimbriae by EM, negative staining with phosphotungstic acid was superior to shadow casting with platinum-carbon in terms of resolution, speed, and reproducibility. The best method consisted of placing the Formvar-coated grid directly on top of the undisturbed, shallow broth culture (or a suspension of purified fimbriae) for 30 sec and then floating the grid on a drop of the phosphotungstic acid stain for 2 min. When the medium contained yeast extract or other materials that remained in suspension, it was necessary to float the grids briefly in distilled water before staining. Formvar grids were used because they are more hydrophobic than those coated with carbon; collodion-coated grids were unsatisfactory.

Estimates of the numbers of fimbriae produced by a particular strain were made on the basis of observations by EM on a minimum of 5,000 cells.

Strains that produced fimbriae, along with the medium found to be most suitable, are listed in Table 1. Of particular interest to us were the fimbriae produced by different strains of *P. solanacearum*, which were produced in very large numbers by strains 8, 81, and 276 (all virulent) as well as by strain B1 (avirulent). On the other hand, strain K60 (virulent) produced very few fimbriae. Strain B1 produced up to 1,000 fimbriae per cell and these appeared to be inserted polarly (Fig. 1). The largest numbers were produced on CPG broth, and the average thickness was about 6 nm (Fig. 2); they were strongly aggregated and appeared to be responsible for "star" formation, whereby several cells were joined at the fimbriated end only.

Other phytopathogenic bacteria that were strongly fimbriated included *P. syringae* pv. *syringae* and *P. s.* pv. *glycinea*, but only when grown in HK2 broth. In this medium, the fimbriae may have been responsible for rapid autoagglutination of the cells.

Relatively low numbers of fimbriae were produced in HK2 broth by the strain of *P. savastanoi* that was tested.

All 14 strains of *A. tumefaciens* produced relatively large numbers of fimbriae when grown in AT minimal medium or HK2 broth (Fig. 3). The fimbriae were very thin (about 2 nm in diameter). Addition of octopine (0.1%) to AT minimal medium did not increase the number of fimbriae, although this compound is a specific inducer of conjugation in octopine plasmid-containing strains of this species (18). The presence of the Ti plasmid did not have a visible effect on the production of fimbriae. Transfer-constitutive mutants, which do not require octopine for conjugation, could not be differentiated from wild type strains in terms of numbers of fimbriae produced in culture.

The production of fimbriae by a Tn*5*-generated mutant of *A.*

TABLE 1. Bacterial strains examined for the presence of fimbriae by electron microscopy

Strain	Source	Fimbriation[a]	Medium[b]
Pseudomonas solanacearum			
K60	L. Sequeira	+	CPG
B1 (Vir$^-$)	L. Sequeira	+++	CPG
8, 81, 276	L. Sequeira	+++	CPG
Q, S210, 334, 335, 283, 135	L. Sequeira	−	...
P. syringae pv. *lachrymans*			
214-6	P. Williams	−	...
P. s. pv. *syringae*			
79-03	C. Morris	++	HK2
79-01, 79-02, S-9-1	C. Morris	−	...
P. s. pv. *glycinea*			
NCPPB 1783	C. Upper	++	HK2
NCPPB 2753, 1134, 1137	C. Upper	−	...
J3-17-2, S-9-1	W. Fett	−	...
P. s. pv. *tagetis*			
C3	R. Durbin	−	...
P. savastanoi			
254	L. Sequeira	+	HK2
Agrobacterium tumefaciens			
AT1, B6, IIBNV6, 15955, C58	J. Kemp	++	AT
C58C1 (pTi$^-$)	J. Tempe	++	AT
A136 (pTi$^-$)	D. Merlo	++	AT
LBA 288 (pTi$^-$)	R. Schilperoort	++	AT
LBA 661 (pTi$^+$, Vir$^+$)	R. Schilperoort	++	AT
LBA 657 (pTi$^+$, Vir$^+$)	R. Schilperoort	++	AT
LBA 1013 (pTi$^+$, Vir$^-$)	R. Schilperoort	++	AT
A723 (Vir$^+$Att$^+$)	C. Douglas	++	AT
A1038 (Vir$^-$Att^{def})	C. Douglas	++	AT
A1038(pRAR205) (Vir$^+$Att$^+$)	C. Douglas	++	AT
Erwinia stewartii			
DC108, RNG, K2RA, DC70, DC71	A. Kelman	−	...
E. chrysanthemi			
SR261, SR120W, SR297, SR30, SR31	A. Kelman	−	...
E. carotovora pv. *carotovora*			
AK9, AK15, AK17	A. Kelman	−	...
E. c. pv. *atroseptica*			
AK16, SR100	A. Kelman	−	...
Xanthomonas campestris pv. *vesicatoria*			
79	L. Sequeira	−	...
X. c. pv. *phaseoli*			
64	L. Sequeira	−	...
X. c. pv. *axonopodis*			
83	L. Sequeira	−	...
X. c. pv. *campestris*			
63	L. Sequeira	−	...
Clavibacter michiganense subsp. *michiganense*			
SSCM 8	S. Slack	−	...
C. m. subsp. *sepedonicum*			
SSCS 43, 38, 13, 15, 20	S. Slack	−	...
Rhizobium trifolii			
126X6, 126P30, 126S32	Nitragin, Inc.	+	BM
T1	E. Appelbaum	−	...
R. meliloti			
F51, 1A113	W. Brill	−	...
Balzac	E. Appelbaum	−	...
R. leguminosarum			
3855, 3841	N. Brewin	++	SH
B151 (pSym$^-$), 16015 (Nod$^-$)	N. Brewin	++	SH
RBL-1	J. Kijne	++	SH
R. (*Bradyrhizobium*) *japonicum*			
SM	W. Brill	−	...
SM106 (Nod$^-$Fix$^-$Att$^-$)	W. Brill	+	BM
USDA 110	E. Appelbaum	−	...

