Partial Characterization of Fimbriae of *Xanthomonas campestris* pv. *hyacinthi*

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***Xanthomonas campestris* pv. *hyacinthi* is a plant-pathogenic bacterium that causes yellow disease in *Hyacinthus*. *X. c.* pv. *hyacinthi* produces monopolarly attached fimbriae with a diameter of approximately 5 nm and a length of at least 6 µm. Fimbriae were purified by acid precipitation and preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis. No hemagglutinating activity of purified fimbriae was found when the fimbriae were tested with several types of erythrocytes. The fimbrial protein subunit had a relative molecular mass of 17 kDa; an isoelectric point was found at pH 4.1. Analysis of the N-terminal amino acid sequence of the fimbrial subunit indicated that *X. c.* pv. *hyacinthi* expresses type 4 fimbriae. A polyclonal rabbit antiserum was raised against the purified fimbriae. This antiserum recognized fimbriae of *X. c.* pv. *hyacinthi* in immunogold electron microscopy and immunoblotting experiments. Immunofluorescence studies showed that *X. c.* pv. *hyacinthi* cells as well as purified native fimbriae were attached to stomata of hyacinth leaves, suggesting a role for these surface antigens in the first stages of yellow disease.**

Additional keywords: immunofluorescent attachment studies.

Xanthomonas campestris pv. *hyacinthi* causes yellow disease in the ornamental bulb plant *Hyacinthus* and in some closely related bulb plants, such as *Scilla tubergeniana* and *Muscari armeniacum* (Janse and Miller 1983). The symptoms brought about by this yellow-pigmented bacterium vary from small lesions at the tops of leaves to ochreous, buttery necrosis of the bulb, depending on the stage of the disease (Beyer 1972).

X. campestris comprises more than 120 pathovars, each of which infects only one or a few closely related hosts. Some of these pathovars seem to be almost identical (Van den Mooter and Swings 1990), but other members of the *campestris* group appear to be less closely related (Vauterin *et al.* 1990, 1991; Hildebrand *et al.* 1990). It appears that the genus *Xanthomonas,* particularly the species *X. campestris,* with its numerous pathovars, consists of a very diverse group of bacteria.

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Most plant-pathogenic bacteria are likely to possess invasive abilities. Some *X. campestris* pathovars, including *X. c.* pv. *hyacinthi*, are known to penetrate their host plants through damaged surfaces or natural openings (stomata and hydathodes) in the stem or leaves (Beyer 1972; Guo and Leach 1989). Which properties of *Xanthomonas* species contribute directly to their pathogenicity is not clear. Several potential virulence factors have been reported. Gene clusters responsible for pathogenicity in *X. c.* pv. *campestris* have been cloned (Daniels *et al.* 1989; Osbourn *et al.* 1990; Bonas *et al.* 1991), and plant-degrading enzymes, such as proteases, polygalacturonate lyases, amylases, and endoglucanases, are produced by *Xanthomonas* species and *X. campestris* pathovars (Tang *et al.* 1987; Dow *et al.* 1987; Daniels *et al.* 1988). Successful infection by pathogens may be preceded by specific recognition of certain host structures followed by attachment to the surface (Romantschuk 1992). Indications of this infection pattern may be found in the closely related plant-pathogenic *Pseudomonas* species, in which surface components such as extracellular polysaccharides and fimbriae are possibly involved in the adherence of several species to plant surfaces (Young and Sequeira 1986; Romantschuk and Bamford 1986).

Knowledge about the role of fimbriae as mediators of specific adherence in the phyllosphere is limited, but they are suspected of playing such a role in *Klebsiella* spp., *Enterobacter* spp., *Erwinia carotovora,* and some other bacteria (Haahtela *et al.* 1985; Korhonen *et al.* 1986; Christofi *et al.* 1979; Vesper 1987; Stemmer and Sequeira 1987).

To develop a good detection method to assay small numbers of *X. c.* pv. *hyacinthi* in hyacinths, we investigated the fimbriae of this bacterium, which might be used as potential target antigens for a detection assay. This paper presents the first description of the fimbriae of *X. campestris* pathovars and *Xanthomonas* spp. Furthermore, immunological and molecular properties of these fimbriae are described, and experiments are presented that indicate that these surface structures may be involved in attachment to stomata of host plants.

RESULTS

Pellicle formation by *X. c.* pv. *hyacinthi* in liquid cultures.

After 1 wk at 28° C in static broth, *X. c.* pv. *hyacinthi* formed a pellicle at the surface of the medium (Fig. 1A). Electron microscopy of negatively stained pellicle material revealed bacterial cells interconnected by long, bundled strands of fibrillar components (Fig. 1B). These strands

