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Biochemical Characterization and Agglutinating Properties of *Xenorhabdus nematophilus* F1 Fimbriae

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***Xenorhabdus* spp., entomopathogenic bacteria symbiotically associated with nematodes of the family *Steinernematidae*, occur spontaneously in two phases. Only the phase I variants of *Xenorhabdus nematophilus* F1 expressed fimbriae when the bacteria were grown on a solid medium (nutrient agar; 24 and 48 h of growth). These appendages were purified and characterized. They were rigid, with a diameter of 6.4 ± 0.3 nm, and were composed of 16-kDa pilin subunits. The latter were synthesized and assembled during the first 24 h of growth. Phase II variants of *X. nematophilus* did not possess fimbriae and apparently did not synthesize pilin. Phase I variants of *X. nematophilus* have an agglutinating activity with sheep, rabbit, and human erythrocytes and with hemocytes of the insect *Galleria mellonella*. The purified fimbriae agglutinated sheep and rabbit erythrocytes. The hemagglutination by bacteria and purified fimbriae was mannose resistant and was inhibited by porcine gastric mucin and *N*-acetyl-lactosamine. The last sugar seems to be a specific inhibitor of hemagglutination by *X. nematophilus*.**

Bacterial symbionts of the entomopathogenic nematodes of the family *Steinernematidae* are members of the family *Enterobacteriaceae* and belong to the genus *Xenorhabdus* (9, 31). The nematode hosts transport their bacterial symbionts into the hemocoel of the insect prey and provide protection for them against the host's immune response during the early stage of infection. Subsequently, these entomopathogenic bacteria induce lethal septicemia and contribute to the symbiotic relationship by providing nutritional requirements for their nematode partners during reproduction in the insect cadavers (8).

Xenorhabdus strains spontaneously produce two colony forms in vitro (1) which have been called phase variants (7). Phase I variants adsorb dyes on agar plates, produce lecithinase, have protoplasmic paracrystalline inclusions, and produce chemical antibiotics that minimize secondary invasion of the cadaver by other microorganisms, while these properties are either apparently absent or greatly reduced in phase II variants (2, 7). Phase I variants are naturally carried within the intestines of the infective juvenile nematodes and provide better conditions than do phase II variants for the reproduction of the nematode within its insect host (1, 6). Infective juvenile nematodes resulting from natural parasitism in insects or from in vitro cultures in the presence of both variants contain only phase I variants (1). Phase I variants produce fimbriae on solid media, while phase II variants do not express these structures (5, 11). The fimbriae probably confer adhesive properties on the phase I variants, which may favor the retention of the phase I cells by the nematode host.

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Biochemical and molecular analyses of fimbriae from a variety of animal and human pathogens have revealed that fimbriae are composed of filamentous surface proteins, which facilitate bacterial adherence to specific host tissues by carbohydrate binding to surface receptors (21, 23, 29). Most of the pathogens in the *Enterobacteriaceae* family express type 1 fimbriae, which mediate mannose-sensitive agglutination of erythrocytes of many species and contribute to mannose-inhibitable adherence to eukaryotic cells (12, 30). Several other types of enterobacterial fimbriae exhibit mannose-resistant hemagglutination (HA) and cell adherence by recognizing either a carbohydrate moiety of membrane glycoprotein (or glycolipid) or a carbohydrate-conjugated mucosal protein (18, 20, 21). Fimbriae have been shown to be an important virulence factor in enterotoxigenic *Escherichia coli* and uropathogenic strains of *E. coli* and *Proteus mirabilis* by enhancing colonization of epithelial cells (14, 21, 28).

In this study, we undertook the biochemical and immunological characterization of *Xenorhabdus nematophilus* phase I fimbriae, and we defined the growth conditions inducing their expression. Furthermore, we investigated the agglutinating properties of *X. nematophilus* cells and fimbriae with mammalian erythrocytes and insect hemocytes as well as HA inhibition to define the specificity of interaction between the fimbriae and the blood cells' surfaces.

MATERIALS AND METHODS

Bacterial strain and growth conditions. Phase I variants of *Xenorhabdus nematophilus* F1 (F1/1) were isolated from the nematode *Steinernema carpocapsae* "Plougastel" from Brittany, France. Phase II (F1/2) variants were selected from in vitro cultures of F1/1.

Bacteria were grown on nutrient agar (NA; 1.5% [wt/vol]; Biomérieux) for 48 h at 28°C or were statically subcultured twice (for 72 h each time at 28°C) in Luria broth (LB) and nutrient broth (NB; Institut Pasteur, Paris, France).

For each subculture, phase status was identified by the differential adsorption of dye when the strains were grown on NBTA (NA supplemented with 25 mg of bromothymol blue and 40 mg of triphenyltetrazolium chloride per liter) and by measuring activity against *Micrococcus luteus* (from the Pasteur Culture Collec-