[a] Fimbriation estimated from the average number of fimbriae per cell (+++ = profuse, 10 or more per cell; ++ = intermediate, 1–9 per cell; + = low, less than 1 per cell; − = not detected).

[b] All strains tested on six different media (see Methods); only the best medium for production of fimbriae is listed.

tumefaciens, which shows reduced attachment to plant cells (9), was compared with that of the wild type, parental strain A723 (vir^+att^+). The mutant strain A1038 (vir^-att^{def}) and its complemented derivative, A1038(pRAR205) (vir^+att^+), produced fimbriae that were morphologically similar to those of the parental strain. There were no apparent differences in numbers of fimbriae produced by the three strains.

Addition of sterile sunflower seedling root exudate or yeast extract (Difco) to AT broth did not increase the number of fimbriae produced by three strains of *A. tumefaciens* (A723, B6, and C58). These strains were also grown in a large volume of AT broth for 3 days at 28 C, followed by vigorous stirring of the cultures for 4 hr at 4 C. After centrifuging, fimbriae were precipitated from the supernatant of all three strains by adding ammonium sulfate to 40% saturation, but not to 25%, as determined by EM.

Three species of *Rhizobium* (*R. japonicum*, *R. leguminosarum*, and *R. trifolii*) produced low to relatively high numbers of fimbriae, but only when grown in BM or in Sherwood's (31) medium. Strain SM106 ($Nod^-Fix^-Att^-$) of *R. japonicum* (*Bradyrhizobium japonicum*) produced very few fimbriae (about 0.1 per cell), but, in this respect, was not substantially different from the parental strain, SM ($Nod^+Fix^+Att^+$). With *R. leguminosarum*, the lack of the Sym plasmid or of effective nodulation genes did not have an observable effect on fimbriation. Strains 3855, B151 ($pSym^-$ derivative of 3855), 3841, and 16015 (Nod^- mutant of 3841) and RBL-1 all produced about five fimbriae per cell in Sherwood's medium. The fimbriae were polar and very thin (about 2 nm diameter) (Fig. 4). When the three strains of *R. trifolii* were grown in BM medium, very few fimbriae were present (about 0.3 per cell). All three strains are commercially available seed inoculants.