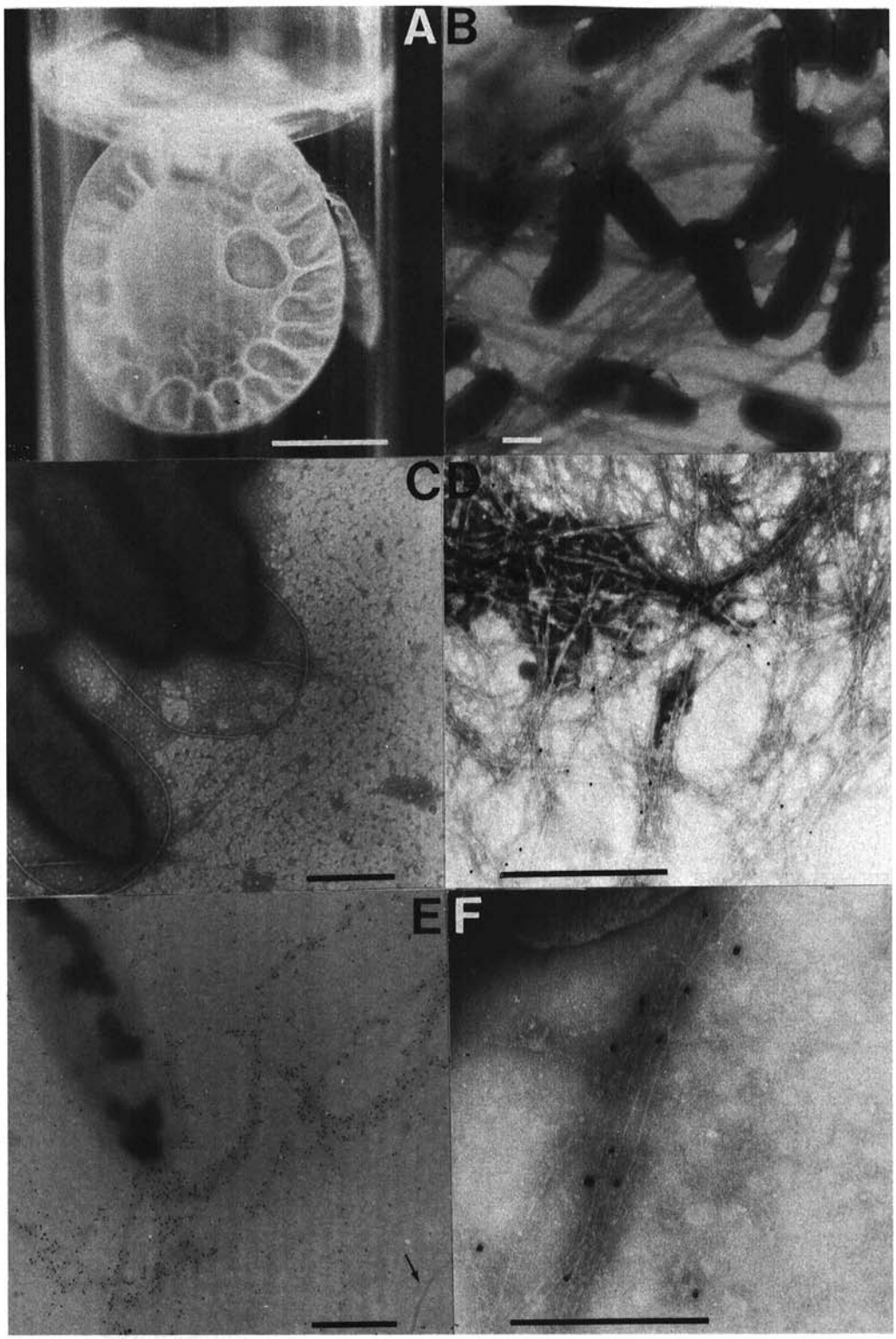

Fig. 1. A, Pellicle formation in a static broth culture of *Xanthomonas campestris* pv. *hyacinthi* strain S148 after 10 days of incubation at 28° C. The pellicle is flipped 90° (bar = 1 cm). **B,** Electron micrograph of pellicle material after staining with 2% ammonium molybdate. Bacterial cells are "caught" in a network of aggregated filaments (bar = 1 μm). **C,** Electron micrograph of cells of strain S148, negatively stained with ammonium molybdate. Polar fimbriae and flagella are visible (bar = 1 μm). **D,** Electron micrograph of fimbriae from strain S148, purified by acid precipitation (bar = 1 μm; the black dots are gold particles, with a diameter of 5 nm). **E,** Immunogold-labeled fimbriae from strain S148, obtained after incubation of bacterial cells with rabbit immunoglobulin G directed against native fimbriae followed by incubation with gold-tagged anti-rabbit conjugate. A piece of an unlabeled flagellum (arrow) is visible in the lower right corner of the micrograph (bar = 1 μm). **F,** Detail of an immunogold-decorated fimbrial strand. The individual antibody-coated filaments are visible (bar = 0.5 μm).

seemed to consist mainly of aggregated fimbriae-like filaments, occasionally intertwined with flagella.

Identification and purification of fimbriae from strain S148.

When viewed with an electron microscope, most *X. c.* pv. *hyacinthi* cells were uniflagellated and appeared to have fimbriae located at the same pole as the flagellum (Fig. 1C). The thin and flexible fimbriae appeared to have a length of approximately 6 μm. The diameter of the fimbriae (about 5 nm) was clearly less than that of the flagella. The fimbriae were often found to aggregate into much thicker and longer strands (Fig. 1B).

For purification of these fimbriae, the standard procedure for type 4 fimbriae was employed, resulting in almost pure protein preparations, as shown in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2, lane 4). The preparation mainly contained a 17-kDa protein band. In some instances, a small amount of background material was visible in the high molecular weight area after immunoblotting. The purified sample, when viewed with an electron microscope, consisted mostly of fimbriae (Fig. 1D). The apparent molecular mass of the fimbrial subunit was approximately 17 kDa.

Whole *X. c.* pv. *hyacinthi* cells and purified fimbrial samples were incubated with several types of erythrocytes, but no hemagglutination was found.

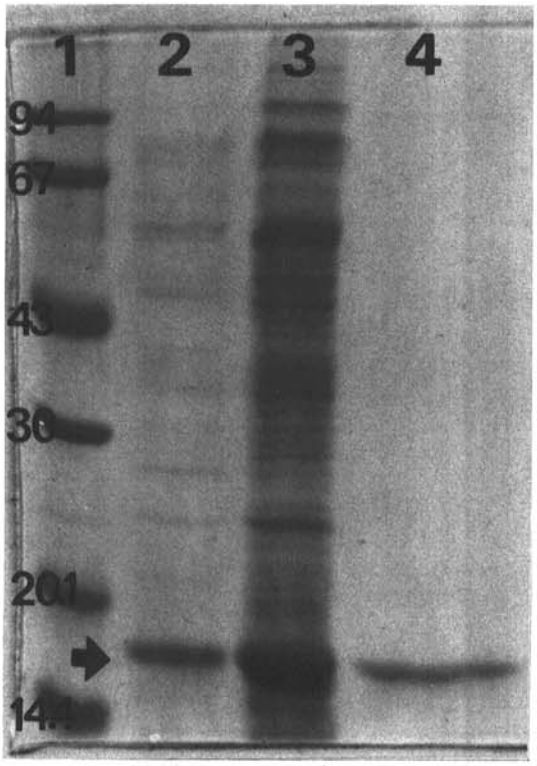

Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of fimbrial fractions from *Xanthomonas campestris* pv. *hyacinthi* strain S148. Lane 1, marker proteins with relative molecular mass in kilodaltons; lane 2, the same fraction as in lane 3, after acid precipitation; lane 3, pooled culture supernatant and shear fraction; lane 4, fraction of lane 2 after gel filtration chromatography. The arrow indicates the fimbrial subunit of 17 kDa.

Isoelectric point of *X. c.* pv. *hyacinthi* fimbriae.

Native fimbrial samples were subjected to horizontal and vertical isoelectric focusing (pH ranges of 3–10 and 3–6, respectively). After immunoblotting and incubation with anti-fimbrial serum, a protein band was visible at approximately pH 4.1 (data not shown).

Immunogold electron microscopy and immunoblotting.

