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NOTES

Purification and Characterization of *Eikenella corrodens* Type IV Pilin

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***Eikenella corrodens* is a gram-negative human pathogen associated with periodontal diseases and soft-tissue infections. Pilin was purified by association-dissociation and fast protein liquid chromatography; it had an apparent molecular mass of about 14.8 kDa and an N-terminal amino acid sequence reflective of type IV pilins. Antibodies to the purified protein reacted with pili on whole cells. This is the first report of purification of type IV pili/pilin from this organism. Other type IV pili are important virulence factors; we are currently investigating the biological role of pili in *E. corrodens*.**

Eikenella corrodens is a gram-negative, rod-shaped, facultative anaerobe which inhabits human mucosal surfaces, particularly in the oral cavity. It has been linked to a variety of disease states (7) including abscesses (11, 24, 35), endocarditis (10, 11, 17), meningitis (4), osteomyelitis (14), keratitis (22), conjunctivitis (35), and cellulitis (9, 15, 33). In addition, it appears that *E. corrodens* also plays a significant role in some periodontal diseases (6, 12, 25, 27, 32, 35). We are interested in genes and gene products that may contribute to the virulence of *E. corrodens*.

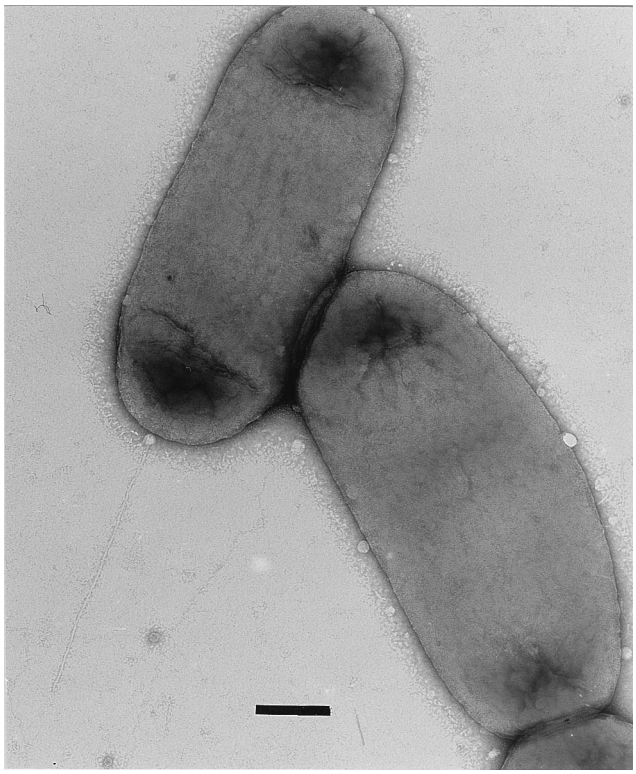

FIG. 1. Electron micrograph of negatively stained *E. corrodens* VA1-2S3 cells showing the presence of pili. Bar = 200 nm.

Piliation has long been recognized as an important factor in bacterial pathogenicity. Early electron microscopic studies appeared to show pili in *E. corrodens* (20); however, other investigators suggested that pili were not present (13, 30). Recently, Rao and Progulske-Fox (31) and Tonjum et al. (37) reported the cloning and sequencing of two type IV pilin genes from *E. corrodens* ATCC 23834 and two from *E. corrodens* 31745, respectively, but neither study examined expression of pilin genes. Here we report the first purification and characterization of type IV pilin from *E. corrodens*, based on protein purification and amino acid sequence data. In addition, we have used immunogold electron microscopy to reveal the presence of pili that react with type IV antipilin antibody.

For this study, we used the clinical isolate *E. corrodens* VA1, variant 2S3. The original isolate, VA1, was obtained from the wound of a patient at the VA Medical Center in Kansas City, Mo. The variant 2S3 forms small, opaque, corroding colonies on solid media (8).

Cells were stained with 1% uranyl acetate and examined for

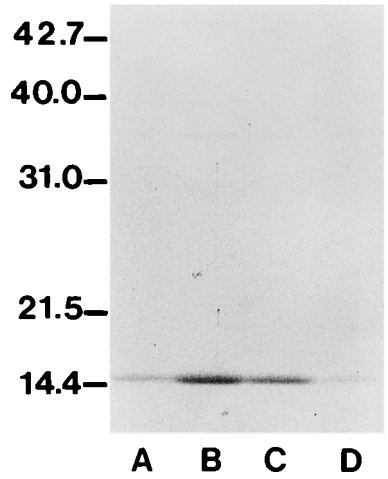

FIG. 2. SDS-PAGE analysis of FPLC fractions containing pilin. The gel was stained with Coomassie blue. Lanes A through D represent samples from fractions constituting the void volume of the column.