Purification of B1 fimbriae. Purification of B1 fimbriae proved difficult because of their insolubility. The high concentration of glucose in CPG medium was found to inhibit the production of fimbriae. When B1 cells were grown in low glucose CPG broth, the numbers of fimbriae per cell (as determined by EM) kept increasing until the culture reached stationary phase (3 or 4 days). The vigorous stirring of bacterial cells in distilled water caused shedding of the fimbriae into the medium. The fimbriae remained in suspension in the culture supernatant but precipitated when ammonium sulfate was added. Upon precipitation, fimbriae

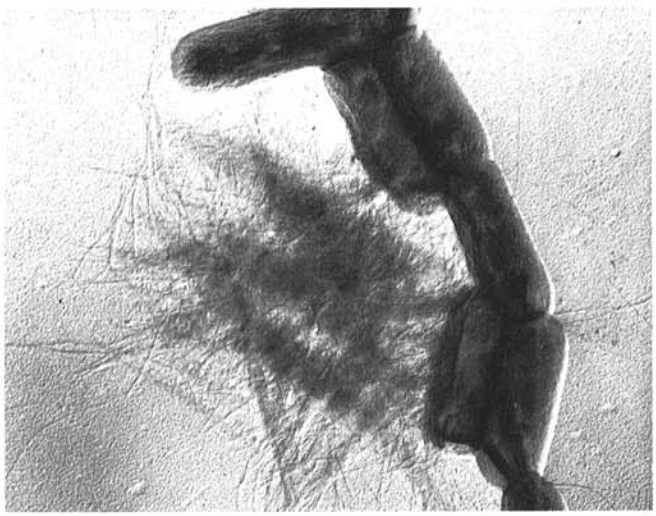

Fig. 1. Fimbriae of *Pseudomonas solanacearum* B1. Cells were grown in CPG broth with low glucose. Note that the fimbriae appear to be inserted polarly. Shadowcast with platinum-carbon. Approximately 17,250×.

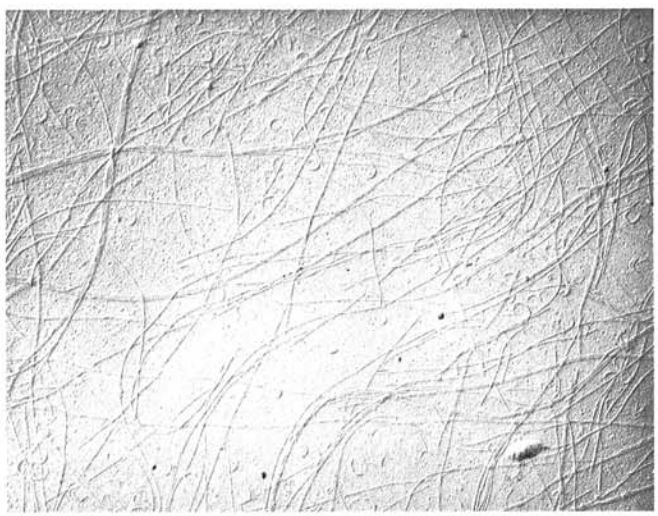

Fig. 2. Fimbriae of *Pseudomonas solanacearum* B1. Loose fimbriae in the medium after bacteria were grown for 3 days. Shadowcast with platinum-carbon. Approximately 27,000×.

Fig. 3. Fimbriae of *Agrobacterium tumefaciens* 15955. The fimbriae are much thinner than flagellae, which are also present. Flagellae have a typical sinusoidal appearance. Cells grown in LB broth (21). Stained with phosphotungstic acid. Approximately 14,250×.

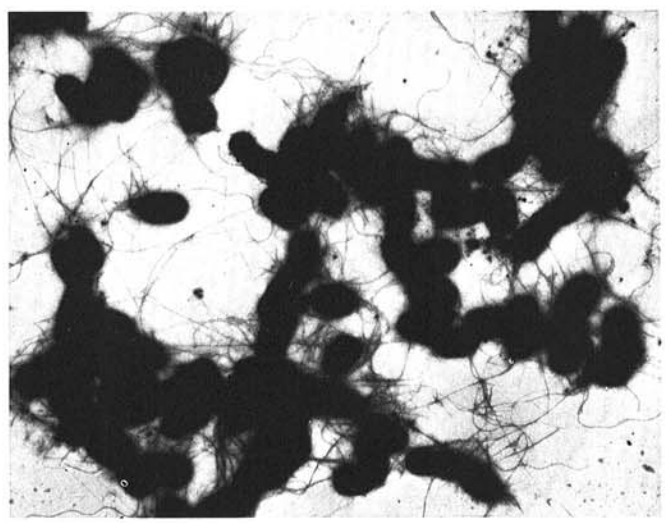

Fig. 4. Fimbriae of *Rhizobium leguminosarum* 3855. The fimbriae are much thinner than flagellae, which are also present. Flagellae have a typical sinusoidal appearance. Cells grown in SH medium. Stained with phosphotungstic acid. Approximately 13,500×.

became aggregated and so insoluble that they precipitated spontaneously when resuspended in distilled water. Further purification was obtained by combining sonication and washing with detergents and various buffers. Based on SDS-PAGE analysis, most of the purification was obtained as a result of the deoxycholate and urea treatments. The fimbriae did not depolymerize when treated with 6 M urea, but they did when treated with 8 M urea.