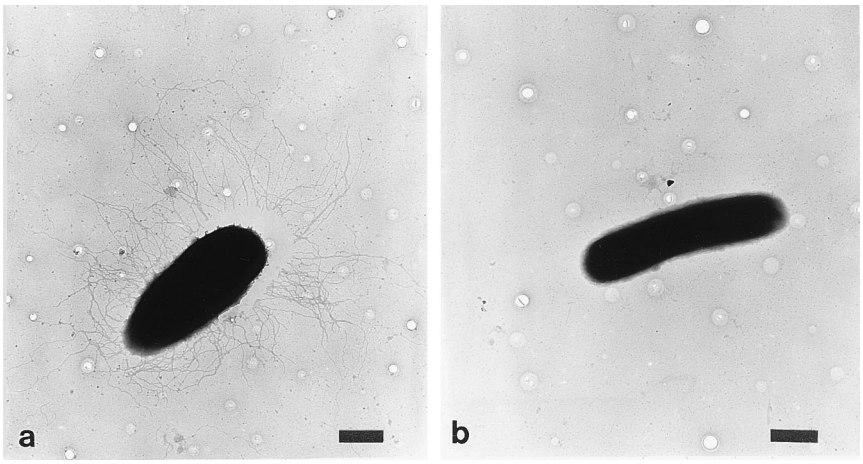

FIG. 1. Transmission electron micrographs (magnification, ×10,000) of a negatively stained (0.5% phosphotungstate) phase I variant of *X. nematophilus* F1 showing fimbriae over the entire surface of the cell (a) and a phase II variant without fimbriae (b). The variants were grown on NA (48 h, 28°C). Bar, 0.5 μm.

tion, Institut Pasteur). Phase I colonies are blue on NBTA and produce agar-diffusible antibiotics, while phase II colonies are red and produce very little or no antibiotic.

Electron microscopy. Whole bacteria applied to collodion-coated 400-mesh grids were fixed with 1.25% glutaraldehyde and 1% paraformaldehyde in 0.015 M sodium cacodylate buffer (pH 7.4; 10 min) and negatively stained with 0.5% phosphotungstate (pH 7). Fimbrial preparations applied to Formvar carbon-coated grids were negatively stained with 1% phosphotungstate. The grids were examined with a JEOL 1200X transmission electron microscope at 70 kV.

Purification of fimbriae. *X. nematophilus* F1/1 cells, from 48-h-old cultures on NBTA plates, were harvested with Ringer's solution and inoculated into 10 large Roux bottles. These bottles contained 150 ml of NA solid medium previously dried to minimize expression of flagella. After a 48-h culture, cells were harvested with 80 ml of 5 mM Tris HCl (pH 8). Fimbriae were unbound from the bacterial cell walls by homogenization with an Ultraturrax homogenizer twice for 5 min each time at 4°C. Cellular debris was removed by centrifugation at 6,000 × *g* (30 min, 4°C), and the supernatant was saturated with 20% ammonium sulfate and incubated overnight at 4°C. After centrifugation at 20,000 × *g* (30 min, 4°C), the pellet was resuspended in 1 ml of 5 mM Tris HCl (pH 8). Fimbriae were separated by sucrose gradient (10 to 60% in 5 mM Tris HCl) centrifugation at 60,300 × *g* (16 h, 15°C) in a Beckman SW28-1 rotor. Fractions of 1 ml each were harvested and dialyzed against 5 mM Tris HCl (pH 8). Proteins were further precipitated with 20 mM Tris HCl (pH 7.5) containing 0.3 M NaCl and 0.2 M $MgCl_2$ overnight at 4°C. After centrifugation (15,000 × *g*, 4°C), fractions were dialyzed as described above.

Fractions were examined by electron microscopy and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions with pure fimbriae were pooled, concentrated by $MgCl_2$ precipitation, and dialyzed as described above.

The protein concentration was estimated by the bicinchoninic acid (Mallet SA; Pierce) method with bovine serum albumin as the standard.

Preparation of membrane extracts. In order to prepare membrane extracts, depiliated bacteria obtained after two successive treatments with the Ultraturrax homogenizer were washed with 5 mM Tris HCl (pH 8) and centrifuged at 5,000 × *g*. The absence of fimbriae was checked by electron microscopy. The pellet was resuspended in sucrose buffer (10 mM EDTA, 100 mM Tris HCl [pH 8], 20% sucrose) containing 100 μg of lysozyme per ml (20 min, 4°C). The spheroplasts obtained were centrifuged at 5,000 × *g*, washed in sucrose buffer, and resuspended in sucrose buffer containing 20 mM $MgCl_2$ and 50 μg of DNase per ml. The spheroplasts were lysed by the addition of ice-cold water followed by four freeze-thaw cycles. Membranes were pelleted by centrifugation at 15,000 × *g*.

Preparation of antipilin antiserum. Specific polyclonal antiserum against the purified and denatured pilin protein was prepared. Pilin bands (about 100 μg) were excised from SDS-PAGE gels, broken in 1 ml of sterile 0.9% saline solution, and emulsified with 1 ml of complete Freund's adjuvant. A rabbit was immunized twice with a 1-week interval at multiple subcutaneous sites with 1 ml of this mixture. One week later, the rabbit was given a booster with the same quantity of proteins in 1 ml of sterile saline solution containing an equal volume of incomplete Freund's adjuvant. After 4 weeks, the rabbit was bled and the presence of antibodies in the serum was tested by Western blotting (immunoblotting).