Immunoglobulin G (IgG) directed against native fimbriae (anti-F) and IgG against the denatured subunit (anti–17 kDa) were used in immunoblotting experiments (Fig. 3) as well as

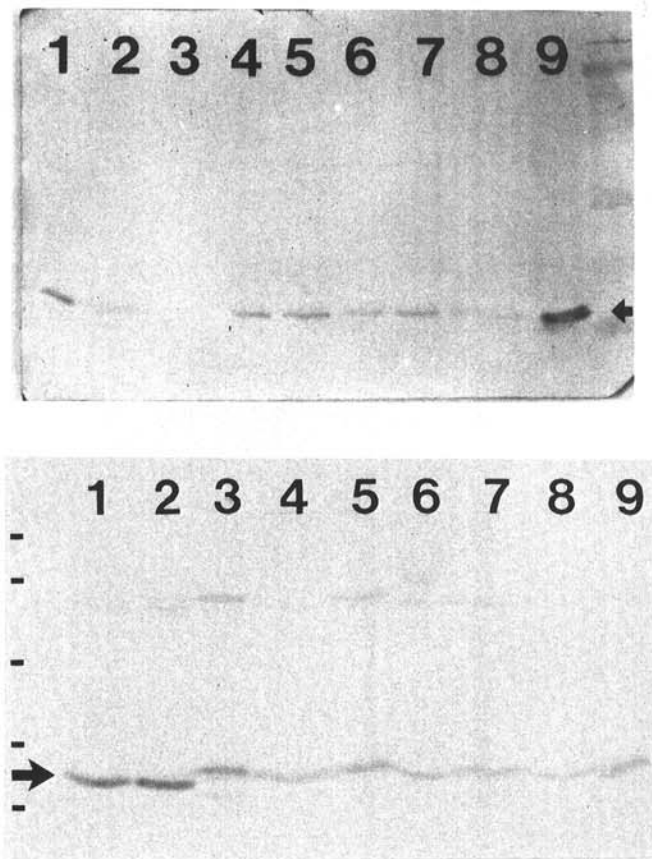

Fig. 3. A, Western blot of shear fractions of different isolates of *Xanthomonas campestris* pv. *hyacinthi* (strains 113, 133, 169, 170, 171, 172, 177, and S148) and a strain of *Erwinia carotovora*. Equal amounts of protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose filter paper, and then developed with absorbed anti–fimbrial subunit immunoglobulin G (IgG) and horseradish peroxidase–conjugated anti-rabbit serum. The arrow indicates the fimbrial subunit. Lane 1, strain 113; lane 2, strain 133; lane 3, *E. carotovora;* lane 4, strain 177; lane 5, strain 172; lane 6, strain 171; lane 7, strain 170; lane 8, strain 169; lane 9, test strain S148. **B,** Western blot of fimbrial fractions (equal amounts of protein) from different *Xanthomonas* species and *X. campestris* pathovars, developed with anti–fimbrial subunit IgG as described. The arrow indicates the fimbrial subunit; the bars represent protein molecular weight markers (from top to bottom): bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.3 kDa). Lane 1, *X. fragariae;* lane 2, *X. maltophilia;* lane 3, *X. c.* pv. *holcicola;* lane 4, *X. c.* pv. *pruni;* lane 5, *X. c.* pv. *citri;* lane 6, *X. axonopodis;* lane 7, *X. ampelina* (*Xylophilus ampelinus*); lane 8, X. *c.* pv. *phaseoli;* lane 9, *X. albilineans.*

for immunoelectron microscopy studies (Fig. 1). In immunogold electron microscopy, only anti-F IgG gave good labeling of native fimbriae, as shown in Figure 1E and F. Fimbrial structures were clearly tagged with 10-nm gold particles; flagella were not labeled (Fig. 1E).

Both types of serum were used for immunoblotting. Both sera reacted with the fimbrial subunit from *X. c.* pv. *hyacinthi* isolates (Fig. 3A). Only the anti–17 kDa IgG reacted with a 17-kDa protein band from *X. campestris* pathovars and *Xanthomonas* spp. Sonicated cell fractions of *E. carotovora* (Fig. 3A) and *P. marginata* (not shown) did not react with the anti–17 kDa IgG. These results indicated that fimbriae are present among these xanthomonads. Small differences in the relative molecular masses of the subunits were visible (Fig. 3B); *X. albilineans, X. populi,* and *X. c.* pvs. *begoniae, manihotis, oryzicola, campestris,* and *vesicatoria* also showed a protein band of approximately 17 kDa when subjected to immunoblotting (not shown). The presence of fimbriae was confirmed in *X. c.* pvs. *holcicola, gummisudans, pelargonii,* and *oryzicola* by electron microscopy (Fig. 4); immunofluorescence microscopy showed that *X. campestris* pathovars and *Xanthomonas* spp. also reacted with anti-F IgG (Fig. 5 and Table 1). When the same amount of bacterial extract was applied on the blot, the immunological reaction was considerably weaker than that for *X. c.* pv. *hyacinthi* preparations. In immunoblotting experiments, whole-cell preparations of *P. aeruginosa* and *Moraxella bovis* (which produce type 4 fimbriae) did not react with the anti–17 kDa IgG. Purified pili from *Neisseria gonorrhoeae* also did not react with the anti–17 kDa IgG (data not shown).

In all immunoblotting experiments, one or two 42- to 44-kDa protein bands in the *Xanthomonas* preparations cross-reacted with the anti–17 kDa IgG (Fig. 3B). This cross-reaction could be inhibited or even prevented by absorption of the IgG with extensively sheared *X. c.* pv. *hyacinthi* cells (Fig. 3A).

Amino acid composition and N-terminal amino acid sequence of the fimbrial subunit of *X. c.* pv. *hyacinthi*.

Table 2 shows the amino acid composition of the fimbrial subunit from *X. c.* pv. *hyacinthi* strain S148. About half of the total number of amino acids are hydrophobic in nature, and high levels of Asx and Glx were found. This is in agreement with results obtained for other type 4 fimbriae (McKern *et al.* 1983; Hermodson *et al.* 1978). The precipitation of *X. c.* pv. *hyacinthi* fimbriae at low pH is also consistent with an acidic protein.