The final distilled water suspension contained large numbers of fimbriae, as determined by EM. These purified fimbriae were approximately 6 nm in diameter. Analysis of this preparation after depolymerization and separation by SDS-PAGE indicated the presence of a major protein band of about 9,500 mol wt and of numerous minor bands (Fig. 5). The final pellet contained large numbers of very short fimbriae (due to extensive sonication), as determined by EM (Fig. 6). Purity was established by the presence of a single band of labeled protein of approximately 9,500 mol wt by SDS-urea-PAGE, as determined by autoradiography. This protein subunit reassembled into long fimbriaelike strands when eluted from the gels and dialyzed against 10 mM Na acetate at pH 4.5, following the procedures developed previously in our laboratory (38). The pure fimbriae were approximately 6 nm in diameter and had a density of 1.27 g/ml in CsCl and 1.22 g/ml in sucrose. The protein subunit had a pI of 4.0 and was particularly rich in hydrophobic amino acids (45%). From the amino acid analysis (Table 2), the calculated molecular weight was 9,590, based on 93 residues per subunit.

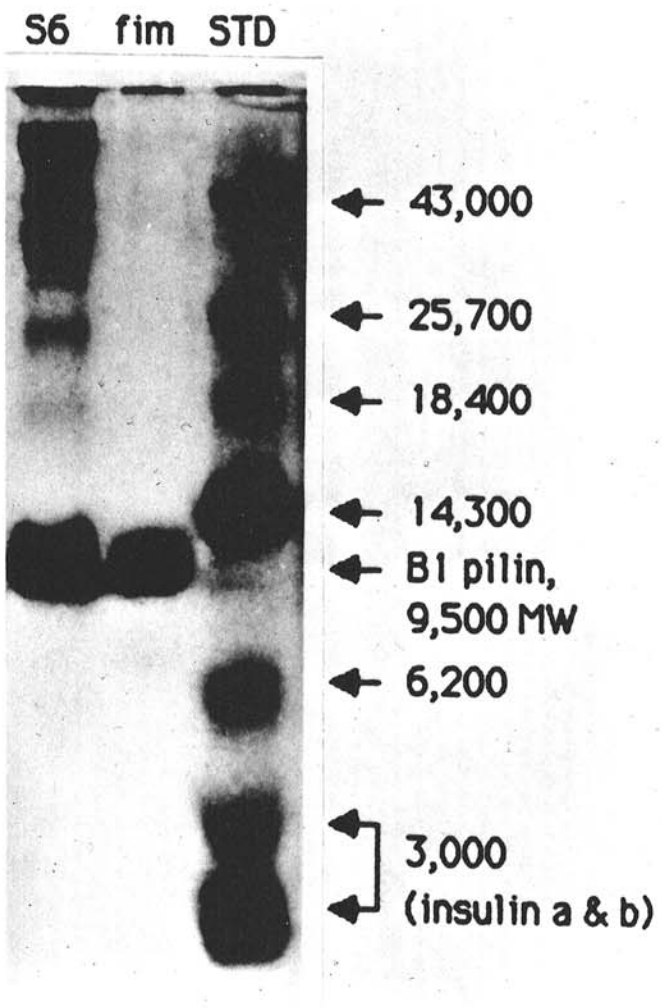

Fig. 5. SDS-Urea-PAGE separation of fimbrial preparations of *Pseudomonas solanacearum* at two stages of purification. Stained with Coomassie Blue. Lane S6: purified fimbriae obtained by precipitating them with ammonium sulfate, followed by sonication and washing with 0.5% Na deoxycholate, citrate, and Tris buffers (100 mM), 6 M urea, and distilled water. Lane fim: pure B1 fimbrial subunit (pilin) obtained after extensive sonication of purified fimbriae and repeated washing procedures as outlined above. Lane STD: Protein standards. Molecular weights are indicated on the right.