SDS-PAGE. Fractions containing fimbriae were analyzed by SDS-PAGE by the method described by Laemmli (25). Whole-cell proteins were prepared by freezing and vortexing bacteria in Laemmli buffer. Samples were resuspended in Laemmli buffer in either the presence or absence of 5% β-mercaptoethanol (100°C, 10 min) before electrophoresis on a 12% polyacrylamide gel. Proteins were stained with Coomassie blue R-250 (Sigma), and protein bands were compared with the following molecular mass standards from the low-molecular-mass calibration kit from Pharmacia: phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

Immunological techniques. Western blotting was carried out as described by Towbin et al. (32). Purified fimbriae and whole-cell proteins from F1/1 and F1/2 bacteria separated by SDS-PAGE were electrophoretically transferred to nitrocellulose (BAS85; Schleicher & Schuell). The nitrocellulose sheet was incubated with a 1:200 dilution of antipilin antiserum and then with a 1:1,000 dilution of goat anti-rabbit antibodies conjugated with peroxidase (Sigma). Proteins bands were detected by using 200 μg of 3-amino-9-ethyl-carbazole (Sigma) per ml as the peroxidase substrate.

For immunogold labelling, the immunoglobulin G of the antiserum was purified by chromatography on a cellulose-protein A column (Protein A Memsep 1000; Millipore). F1/1 bacteria applied to collodion-coated grids were incubated in 150 mM phosphate-buffered saline (PBS) with 0.5% bovine serum albumin (15 min) and then with a 1:50 dilution of antipilin immunoglobulin G (final concentration, 2 μg/ml) for 45 min. After being washed with PBS, the grids were exposed to a 1:50 dilution of protein A conjugated with 10-nm-diameter colloidal gold particles (Sigma) (45 min). After another washing, grids were negatively stained with 0.5% phosphotungstate.

Agglutination assays. Bacterial suspensions (10^9 cells per ml), purified fimbriae, or membrane extracts (50 μl) were serially diluted twofold in 96-well microtiter plates in 150 mM PBS (pH 7.2). An equal volume of 2% fresh human blood group (A, B, AB, or O), rabbit, or sheep erythrocytes washed in PBS was added to the wells. Growth medium and PBS were used as controls. The HA titer was defined as the reciprocal of the highest dilution of the samples causing a visible agglutination.

Agglutination assays with insect hemocytes were performed by collecting hemolymph samples from *Galleria mellonella* larvae (fifth stage) in an anticoagulant buffer (4). Hemocytes (2×10^4 cells per mm^3) were centrifuged, rinsed in 150 mM PBS to remove plasmatic factors, and resuspended with bacterial suspensions (10^8 cells per ml). Suspensions (20 μl) were deposited on slides, and incubation was carried out in a hanging drop (20 min, 4°C). Hemocytes with PBS were used as a control. Agglutination between the slide and coverslip was observed with a light microscope.

Inhibition of HA. For HA inhibition assays, F1/1 bacteria or fimbriae, serially twofold diluted, were incubated with nondiluted antipilin antiserum for 1 h at room temperature before 2% erythrocytes were added. Preimmune serum was used as a control. For inhibition of HA by sugars, bacteria or fimbriae were incubated with various sugars and glycoproteins for 2 h before erythrocytes were added at the concentrations mentioned for each experiment. HA by either the bacteria or the fimbriae was used as a positive control.

RESULTS

Morphological study of *X. nematophilus* F1/1 and F1/2. One morphologic type of fimbriae was observed on F1/1 cells when the cells were examined by electron microscopy (Fig. 1a). Numerous thin fimbriae, no more than 3 μm in length, were distributed over the entire bacterial surface.

TABLE 1. Influence of growth conditions on piliation, expression of pilin, and HA of F1/1 and F1/2 bacteria

Strain	Growth medium	Temp, incubation time	Piliation[a]	Western blot result[b]	Sheep HA titer	Rabbit HA titer	Human (A, B, O, or AB) HA titer
F1/1	NA	28°C, 48 h	+++	+	64	64	64
F1/1	NA	28°C, 24 h	++	+	32	32	ND[c]
F1/1	NB	28°C, 72 h (twice)	+	+	16	16	ND
F1/1	LB	28°C, 72 h (twice)	−	−	8	8	ND
F1/2[d]			−	−	<2	<2	<2

[a] Piliation determined by electron microscopy. +++, all bacterial cells (100%) were piliated; ++, all bacterial cells (100%) were piliated but fewer fimbriae were observed on the cell surface; +, few bacterial cells (<5%) were piliated; −, no bacterial cells (<1%) were piliated.

[b] Western blotting analysis was performed with whole-cell proteins. Blots were reacted with antipilin antiserum diluted 1:200. + or −, presence or absence of the 16-kDa pilin, respectively.

[c] ND, not determined.

[d] F1/2 was tested under the same growth conditions as F1/1 (NA, 24 and 48 h; NB and LB, 72 h [twice]).

To determine the influence of growth conditions on the occurrence of fimbriae, bacteria were also examined by electron microscopy (Table 1). All F1/1 bacteria (100%) grown on solid medium (NA) were piliated, but those grown in static broth cultures (NB and LB) were not. F1/2 bacteria grown on solid medium or statically cultured in liquid medium had neither fimbriae nor other filamentous surface components as shown by negative staining (Fig. 1b).

Purification of F1/1 fimbriae. Since electron microscopy showed that the F1/1 bacteria were piliated when cultivated on solid medium, this medium was used to obtain large numbers of fimbriae. Concentrated ultracentrifuge fractions were examined by electron microscopy, revealing that large numbers of fimbriae were accumulated in the fractions containing around 40% sucrose (Fig. 2A). The fimbriae were rigid, with diameters of 6.4 nm ± 0.3 nm. Aggregation of fimbriae was often observed. At this stage, fimbrial preparations appeared to be free of any contaminant visible by electron microscopy. SDS-PAGE of purified fimbriae boiled for 10 min in the presence or absence of 5% β-mercaptoethanol showed a single band with an apparent molecular mass of 16 kDa as determined by Coomassie blue staining (Fig. 2B).