The N-terminal amino acid sequence of the fimbrial subunit showed strong homology with those from several other pilins (Fig. 6), suggesting that *X. c.* pv. *hyacinthi* pro-

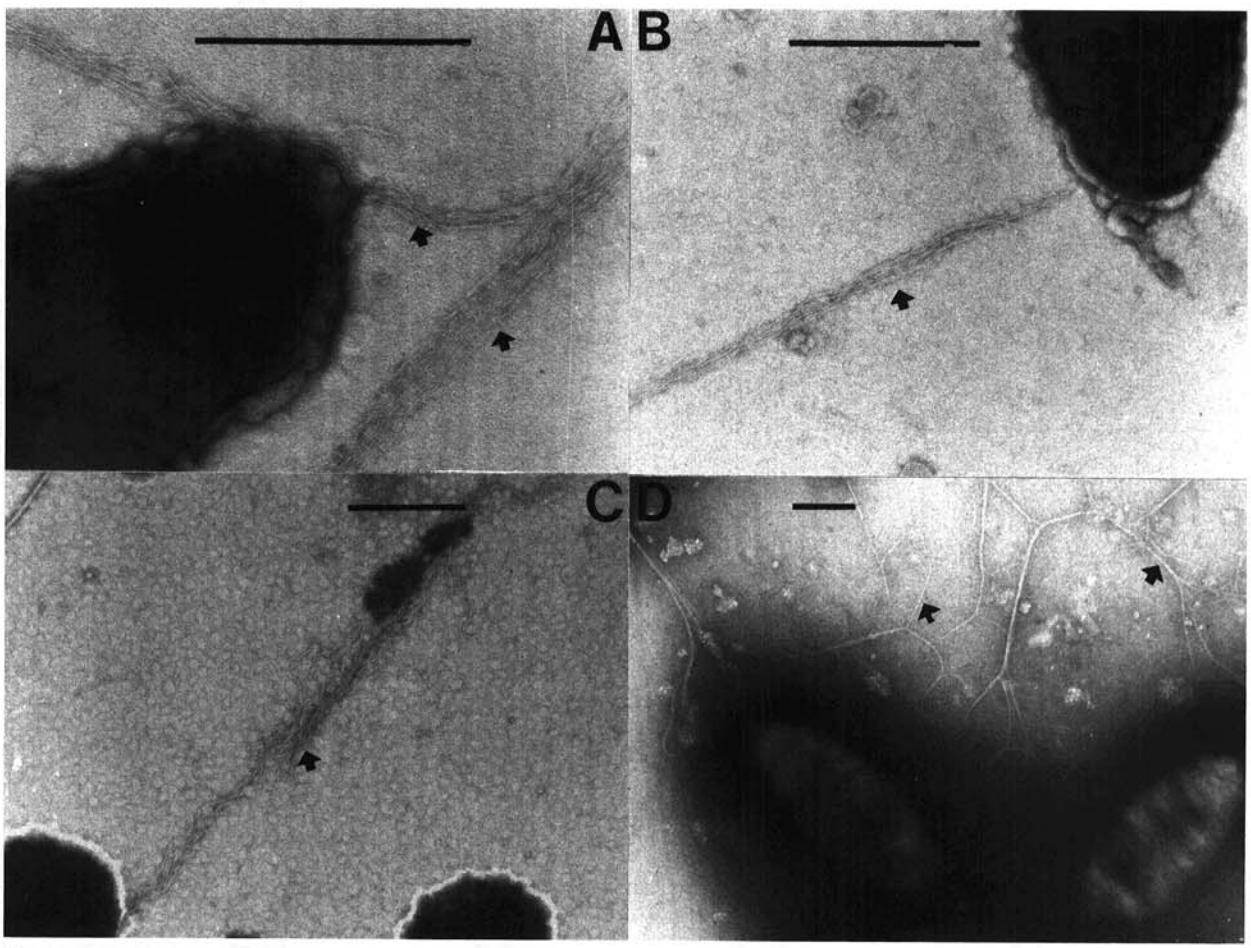

Fig. 4. Electron micrographs of negatively stained *Xanthomonas campestris* pv. *oryzicola* (**A**), *X. c.* pv. *holcicola* (**B**), *X. c.* pv. *gummisudans* (**C**), and *X. c.* pv. *pelargonii* (**D**). Arrows point to fimbrial filaments (bars = 0.1 μm).

duces type 4 or *N*-methylated phenylalanine (N-MePhe) fimbriae.

Attachment of *X. c.* pv. *hyacinthi* to hyacinth stomata.

To study the interaction of *X. c.* pv. *hyacinthi* with plant material, leaves from hyacinths grown in tissue culture were incubated with *X. c.* pv. *hyacinthi* labeled with fluorescein isothiocyanate (FITC) (Fig. 7A). The bacteria became associated mainly with stomata (Fig. 7A). Sheared cells of *X. c.* pv. *hyacinthi* (not shown), FITC-labeled *X. c.* pv. *campestris* (Fig. 7B), and *Escherichia coli* HB101 (not shown) did not adhere significantly to the stomata. As viewed by fluorescence microscopy, purified native fimbriae of the *X. c.* pv. *hyacinthi* strain also gave a strong signal at and about the stomata (Fig. 7C), which was inhibited by anti-F IgG and Fab fragments prepared from anti-F IgG. No signal was detected when stomata were incubated with antisera and FITC-labeled anti-rabbit IgG (Fig. 7F). Fluorescent microparticles coated with fimbriae (Fig. 7E) were also found in association with stomata (Fig. 7D). Microparticles coated with bovine serum