DISCUSSION

Of the 77 strains of plant-associated bacteria that were examined by EM, 31 were found to produce fimbriae in culture. This is the first report of the presence of fimbriae in the plant-pathogenic bacteria, *P. savastanoi*, *P. syringae* pv. *syringae* and pv. *glycinea*, and *A. tumefaciens*. Our work has confirmed the presence of fimbriae in *P. solanacearum* and in several strains of the symbionts *R. trifolii*, *R. leguminosarum*, and *R. japonicum* (*Bradyrhizobium japonicum*).

Fimbriae appear to be as common in plant-associated bacteria as they are in bacterial pathogens of mammals. Most of these fimbriae had diameters ranging from 2 to 6 nm and were polar in many of the bacteria we examined (data not included). The presence of polar fimbriae in strains of *Rhizobium* and *Agrobacterium* is interesting in view of the reports of polar attachment of these organisms to plant cell walls (26).

Fimbriae were produced most abundantly when cells were grown in broth under static, highly aerobic conditions. These culture conditions are similar to those reported as most suitable for the production of fimbriae by Enterobacteriaceae (25). There was considerable variation in the type of medium required by the different species of bacteria that we tested. For some strains, minimal media were much better than complex, rich media; for others, the reverse was true. The repeated transfer of the pellicle that we used for subculturing appeared to create a selective enrichment for fimbriated bacteria. This enrichment can only occur if there is genetic diversity in fimbriation in the original cultures, a phenomenon that has been reported frequently for bacterial pathogens of mammals.

Of particular interest was the finding that the presence of the Ti or Sym plasmids in *A. tumefaciens* and *R. leguminosarum*, respectively, was not required for the production of fimbriae. Thus, the genes for fimbrial production are likely to be chromosomally encoded. The conjugation inducer, octopine, did not have a significant effect on the formation of fimbriae by octopine strains of *A. tumefaciens*. Because conjugative fimbriae (F pili) are encoded by specific plasmids and are essential for conjugation in the Enterobacteriaceae (3), it seemed possible that

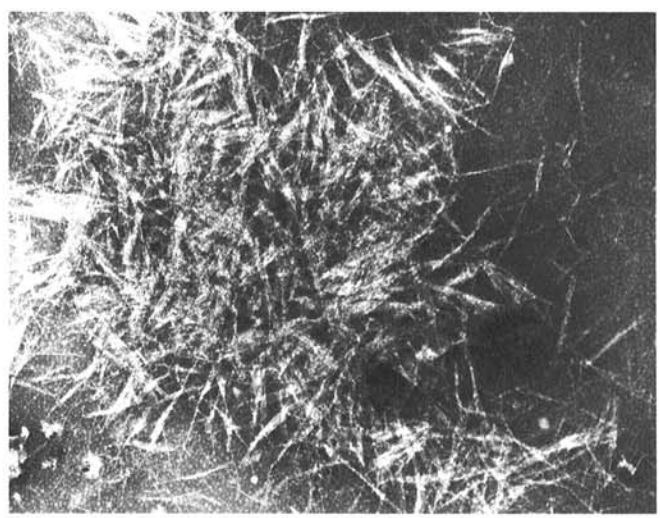

Fig. 6. Purified fimbriae of *Pseudomonas solanacearum* B1. Note that the purified fimbriae are much shorter than native fimbriae, presumably the result of repeated sonication during purification. At this stage in purification, the fimbriae form semicrystalline aggregates. Stained with phosphotungstic acid. Approximately 24,000×.

similar structures would be formed by *A. tumefaciens* under appropriate conditions for conjugation.

The results presented here do not support the concept that fimbriae are involved in attachment of plant-associated bacteria to cell walls. The presence of equivalent numbers of fimbriae in the wild type strain of *A. tumefaciens*, A723, and in the attachment-deficient mutant A1038 as well as in the complemented strain, A1038(pRAR205), suggests that these fimbriae are not important in attachment of this bacterium to plant cell walls. Similarly, the attachment-deficient mutant of *R. japonicum*, SM106, produced fimbriae in culture. Finally, the concept that avirulence in *P. solanacearum* is correlated with attachment and, thus, with fimbriation in strains such as B1 (10,37) did not appear to be supported when additional strains were examined. Several virulent strains were found to be strongly fimbriated.