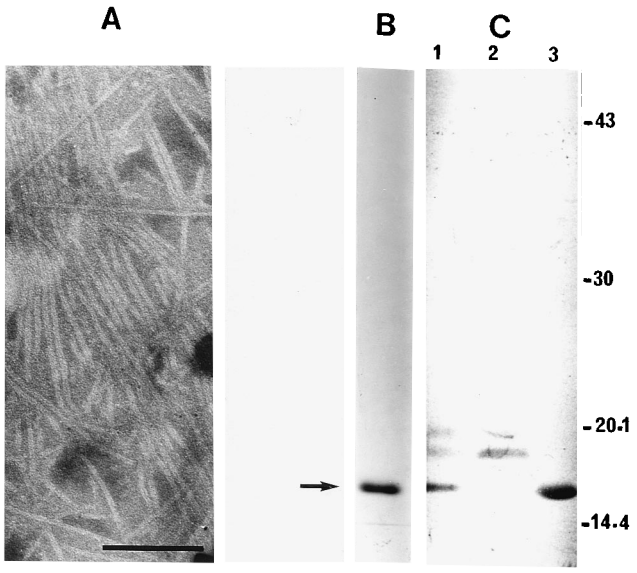

FIG. 2. Purified fimbriae of *X. nematophilus* and Western blot analysis of pilin synthesis of F1 variants. (A) Transmission electron micrograph of negatively stained (1% phosphotungstate) purified fimbriae (magnification, ×37,500). Bar, 0.1 μm. (B) SDS-PAGE (12% polyacrylamide gel) of purified fimbriae (5 μg, with 5% β-mercaptoethanol) stained with Coomassie blue. Standard proteins used here were from Pharmacia (molecular size markers, in kilodaltons, are indicated on the right). (C) Western blot analysis of F1/1 whole-cell proteins (lane 1) and F1/2 whole-cell proteins (lane 2) from NA cultures (48 h, 28°C) and purified fimbriae (lane 3) detected with antipilin antiserum diluted 1:200. The arrow points to the 16-kDa pilin.

Immunological studies. In order to study the expression of pilin by phase I and phase II variants under different growth conditions, a specific rabbit antiserum against the denatured 16-kDa pilin was produced. The reactivity of this antiserum was analyzed by immunogold labelling of F1/1 variants grown on NA (48 h, 28°C) and by Western blotting of whole-cell proteins from F1/1 NA cultures (48 h, 28°C) and of purified fimbriae.

Immunogold labelling of F1/1 variants confirmed the specificity of antipilin antibodies: gold particles bound to the fimbriae but not to the flagella (Fig. 3). This result also demonstrated that antibodies against denatured pilin reacted with native fimbriae. In Western blotting, antipilin antiserum reacted with the 16-kDa pilin of purified fimbriae (Fig. 2C, lane 3) and revealed one 16-kDa band in the lane with F1/1 whole-cell proteins (Fig. 2C, lane 1). This antiserum cross-reacted weakly with two other proteins of about 20 and 19 kDa (Fig. 2C, lane 1); these two bands were also revealed by preimmune serum, while the 16-kDa pilin band was not.

Western blotting with the antipilin antiserum was also performed with whole-cell proteins from phase I and phase II variants grown under different conditions (Table 1). Pilin was detected in whole-cell proteins from 24-h NA cultures and static NB cultures of F1/1 variants, whereas it was absent when the bacteria were statically cultured in LB. Pilin was not detected in protein extracts of depiliated F1/1 bacteria (48 h, 28°C) (data not shown). The pilin was never detected in the F1/2 whole-cell proteins under any growth conditions used (Fig. 2C, lane 2).

Agglutination assays. In order to study the agglutinating properties of *X. nematophilus* fimbriae, whole bacteria were first tested with erythrocytes of different species and insect hemocytes. Phase I bacteria grown on NA strongly agglutinated sheep, rabbit, and human blood type (A, B, AB, or O) erythrocytes with identical HA titers, while the level of HA by F1/1 bacteria statically cultured in liquid medium was much lower. No hemagglutinating activity was observed with F1/2 bacteria (Table 1). A strong agglutination by phase I bacteria (grown on NA) was observed with the different types of *G. mellonella* hemocytes (Fig. 4a). A few free phase I bacteria were observed in the medium (Fig. 4a), and numerous phase I cells adhered to hemocytes (Fig. 4b). Phase II and nonpiliated phase I bacteria did not agglutinate hemocytes (Fig. 4c and d) and remained free in the hanging drop (Fig. 4c and d). The