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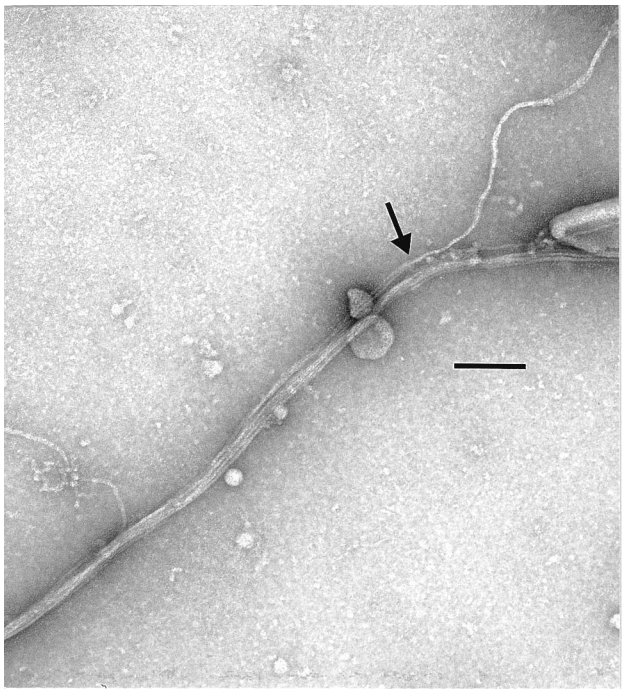

FIG. 3. Electron micrograph of negatively stained reassembled pilin after FPLC purification. Bar = 100 nm. The arrow indicates a single filament.

the presence of pili with a JEOL 1200 transmission electron microscope. Negatively stained VA1-2S3 cells had surface appendages that appeared to be pili (Fig. 1). The structures were 6.5 to 7.5 nm in diameter and varied considerably in length. Generally, one or only a few pili per cell were observed.

Pilin purification was achieved by a modification of published procedures that have been used to purify type IV pilin from *Neisseria gonorrhoeae* (5, 28). Overnight surface growth from 50 chocolate agar plates was scraped into about 20 ml of 150 mM ethanolamine buffer, pH 10.5 (EA buffer). The pili were sheared from the cells by using an Omnimixer at setting 5 for 2 min; cellular debris was removed by centrifugation at 10,000 × *g* for 10 min. Pilin in the supernatant was then precipitated by adjusting the mixture to 10% ammonium sulfate and rocking gently for 4 h at 4°C. The precipitated pilin was then pelleted by centrifugation at 10,000 × *g* for 30 min. The pellet was resuspended in 4 ml of EA buffer, and residual ammonium sulfate was removed by overnight dialysis against 2 liters of the same buffer. Finally, the pilin solution was centrifuged at 10,000 × *g* for 10 min to pellet nonpilin particulates. The resulting pilin supernatant was subjected to a second cycle of ammonium sulfate precipitation, centrifugation, dialysis, and centrifugation.

The partially purified pilin was applied to a Sephadex 75 size exclusion fast protein liquid chromatography (FPLC) column (Pharmacia, Piscataway, N.J.) and eluted with EA buffer. The pilin eluted in the void volume due to its tendency to aggregate. A sample of the pilin from the FPLC void volume fractions was analyzed on a 5 to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gradient gel, using standard reducing buffer conditions (23); the major band, presumed to be pilin, ran at approximately 14.8 kDa (Fig. 2). Only very minor contaminating protein bands were observed. Approximately 1 to 2 mg of essentially pure pilin was obtained from a 50-plate preparation. An electron micrograph of the purified material is shown in Fig. 3.

To confirm that the purified protein was pilin and to determine that it was type IV, the 14.8-kDa band was electrophoretically transferred to a polyvinylidene difluoride membrane and the N-terminal region was sequenced (26) with an Applied Biosystems Sequencer (Applied Biosystems, Foster City, Calif.). Results from sequencing the first 32 amino acids are shown in Fig. 4, along with several other type IV pilin sequences. The first amino acid residue could not be identified by amino acid analysis, but it was identified as phenylalanine (Phe) by examination of the translated DNA sequence from strain VA1-2S3 (19). We assume that modification of Phe to *N*-methyl-phenylalanine occurs in *E. corrodens* pilin as it does in other type IV pilins; this would explain the failure to identify this residue by standard amino acid analysis procedures. Residue 31 was identified as alanine from the DNA sequence since the amino acid analysis was not conclusive.