This report and previous work from this laboratory (38) give adequate proof that fimbriae are produced by *P. solanacearum*. This proof was obtained by purification and biochemical characterization of the fibers that had been detected previously by EM. Characterization of the fimbriae produced by other plant-pathogenic bacteria should also be carried out. Microscopic observations can be misleading because bacteria produce polysaccharides that aggregate as fibers and can be mistaken for fimbriae. This is not a trivial problem, for purification of fimbriae is particularly difficult due to their large size and insolubility.

The new procedure for purification of the *P. solanacearum* B1 fimbriae reported here is a useful complement to the procedures reported previously (38). The latter procedures depended on precipitation of the fimbriae by Ca^{2+}, followed by SDS-PAGE, elution of the major protein band, and reassembly of the fimbrial subunit. In the procedures reported here, the fimbriae were purified first, before SDS-PAGE was attempted. Use was made of the insolubility of these fimbriae in distilled water; purification was achieved by washing with detergents and various buffers, in combination with extensive sonication. Both procedures yielded a protein subunit of about 9,500 mol wt, which was capable of reaggregating into complete fimbriae under appropriate conditions. We have no explanation, however, for some differences in the relative content of certain amino acids in the subunit (e.g. threonine, serine, alanine, and glycine) that are apparent in the two reports. We are attempting to establish the reasons for these differences.

The molecular weight of 9,500 for the protein subunit of B1 fimbriae is smaller than average. Only the subunits of *Caulobacter crescentus* fimbriae (8,000 mol wt; ref. 32) and of the F pilus of *E. coli* (7,200 mol wt; ref. 12) are known to be smaller. The amino acid composition of the B1 subunit is similar to that reported for other fimbriae, for example, the high levels of aspartic and glutamic acids and the high proportion of hydrophobic amino acids. The precipitation of B1 fimbriae at low pH is consistent with a protein with relatively low pI (4.0). The low pI may also explain the lack of staining with silver reagents.

TABLE 2. Amino acid composition of the fimbriae of *Pseudomonas solanacearum*

Amino acid	Mole (%)	Residues per subunit[a]
Aspartic acid	11.7	11
Threonine	12.1	12
Serine	5.7	5
Glutamine	12.4	12
Proline	3.5	3
Glycine	6.2	6
Alanine	17.5	17
Valine	2.7	2
Cystine (half)	0.0	0[b]
Methionine	4.7	4
Isoleucine	2.3	2
Leucine	6.2	6
Tyrosine	1.5	1
Phenylalanine	3.8	3
Lysine	5.2	5
Histidine	1.4	1
Arginine	3.1	3
Total	100.0	93
Subunit mol wt (93 residues/subunit = 9,590		
Hydrophobic amino acids 42/93 = 45%		

[a] Based on a molecular weight of 9,500 estimated by SDS-PAGE.
[b] Cystine recoveries were difficult to estimate.

Our EM studies showed that the fimbriae of *P. solanacearum* B1 interconnect cells and that they may be involved in "star" formation and in autoagglutination. Other evidence also supports the role of fimbriae in autoagglutination. B1 cells are agglutinated by divalent cations (Ca^{2+} or Zn^{2+}) and fimbriae are also precipitated by similar concentrations of these cations (37). Thus, agglutination is probably mediated by fimbriae, but the further extrapolation that this phenomenon is related to the behavior of virulent and avirulent strains *in planta* may not be justified on the basis of the results reported here. The relationship of fimbriae to attachment in this species arose from observations that strain B1 is readily immobilized on tobacco cell walls, whereas the virulent, parental strain (K60), which produces very few fimbriae, does not attach to cell walls (10). We have now shown that several virulent strains of *P. solanacearum* are heavily fimbriated and, in addition, strains such as S210, which attach rapidly to tobacco cell walls (30), do not produce fimbriae in culture. Because bacteria produce different types of fimbriae under different environmental conditions, however, the relationship of these structures to attachment and/or avirulence in *P. solanacearum* cannot be resolved by examination of the natural variability within the species. A genetic approach is required in which the behavior of fimbriated and nonfimbriated mutants can be compared. We have followed such an approach, and the results will be the subject of a second paper in this series.

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