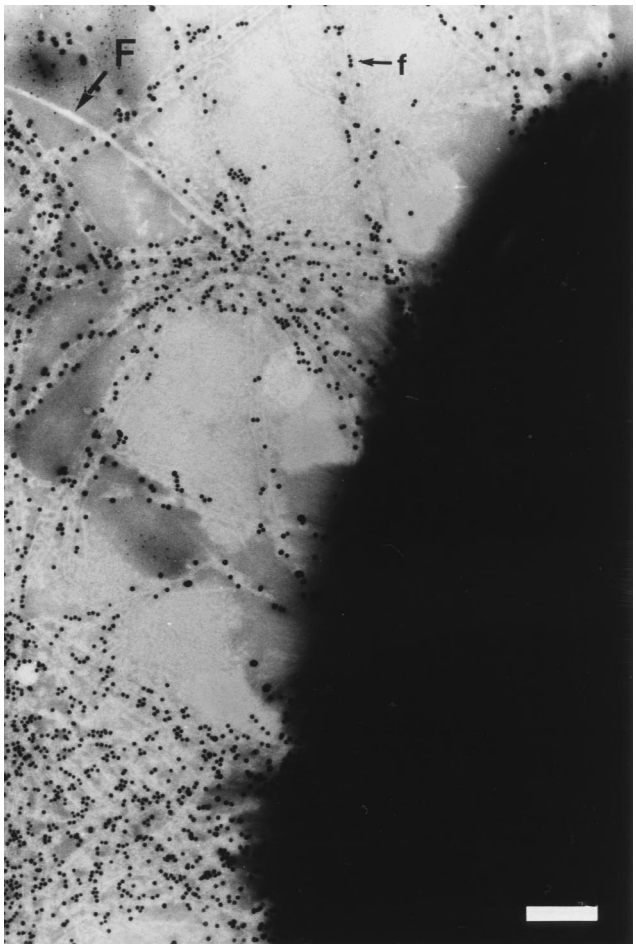

FIG. 3. Transmission electron micrograph (magnification, ×27,900) of F1/1 bacteria immunogold labelled with antipilin immunoglobulin G and protein A conjugated with 10-nm-diameter colloidal gold particles. Bacteria were grown on NA (48 h, 28°C). Bar, 0.17 μm. f, fimbriae; F, flagellum.

weak autoagglutination of hemocytes observed in the presence of phase II and nonpiliated phase I bacteria (Fig. 4c and d) was equal to that of the control with hemocytes in PBS (Fig. 4e).

The hemagglutinating properties of F1/1 bacteria were analyzed by testing sheep and rabbit erythrocytes with the purified fimbriae, the depiliated F1/1 bacteria, and the membrane extracts from depiliated F1/1 bacteria (Table 2). Purified fimbriae agglutinated these erythrocytes when the fimbriae were at concentrations up to 30 μg/ml. The level of HA by depiliated F1/1 bacteria was lower than the level of HA by piliated bacteria; the membrane extracts had no hemagglutinating activity. Incubation of F1/1 bacteria with nondiluted antipilin antiserum, before sheep erythrocytes were added, completely inhibited HA (Table 2). Preimmune serum did not inhibit the HA of F1/1 bacteria.

Inhibition of sheep HA by sugars and glycoproteins. In order to determine the nature of the interaction between *X. nematophilus* fimbriae and the erythrocyte surface, the inhibitory activities of various sugars and glycoproteins on sheep HA were analyzed. Only two compounds significantly inhibited HA of F1/1 bacteria: an inhibition of 50% was observed with *N*-acetyl-lactosamine at 13 mM and with porcine gastric mucin at 0.25 mg/ml (Table 3). These compounds also inhibited HA mediated by purified fimbriae at similar concentrations. Mannose (500 mM) did not inhibit fimbrial HA.

DISCUSSION

X. nematophilus F1 fimbriae were biochemically characterized and the expression of the pilin was analyzed under different growth conditions. Only the phase I variants of *X. nematophilus* F1 express fimbriae when grown on solid medium, as previously described for phase I variants of *X. nematophilus* A24 (5). Negative staining of bacteria showed one type of fimbriae, which were rigid like the enterobacterial type 1 fimbriae or *E. coli* P fimbriae (12, 21). *X. nematophilus* fimbriae were 6 to 7 nm in diameter, as were type 1 or P fimbriae (7-nm diameter). Because of this similarity, the *X. nematophilus* fimbriae were purified according to a protocol previously used for the purification of *E. coli* P fimbriae (22). This method, which involves sucrose gradient ultracentrifugation, allows the separation of fimbriae from other similar structural components expressed by F1/1 bacteria, such as flagella (16) or the spontaneously produced phage tail-like bacteriocins (10). SDS-PAGE analysis showed that fimbriae are mainly composed of 16-kDa subunits. This size is close to those of fimbriae isolated from many bacterial species (12). The subunits were completely dissociated by boiling in the absence of β-mercaptoethanol, suggesting that *X. nematophilus* fimbriae are likely to be composed of noncovalently associated subunits. Although bacterial fimbriae contain cysteine, their subunits are generally associated by hydrophobic interactions (18, 29).

X. nematophilus F1 phase II variants were shown to be unpiliated cells as previously described (11). Pilin subunits were undetectable in F1/2 whole-cell proteins by immunoblot analysis. The loss of flagellation of F1/2 bacteria has also been described previously (15). The absence of pilin (discussed in this report) and flagellin synthesis (15) in F1/2 cells suggests that (i) a common mechanism regulates the genes encoding these proteins and (ii) it is unlikely that a defect in subunit assembly occurs.

Piliation of phase I bacteria varied depending on growth conditions as determined by electron microscopy: piliation was observed for bacteria grown on solid medium for 24 and 48 h, while F1/1 bacteria grown in liquid medium did not possess fimbriae. Immunoblotting analysis of pilin synthesis in bacteria grown on solid medium and the presence of numerous fimbriae at the surfaces of 24-h-old cells suggest that the fimbrial elements were mainly produced and assembled during the first 24 h of culture. Thus, the pattern of the synthesis of *X. nematophilus* fimbriae appears to be similar to the pattern of the synthesis of *E. coli* type 1 fimbriae (13). Pilin was expressed by F1/1 bacteria in NB and not in LB, suggesting that specific nutrients are required for its synthesis.