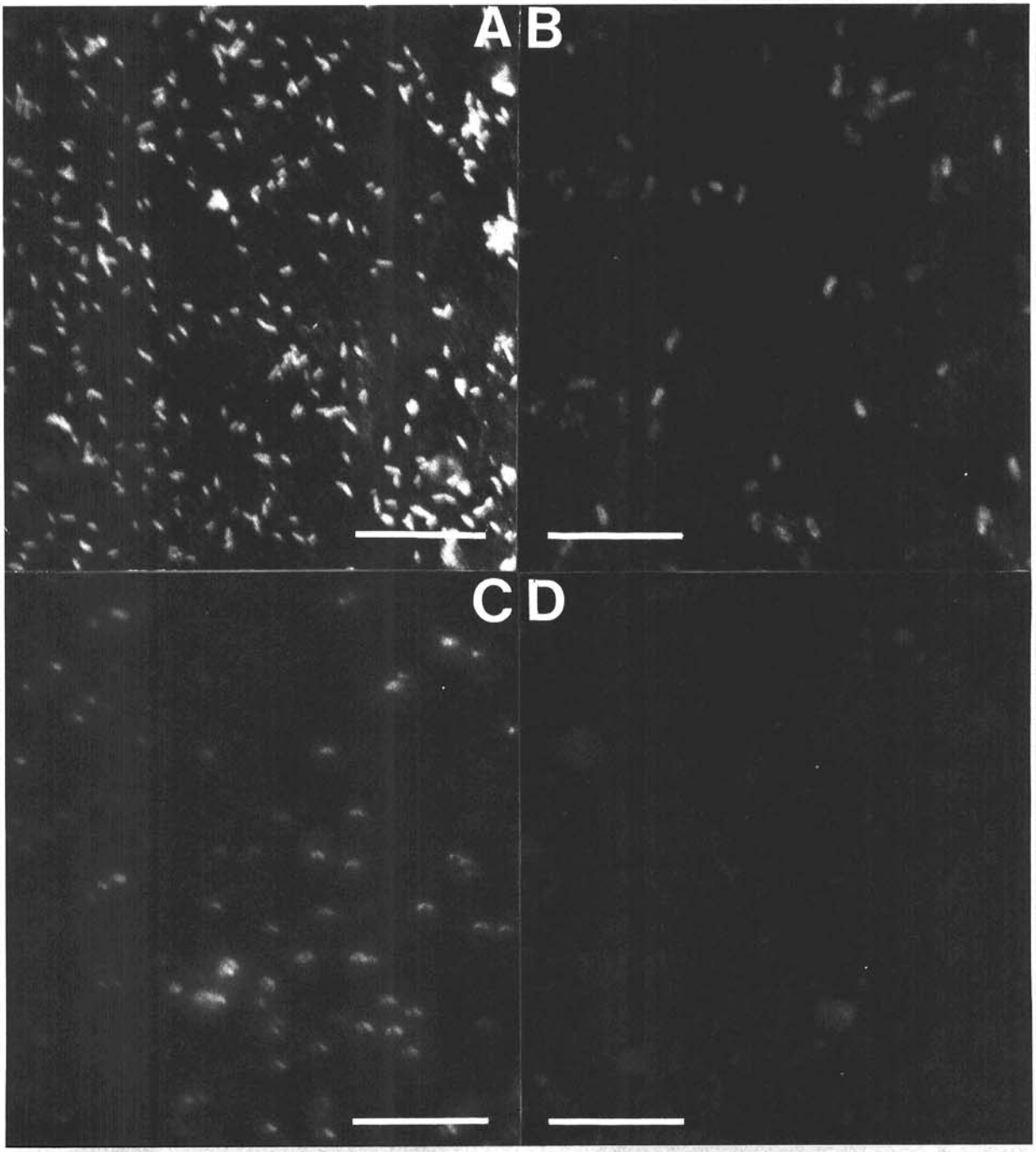

Fig. 5. Fluorescence of *Xanthomonas* cells (**A–C**) resulting from indirect fluorescent labeling of fimbriae after incubation with polyclonal anti-fimbriae immunoglobulin G (IgG) followed by incubation with anti-rabbit IgG conjugated with fluorescein isothiocyanate (bar = 10 μm). **A,** *X. campestris* pv. *hyacinthi* strain S148. **B,** *X. c.* pv. *citri.* **C,** *X. c.* pv. *graminis.* **D,** *Escherichia coli* HB101 (negative control).

albumin (BSA) did not attach themselves to stomata (data not shown). These results suggested a function for this type of fimbriae in mediating the adherence of bacteria to the plant surface, in particular to stomata.

DISCUSSION

Type 4 or MePhe fimbriae are well characterized (Elleman 1988; Dalrymple and Mattick 1987). Bacteria known to be able to produce these polar fimbriae are, for instance, *Dichelobacter nodosus* (*Bacteroides nodosus*), *P. aeruginosa,* and *N. gonorrhoeae.* Our results show that *X. c.* pv. *hyacinthi* produces a similar type of fimbria. Not only the conserved N-terminal region of the *X. c.* pv. *hyacinthi* fimbrial subunit indicate that they belong to this type of fimbria, but other characteristics, including polar location on the cell, diameter, molecular mass of the composing subunits, and isoelectric point, also fit within the definition of type 4 fimbriae (Ottow 1975). Immunoblotting experiments showed that other *X. campestris* pathovars also reacted with the antiserum raised against purified fimbriae from strain S148. The fimbrial antisera did not cross-react with purified gonococcal fimbriae or with shear fractions from *M. bovis* and *P. aeruginosa*; in some cases this has been found for bacteria producing type 4 fimbriae (Patel *et al.* 1991). In general, type 4 fimbrial subunits contain (besides a conserved region in the N-terminal part and a semiconserved part in the C-terminal sequence) a central sequence of amino acid residues that can be highly variable, even among strains of the same species (Dalrymple and Mattick 1987; Sastry *et al.* 1985). Variation

Table 1. Immunofluorescence of bacterial cells after incubation with polyclonal anti-fimbriae antibodies followed by anti-rabbit immunoglobulin G (conjugated with fluorescein isothiocyanate)

	Immunofluorescence[a]
Xanthomonas campestris pv. *hyacinthi*	+++
X. axonopodis	+
X. fragariae	+
X. maltophilia	+/++
X. c. pv. *citri*	+
X. c. pv. *graminis*	+/++
X. c. pv. *holcicola*	+
X. c. pv. *pelargonii*	+
X. c. pv. *phaseoli*	+
X. c. pv. *pruni*	+
X. c. pv. *vesicatoria*	+
Escherichia coli HB101	−

[a]Qualitative immunofluorescence values, obtained by immunofluorescence microscopy: +++ = strong fluorescent signal; +/++ = intermediate fluorescent signal; + = weak but positive fluorescent signal; − = no fluorescence found.

Table 2. Amino acid composition of the 17-kDa subunit from fimbriae of *Xanthomonas campestris* pv. *hyacinthi*[a]

Amino acid	Residues per molecule
Asx	12.1
Thr	23.7
Ser	8.7
Glx	16.7
Pro	4.7
Gly	12.7
Ala	27.8
Cys	2.7
Val	12.2
Met	1.5
Ile	9.9
Leu	9.8
Tyr	2.6
Phe	0.3
Lys	4.5
His	0.2
Arg	5.1
Trp	...[b]
Total amount	150.1

[a]The M_r of 17 kDa for the major fimbrial subunit was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.
[b]Not determined.