The agglutinating activity of *X. nematophilus* F1/1 with sheep, rabbit, and human erythrocytes and insect hemocytes was the first demonstration of the adhesive properties of *X. nematophilus* phase I cells. Fimbriae were shown to mediate sheep and rabbit HA. Some carbohydrates were observed to have an inhibitory effect on sheep HA by bacteria and purified fimbriae. Therefore, the fimbrial agglutinin of *X. nematophilus* appears to be a lectin. The agglutinating site of *E. coli* type 1 and P fimbriae has been shown to be localized on an adhesin, distinct from the pilin subunit, at the tip of the filament (19, 24). HA by purified *X. nematophilus* fimbriae requires high concentrations of aggregated proteins like those of type 1 or P fimbriae (26, 30). Incubation of F1/1 fimbriae with nondiluted and diluted antipilin antibodies (1:10 or 1:50) increased the HA (unpublished data); this could be due to cross-linking of fimbriae by antibodies, enhancing their aggregation (30). These results suggest that the agglutinating sites of *X. nematophilus* fimbriae are localized not on the pilin but on a distinct agglutinin subunit. This hypothesis should be confirmed by genetic characterization of the agglutinin and the pilin.

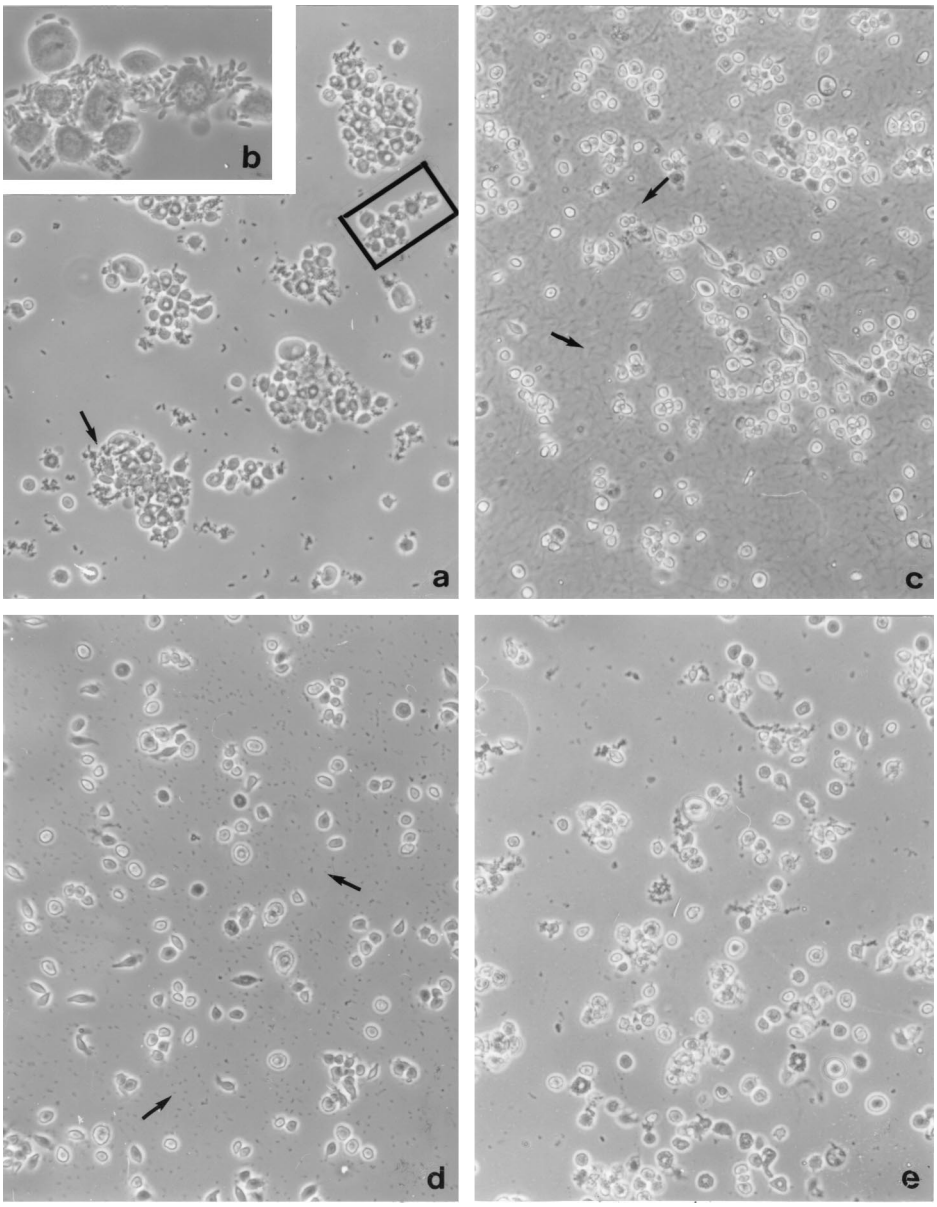

FIG. 4. Light microscopy (magnification, ×100) of agglutination of *G. mellonella* hemocytes by *X. nematophilus*. (a) Piliated F1/1 bacteria grown on NA (48 h, 28°C). The arrow indicates bacteria on hemocytes. (b) High magnification (×400) of the area outlined in black. (c) Nonpiliated F1/1 bacteria statically grown in LB (twice for 72 h each time at 28°C). Arrows indicate free motile bacteria. (d) F1/2 bacteria grown on NA (48 h, 28°C). Arrows indicate free bacteria. (e) Hemocytes in PBS used as a control. A weak autoagglutination similar to that in panels c and d can be observed.