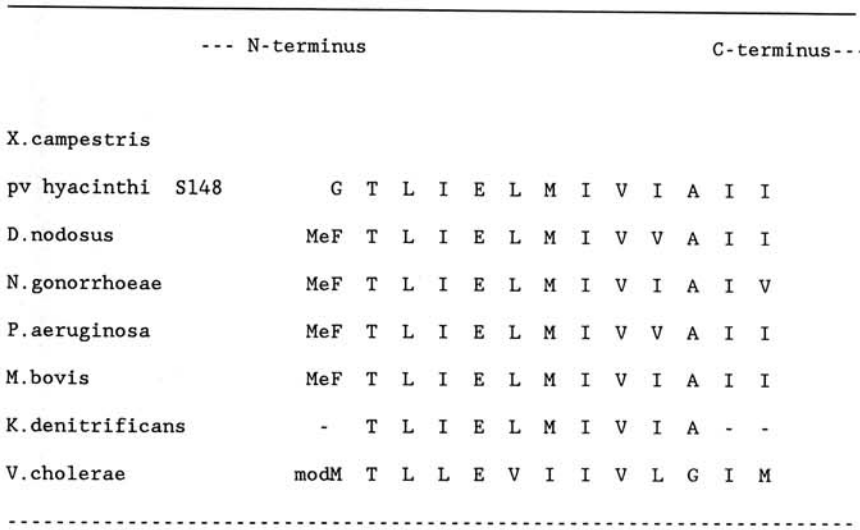

Fig. 6. Comparison of the N-terminal amino acid sequence of the fimbrial subunit from *Xanthomonas campestris* pv. *hyacinthi* strain S148 with the fimbrial N-terminal sequences of *Dichelobacter nodosus, Neisseria gonorrhoeae, Pseudomonas aeruginosa, Moraxella bovis* (Dalrymple and Mattick 1987), *Kingella denitrificans* (Weir and Marrs 1992), and *Vibrio cholerae* (Taylor *et al.* 1987). The N-terminal amino acid residue (glycine) detected in the *X. c.* pv. *hyacinthi* fimbrial subunit might be the result of residual glycine in the sample used for determination of the sequence. MeF = methylated phenylalanine; modM = modified methionine; dashes indicate amino acids not determined.

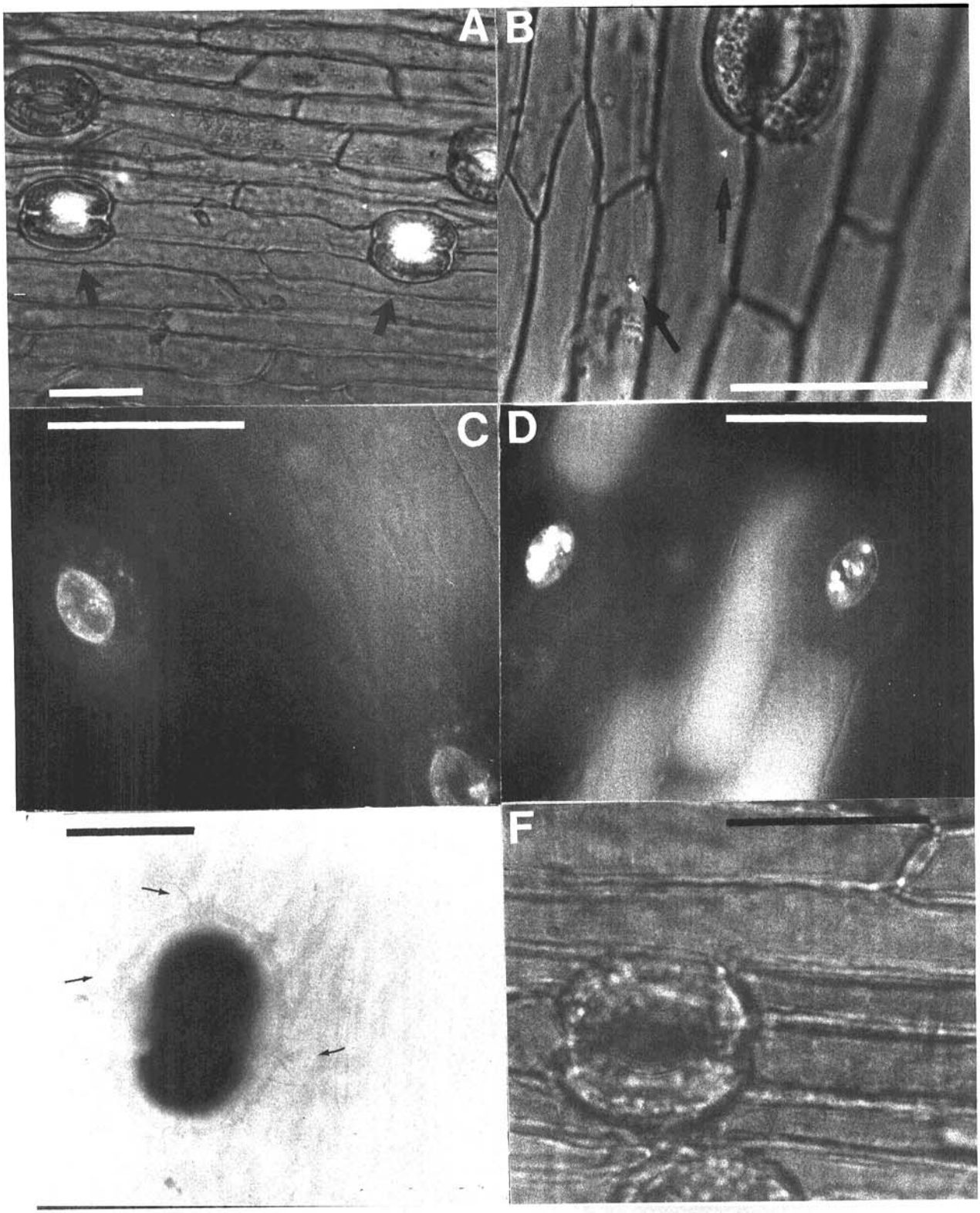

Fig. 7. Fluorescent cells and isolated fimbriae of *Xanthomonas campestris* pv. *hyacinthi* strain S148 incubated with leaves of hyacinth cultivar Pink Pearl. **A,** S148 cells labeled with fluorescein isothiocyanate (FITC) associated with stomata (bar = 50 μm). The arrows point to the fluorescent bacteria in and about stomata. **B,** FITC-labeled cells of *X. c.* pv. *campestris*, indicated by arrows (bar = 25 μm). No bacteria were found to attach to the stomata. **C,** Fluorescent S148 fimbriae associated with stomata, visualized by indirect immunofluorescent labeling with FITC-conjugated anti-fimbriae rabbit immunoglobulin G (IgG) (bar = 25 μm). **D,** Fluorescent microbeads coated with purified native fimbriae, associated with stomata (bar = 25 μm). **E,** Electron micrograph of two adjacent fluorescent microbeads, coated with fimbriae (arrows). The microbeads have a diameter of 0.65 μm (bar = 1 μm). **F,** Control experiment, in which stomata remained unstained after incubation with FITC-conjugated anti-fimbriae rabbit IgG (bar = 25 μm).

in immunoreactivity and molecular mass of the fimbrial subunit was found for different *X. campestris* pathovars and *Xanthomonas* spp. (Fig. 3B). This has also been found for *D. nodosus* and *N. gonorrhoeae* (Elleman 1988). However, the lower immunoreactivity in immunoblots might be caused by a lower expression of fimbriae compared with that of *X. c.* pv. *hyacinthi* strain S148. Also, phase variation is known among bacteria producing type 4 fimbriae, often correlated with altered colony morphology (Marrs *et al.* 1985). Differences in colony morphology have been found among xanthomonads, including *X. c.* pv. *hyacinthi* (Peros 1988; Smith 1901). However, no clear differences were found for strain S148.

The function of type 4 fimbriae in general is not clear. For the bacterial species mentioned above they might function in adherence to epithelial surfaces (Dalrymple and Mattick 1987). Fimbriae of *N. gonorrhoeae* and *P. aeruginosa* mediate, for instance, binding to lactosylceramide (Karlsson 1989). However, the role of receptor binding of fimbriae in the pathogenicity of *D. nodosus* is not clear (Elleman 1988). Other putative functions of type 4 fimbriae have been proposed, such as involvement in DNA uptake (Breitling and Dubnan 1990) and the "twitching" motility of cells (Henrichsen 1983). Coaggregation of bacterial cells might be mediated by type 4 fimbriae–like proteins, as was found for an *E. coli* strain (Giron *et al.* 1991). Besides the striking homology among the type 4 fimbrial amino-terminal sequences, there is a strong resemblance to other proteins involved in the export of molecules in eubacteria (Dums *et al.* 1991; Whitchurch *et al.* 1990). This similarity might explain the cross-reacting 42- to 44-kDa protein band in the immunoblotting experiments (Fig. 3).

Table 3. Strains used in this study

	Strain designation	Source[a]
Xanthomonas albilineans	494	LMG
X. axonopodis	982	LMG
X. campestris		
pv. *begoniae*	7189	LMG
pv. *campestris*	372	CPRO
pv. *citri*	409	LMG
pv. *graminis*	S168	BRC
pv. *gummisudans*	S136	BRC
pv. *holcicola*	736	LMG
pv. *hyacinthi*	S148, 133, 169, 170, 171, 172, 177, 113	BRC
pv. *manihotis*	784	LMG
pv. *oryzicola*	797	LMG
pv. *pelargonii*	7314	LMG
pv. *phaseoli*	7455	LMG
pv. *pruni*	852	LMG
pv. *vesicatoria*	512	CPRO
X. fragariae	708	LMG
X. maltophilia	958	LMG
X. populi	5746	LMG
Xylophilus ampelinus (*Xanthomonas ampelina*)	5856	LMG
Erwinia carotovora	I155	BRC
Escherichia coli	HB101	Clontech
Moraxella bovis	986	LMG
Pseudomonas aeruginosa	1242	LMG
P. marginata	I570	BRC

[a]BRC = Bulb Research Centre, Lisse, Netherlands. Clontech = Clontech, Palo Alto, CA. CPRO = Centre for Plant Breeding and Reproduction Research, Wageningen, Netherlands. LMG = culture collection of the Laboratory for Microbiology, Rijksuniversiteit Gent, Gent, Belgium.

Functions for fimbriae among plant-pathogenic *Pseudomonas* species have been proposed (Romantschuk 1992; Romantschuk and Bamford 1986). Avirulent *P. solanacearum* produces many fimbriae, of which the subunit has a molecular mass of about 9,500 Da, and which cause the bacterium to adhere to the leaf surface of the host plant. In contrast, *P. syringae* pv. *phaseolicola* expresses only a few fimbriae per cell, which are correlated with the ability of this bacterium to adhere selectively to stomata of bean. It is very likely that both types of fimbriae do not belong to the type 4 group, suggesting the possibility that different types of fimbriae function as virulence or avirulence factors.

In static broth, *X. c.* pv. *hyacinthi* formed a pellicle which consisted mainly of fimbrial aggregates. The function of this pellicle *in vivo* might be to protect bacteria during dry periods on the leaf surface. A specific adhesion mechanism may enhance entrance into the host plant when conditions are favorable (high humidity). The attachment studies indicated that *X. c.* pv. *hyacinthi* can associate with stomata of a hyacinth cultivar that was grown in tissue culture. Entrance via hydathodes or stomata seems to be a crucial step in infection, because hybrid hyacinths that produce smaller stomata have been found to be significantly more resistant to infection with yellow disease (Van Tuyl and Toxopeus 1980). The fimbriae of *X. c.* pv. *hyacinthi* may be involved in the first stage of infection. Fimbriae-coated beads were found to associate predominantly with stomata, whereas antifimbrial serum inhibited the attachment. *X. c.* pv. *hyacinthi* mutants defective in expressing fimbriae should give a more conclusive answer about the role of the type 4 fimbriae of *X. c.* pv. *hyacinthi.*

MATERIALS AND METHODS

Strains and media.

X. c. pv. *hyacinthi* strain S148 was isolated from hyacinths with yellow disease and used as reference strain. The other *Xanthomonas* strains used in this study are listed in Table 3. The *Xanthomonas* isolates were grown on nutrient agar plates supplemented with yeast extract (5 g/l) (Difco Laboratories, Detroit, MI) or, for liquid cultures, nutrient broth supplemented with yeast extract (5 g/l). Pathogenicity was tested by spraying leaf tops of healthy plants (*Hyacinthus* cultivars Pink Pearl and Anna Marie) with strain S148 cell suspensions, diluted with sterilized tap water (10^7–10^8 cfu/ml). The plants were maintained in a greenhouse at 21° C and examined for lesions after 2 or 3 wk.

Isolation of fimbriae.

For the isolation of fimbriae, the acid precipitation method was used essentially as described for the purification of type 4 fimbriae of *D. nodosus* (Every 1979). *X. c.* pv. *hyacinthi* cells, cultured on plates for 3–4 days at 28° C, were harvested and resuspended in phosphate-buffered saline (PBS) (approximately 5 g, wet weight, in 50 ml of buffer). After centrifugation, the culture supernatant fraction was kept, and the bacteria were resuspended in ice-cold 0.01 M phosphate buffer (pH 7.5) with 0.5 M NaCl. The cells were sheared three times for 2 min at 2-min intervals in a Sorvall Omni-mixer (Sorvall, Norwalk, CT). The sheared cells were pelleted by centrifugation (20 min at 12,000 × *g*), and the supernatant fractions were combined. Next, 1 M citric acid was slowly added to a

final concentration of about 25 mM, until aggregates became visible. After centrifugation (15 min at 12,000 × *g*), the precipitate was washed with distilled water, redissolved in PBS, and precipitated again with citric acid. This procedure was repeated several times, until (as checked by SDS-PAGE) a partially purified fimbrial preparation was obtained. Gel filtration chromatography (Sepharose CL-4B column, 1 × 35 cm, Pharmacia Fine Chemicals, Uppsala, Sweden) was used for further purification (Van Doorn *et al.* 1987). Preparative SDS-PAGE was used to obtain purified denatured fimbrial subunits (Van Doorn *et al.* 1987). The protein band corresponding to the potential fimbrial subunit was visualized by staining the gels with 0.01% (w/v) Coomassie Brilliant Blue (Bio-Rad Laboratories, Richmond, CA) in distilled water. This protein band was cut out of the gel, and the protein was eluted by electroelution (Van Doorn *et al.* 1987). This protein preparation was then used to immunize rabbits and to determine the amino acid composition after the removal of SDS (Amons and Schrier 1981). Purified pili from *N. gonorrhoeae* strain MS1 were used in immunoblotting experiments and were kindly donated by Jos van Putten, Max-Planck-Institut fur Biologie (Infektionsbiologie), Tübingen, Germany.