Fimbrial adhesins are generally known to be lectins which mediate mannose-sensitive (type 1 fimbriae) or mannose-resistant HA of bacteria (18). *X. nematophilus* fimbriae are probably different from type 1 fimbriae because they show a mannose-resistant hemagglutinating activity. HA of sheep erythrocytes by F1/1 bacteria and purified fimbriae was inhibited by *N*-acetyl-lactosamine and porcine stomach mucin, demonstrating that the interaction between *X. nematophilus* fimbriae and erythrocytes might be due to binding to an oligosaccharidic complex similar to *N*-acetyl-lactosamine in the erythrocyte membrane. This sugar, as an inhibitor of HA, has not been documented for bacteria and seems to be unique for *X. nematophilus*.

X. nematophilus fimbriae were apparently involved in the agglutination of *G. mellonella* hemocytes by phase I bacteria: strong adhesion between piliated bacteria and hemocyte surfaces was

TABLE 2. HA of different cellular extracts of F1/1 and inhibition of sheep HA by antipilin antiserum

Cellular extract	HA titer		
	In sheep erythrocytes	In rabbit erythrocytes	With undiluted antipilin antiserum in sheep erythrocytes[a]
F1/1 (10^9 cells/ml)	64	64	<2
Depiliated F1/1	16	16	ND[b]
Purified fimbriae (500 μg/ml)	16	16	ND
Membrane extracts[c]	<2	<2	ND

[a] Inhibition was tested only with HA of sheep erythrocytes.
[b] ND, not determined.
[c] Prepared from depiliated F1/1.

TABLE 3. Inhibition of sheep HA of F1/1 bacteria[a] by different sugars and glycoproteins

Sugar or glycoprotein	IC_{50}[b]
D-Glucose	–
D-Galactose	–
L-Fucose	–
D-Mannose	–
L-Rhamnose	–
L-Arabinose	>250 mM
D-Trehalose	–
L-Lactose	–
L-Sorbose	–
D-Saccharose	–
D-Fructose	–
Stachyose	–
Raffinose	–
Melibiose	–
D-Gluconic acid	>250 mM
D-Glucuronic acid	>250 mM
Methyl-α-D-glucoside	–
Methyl-α-D-galactoside	–
D-Glucosamine	–
D-Galactosamine	–
D-Mannosamine	–
Maltose	–
Mannitol	–
N-Acetyl-D-glucosamine	–
N-Acetyl-D-galactosamine	–
N-Acetyl-D-lactosamine	13 mM
N-Acetyl muramic acid	–
N-Acetyl neuraminic acid	–
Fetuin (Sigma)	5 mg/ml
Porcine gastric mucin (Sigma)	0.25 mg/ml

[a] 10^8 cells per ml; agglutination titer before inhibition was 8.
[b] Concentration giving a 50% inhibition. –, no inhibition was detected at a concentration of 500 mM.

observed. Since *X. nematophilus* is known to be an insect pathogen (3), this positive agglutination suggests that fimbriae have an important role in hemolymph colonization by bacteria.

Fimbrial adhesins of pathogenic bacteria have been shown to be involved in pathogenesis by promoting specific adhesion to the host tissues (21, 23). They may also interact with vertebrate immune cells and induce an inflammatory response (27) and phagocytosis of bacteria (17). A study of *X. nematophilus* virulence for insects is in progress in order to investigate the action of fimbriae on insect hemocytes with respect to defense reactions. Moreover, in vivo studies of the *X. nematophilus* F1/1 adhesive properties in the intestinal epithelium of the nematode host should also evidence the role of fimbriae in the specificity of helminthic bacterium symbiosis.

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REFERENCES

1. **Akhurst, R. J.** 1980. Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. J. Gen. Microbiol. **121:**303–309.
2. **Akhurst, R. J.** 1982. Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the families *Heterorhabditidae* and *Steinernematidae*. J. Gen. Microbiol. **128:**3061–3065.
3. **Akhurst, R. J., and N. E. Boemare.** 1990. Biology and taxonomy of *Xenorhabdus*, p. 75–90. *In* R. R. Gaugler and H. K. Kaya (ed.), Entomopathogenic nematodes in biological control. CRC Press, Boca Raton, Fla.
4. **Anggraeni, T., and N. A. Ratcliffe.** 1991. Studies on cell-cell co-operation during phagocytosis by purified haemocyte populations of the wax moth, *Galleria mellonella*. J. Insect Physiol. **37:**453–460.
5. **Binnington, K. C., and L. Brooks.** 1993. Fimbrial attachment of *Xenorhabdus nematophilus* to the intestine of *Steinernema carpocapsae*, p. 147–155. *In* R. Bedding, R. Akhurst, and H. Kaya (ed.), Nematodes and the biological control of insect pests. CSIRO Publications, Melbourne, Australia.
6. **Bird, A. F., and R. J. Akhurst.** 1983. The nature of the intestinal vesicle in nematodes of the family *Steinernematidae*. Int. J. Parasitol. **13:**599–606.
7. **Boemare, N. E., and R. J. Akhurst.** 1988. Biochemical and physiological characterization of colony form variants in *Xenorhabdus* spp. (*Enterobacteriaceae*). J. Gen. Microbiol. **134:**1835–1845.
8. **Boemare, N. E., and R. J. Akhurst.** 1990. Physiology of phase variation in *Xenorhabdus* spp., p. 208–212. *In* Society for Invertebrate Pathology (ed.), Vth International Colloquium on Invertebrate Pathology and Microbial Control. Society for Invertebrate Pathology, Adelaide, Australia.
9. **Boemare, N. E., R. J. Akhurst, and R. G. Mourant.** 1993. DNA relatedness between *Xenorhabdus* spp. (*Enterobacteriaceae*), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. Int. J. Syst. Bacteriol. **43:**249–255.
10. **Boemare, N. E., M.-H. Boyer-Giglio, J.-O. Thaler, R. J. Akhurst, and M. Brehélin.** 1992. Lysogeny and bacteriocinogeny in *Xenorhabdus nematophilus* and other *Xenorhabdus* spp. Appl. Environ. Microbiol. **58:**3032–3037.
11. **Brehélin, M., A. Cherqui, L. Drif, J. Luciani, R. Akhurst, and N. Boemare.** 1993. Ultrastructural study of surface components of *Xenorhabdus* sp. in different cell phases and culture conditions. J. Invertebr. Pathol. **61:**188–191.
12. **Clegg, S., and G. F. Gerlach.** 1987. Enterobacterial fimbriae. J. Bacteriol. **169:**934–938.
13. **Dodd, D. C., and B. I. Eisenstein.** 1984. Kinetic analysis of the synthesis and assembly of type 1 fimbriae of *Escherichia coli*. J. Bacteriol. **160:**227–232.
14. **Gaastra, W., and F. K. de Graaf.** 1982. Host-specific fimbrial adhesins of noninvasive enterotoxigenic *Escherichia coli* strains. Microbiol. Rev. **46:**129–161.
15. **Givaudan, A., S. Baghdiguian, A. Lanois, and N. Boemare.** 1995. Swarming and swimming changes concomitant with phase variation in *Xenorhabdus nematophilus*. Appl. Environ. Microbiol. **61:**1408–1413.
16. **Givaudan, A., and N. Moureaux.** Unpublished data.
17. **Goetz, M. B., S. M. Kuriyama, and F. J. Silverblatt.** 1987. Phagolysosome formation by polymorphonuclear neutrophilic leukocytes after ingestion of *Escherichia coli* that express type 1 pili. J. Infect. Dis. **156:**229–233.
18. **Graaf, F. K. D., and F. R. Mooi.** 1986. The fimbrial adhesins of *Escherichia coli*. Adv. Microb. Physiol. **28:**65–143.
19. **Hanson, M. S., and C. C. Brinton.** 1988. Identification and characterization of *Escherichia coli* type 1 pilus tip adhesion protein. Nature (London) **332:**265–268.
20. **Hultgren, S. J., S. Abraham, M. Caparon, P. Falk, J. W. St. Geme III, and S. Normark.** 1993. Pilus and nonpilus bacterial adhesins: assembly and function in cell recognition. Cell **73:**887–901.
21. **Johnson, J. R.** 1991. Virulence factors in *Escherichia coli* urinary tract infection. Clin. Microbiol. Rev. **4:**80–128.
22. **Korhonen, T. K., E.-L. Nurmiaho, H. Ranta, and C. Svanborg Edén.** 1980. New method for isolation of immunologically pure pili from *Escherichia coli*. Infect. Immun. **27:**569–575.
23. **Krogfelt, K. A.** 1991. Bacterial adhesion: genetics, biogenesis and role in pathogenesis of fimbrial adhesins of *Escherichia coli*. Rev. Infect. Dis. **13:**721–735.
24. **Kuehn, M. T., J. Heuser, S. Normark, and S. J. Hultgren.** 1992. P pili in uropathogenic *E. coli* are composite fibres with distinct fibrillar adhesive tips. Nature (London) **356:**252–255.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
26. **Lindberg, F. P., B. Lund, and S. Normark.** 1984. Genes of pyelonephritogenic *E. coli* required for digalactoside-specific agglutination of human cells. EMBO J. **3:**1167–1173.
27. **Linder, H., I. Engberg, I. M. Baltzer, K. Jann, and C. Svanborg-Edén.** 1988. Induction of inflammation by *Escherichia coli* on the mucosal level: requirement for adherence and endotoxin. Infect. Immun. **56:**1309–1313.
28. **Massad, G., C. V. Lockatell, D. E. Johnson, and H. L. T. Mobley.** 1994. *Proteus mirabilis* fimbriae: construction of an isogenic *pmfA* mutant and analysis of virulence in a CBA mouse model of ascending urinary tract infection. Infect. Immun. **62:**536–542.
29. **Paranchych, W., and L. S. Frost.** 1988. The physiology and biochemistry of pili. Adv. Microb. Physiol. **29:**53–114.
30. **Sharon, N., and I. Ofek.** 1986. Mannose specific bacterial surface lectins, p. 55–81. *In* D. Mirelman (ed.), Microbial lectins and agglutinins. John Wiley and Sons, New York.
31. **Thomas, G. M., and G. O. Poinar, Jr.** 1979. *Xenorhabdus* gen. nov., a genus of entomopathogenic, nematophilic bacteria of the family *Enterobacteriaceae*. Int. J. Syst. Bacteriol. **29:**352–360.
32. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.