Determination of the isoelectric point of *X. c.* pv. *hyacinthi* fimbriae.

Isoelectric focusing was carried out in 1) ultrathin polyacrylamide isoelectric focusing gels (Servalyte Precotes, pH 3–10, Serva, Heidelberg, Germany) on an LKB Multiphor-II apparatus and 2) a mini-vertical electrophoresis system (ABN, Hayward, CA) as described by Robertson *et al.* (1987). After focusing, proteins were blotted on nitrocellulose membranes. The immunoblots were incubated with antisera raised against the *X. c.* pv. *hyacinthi* fimbriae and developed with anti-rabbit horseradish peroxidase conjugate (Bio-Rad) as described by Towbin *et al.* (1979).

Sample preparation for SDS-PAGE.

Bacterial samples (disrupted *Xanthomonas* cells or fimbrial preparations) were applied on 12% SDS-polyacrylamide gels (Laemmli 1970) in a Bio-Rad minigel system. Cells (10^8–10^9/ml) were resuspended in PBS and disrupted by ultrasonication (three times, for 1 min each, with a microtip) (Branson, Danbury, CT) or by 0.1-mm glass beads (5 min at maximal power) in a Mini-Beadbeater (Biospec Products, Bartlesville, OK).

Antisera and immunological methods.

Two rabbits were immunized subcutaneously on days 0, 10, and 20 with a 1:1 mixture of 0.25 mg of purified native fimbriae or fimbrial subunit (17-kDa protein, 0.10 mg), respectively, and Freund's incomplete adjuvant. After a booster injection (0.5 mg and 0.20 mg, respectively, in Freund's incomplete adjuvant) 10 ml of serum was taken from each rabbit by bleeding from the marginal vein of the ear. The IgG fraction of the sera was isolated (Steinbuch and Audran 1969) and used in immunoblotting and immunogold electron microscopy experiments.

The Fab fragments of IgG directed against the native fimbriae of strain S148 were prepared by digestion of immunoglobulins by immobilized papain (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. Immunoblotting was carried out with a semidry blot apparatus (Novablot, LKB, Bromma, Sweden), and the nitrocellulose blots (Schleicher & Schuell, Dassel, Germany) were developed with antiserum and anti-rabbit horseradish peroxidase conjugate (Bio-Rad) as described by Towbin *et al.* (1979). In some immunoblotting experiments the antiserum was absorbed with sheared S148 cells (fimbriae-free) in order to minimize background reactions. For immunogold labeling of electron microscopic preparations, gold-tagged goat anti-rabbit IgG (GAR10, Janssen Life Sciences Products, Beerse, Belgium) was used after incubation with fimbrial antiserum (Van Doorn *et al.* 1987).

Hemagglutination.

Several types of erythrocytes were used in a microtiter plate assay, essentially as described before (Van Doorn *et al.* 1987). Fresh chicken and guinea pig erythrocytes and glutaraldehyde-stabilized human (group O) and bovine red blood cells (Sigma, St. Louis, MO) were incubated with whole bacterial cells or purified fimbriae from strain S148 at 0, 4, or 37° C.

Electron microscopy.

Bacterial and fimbrial preparations were negatively stained with 2% ammonium molybdate (pH 6.8) and viewed at 60 kV with a Philips 201 electron microscope.

Amino acid composition and N-terminal amino acid sequence of the fimbrial subunit of *X. c.* pv. *hyacinthi*.

The purified 17-kDa protein was hydrolyzed with 5.7 N HCl for 60 hr at 110° C and analyzed by high-pressure liquid chromatography (Eurosequence, Groningen, Netherlands) for determination of the amino acid composition. The N-terminal amino acid sequence was determined at the Gas Phase Sequenator Facility of the Department of Medical Biochemistry, University of Leiden, Leiden, Netherlands. The 17-kDa protein (0.1 mg) was electroblotted from SDS-PAGE onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA) and analyzed by means of an Applied Biosystems Model 470A Protein Sequencer, equipped on-line with a model 120A PTH Analyser.

Adherence of bacteria to hyacinth leaves and immunofluorescence.

Adherence experiments were carried out according to Romantschuk and Bamford (1986) with some modifications. Leaves from sterile tissue cultures of Pink Pearl hyacinth, grown as described by Van der Linde *et al.* (1988), were incubated with bacterial suspensions in PBS at room temperature. Subsequently, the incubated leaves were washed several times with a mixture of PBS and 0.1% Tween 20 and incubated in the presence of 0.25% ovalbumin (Sigma) with rabbit antisera raised against whole bacterial cells or native fimbriae. Normally, leaf pieces of about 2 cm were incubated in test tubes with 3 ml of buffer in the presence of bacteria (about 10^7 ml^{-1}) for 1 hr; antisera were used at a dilution of 1:100 (0.05 mg of IgG per milliliter). Fab fragments of anti-F IgG were used at a concentration of 0.05 mg/ml. In order to visualize nonfluorescent bacteria or fimbriae attached to the plant epidermis, FITC-conjugated anti-rabbit IgG (Sigma) was used. In some instances, bacterial cells were directly

labeled with FITC (Sigma) (Nowicki *et al.* 1986). In some experiments, hyacinth leaves were preincubated with anti-F IgG as a control or to inhibit the attachment of bacteria or fimbriae. To study the function of purified fimbriae, fluorescent beads (Covaspheres Reagent, 0.65 μm, Duke Scientific, Palo Alto, CA) were coated with fimbriae or BSA according to the manufacturer's instructions and used according to Westerlund (1991). The coating of microparticles with fimbriae was assayed by electron microscopy. To visualize fluorescent signals under a UV light microscope (Leitz Wetzlar, Wetzlar, Germany), the epidermal layer of the incubated hyacinth leaves was stripped and mounted on a microscope slide.

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