As expected, there was a strong similarity between the amino acid sequence of our purified protein and the amino

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EcpE  : F T L I E L M I V I   A I I G I L A A I A   L P A Y Q D Y T A R   A Q
EcpA  : F . . . . . . . . .   . . . . . . . . . .   . . L . . . . I S K   S .  (84)
EcpB  : F . . . . . . . . .   . . . . . . . . . .   . . L . . . H M . K   . .  (84)
EcpC  : F . . . . . . . . .   . . . . . . . . . .   . . . . . . . V . .   S .  (94)
EcpD  : F . . . . . . . . .   . . . . . . . . . .   . . . . . S . . . .   . .  (97)
PilA  : F . . . . . . . . V   . . . . . . . . . .   I . Q . . N . V . .   S E  (78)
TfpQ  : F . . . . . . . . .   . . . . . . . . . .   . . . . . . . I S K   S .  (88)
TfpA  : F . . . . . . . . .   . . . . . . . . . .   . . . . . . . I . .   . .  (97)
FimA  : F . . . . . . . . V   . . . . . . . . F .   I . . . N . . I . .   S .  (81)
PilE1 : F . . . . . . . . .   . . V . . . . . V .   . . . . . . . . . .   . .  (94)
PilE2 : F . . . . . . . . .   . . V . . . . . V .   . . . . . . . . . .   . .  (94)
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FIG. 4. N-terminal amino acid sequences of type IV pilins. EcpE is *E. corrodens* VA1-2S3 (this study); EcpA is the translated sequence of *ecpA* from *E. corrodens* ATCC 23834 (31); EcpB is the translated sequence *ecpB* of *E. corrodens* ATCC 23834 (31); EcpC is the translated sequence of *ecpC* of *E. corrodens* 31745 (37); EcpD is the translated sequence of *ecpD* of *E. corrodens* 31745 (37); PilA is from *Pseudomonas aeruginosa* PAK (21); TfpQ is from *Mycobacterium bovis* EPP63 (16); TfpA is from *M. nonliquefaciens* NCTC 7784 (36); FimA is from *Dichelobacter nodosus* AC6 (3); PilE1 is from *N. gonorrhoeae* MS11 (2); and PilE2 is from *Neisseria meningitidis* C311 (29). The number in parentheses at the end of each sequence is the percent similarity to EcpE.

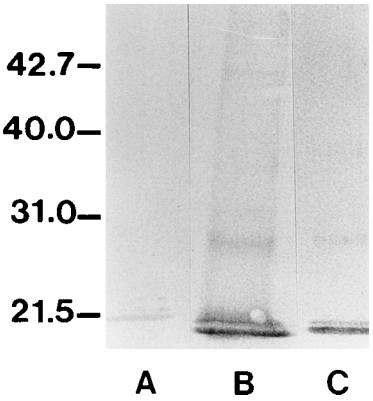

FIG. 5. Immunoblots of partially purified pilin reacted with polyclonal antiserum against *E. corrodens* VA1-2S3 pilin. Lanes: A, preimmune serum; B, rabbit antipilin serum; C, rabbit antipilin serum after absorption with an acetone powder of depiliated *E. corrodens* VA1-2S3.

acid sequences predicted from the nucleotide sequence data of related *E. corrodens* pilin genes. The similarity ranged from 84 to 97%. *E. corrodens* VA1 pilin was more similar to pilins from *E. corrodens* 31745 than to *E. corrodens* ATCC 23834 pilins. Furthermore, there was great similarity between our protein and the type IV pilins of several other organisms, 78 to 97% in the cases examined, with the similarity to *Moraxella nonliquefaciens* being greatest.

Polyclonal rabbit antiserum against *E. corrodens* pilin was produced commercially by Atlantic Antibodies, Windham, Mass., using pilin protein which had been purified by four cycles of association-dissociation. A primary immunization with about 50 μg of pilin was followed at 2-week intervals with two additional immunizations. After 56 days, strongly reactive sera were obtained, as determined by enzyme-linked immunosorbent assay (data not shown). Immunoblots (1) (Fig. 5, lane B) using this polyclonal antiserum revealed a strongly reactive pilin band at 14.8 kDa and several less prominent reactive bands probably due to antibodies against minor protein contaminants in the pilin preparation used as antigen. Silver-stained gels of the pilin antigen preparation showed that lipopolysaccharides were present. Consequently, an acetone powder of depiliated *E. corrodens* VA1-2S3 was used to absorb non-pilin-specific antibodies in the serum (18). The absorbed serum yielded blots with little background and high specificity for pilin (Fig. 5, lane C). Immunogold electron microscopy (34) using the absorbed serum (Fig. 6) showed that it reacted with the pili present on VA1-2S3 cells and confirmed that they were composed of type IV pilin.

The results of this study show conclusively that *E. corrodens* VA1 has the capacity to make pili and, as was suggested by the sequences of cloned pilin genes (19, 31, 37), the pili are composed of type IV pilin. This has been documented here by electron microscopy and protein purification. The production of type IV pili in other *E. corrodens* strains has also been documented (data not shown). The function of these pili, particularly their possible role in pathogenesis, remains to be determined. Regulation of pilin gene expression and the significance of two pilin genes in this organism are currently under investigation in this laboratory and others.

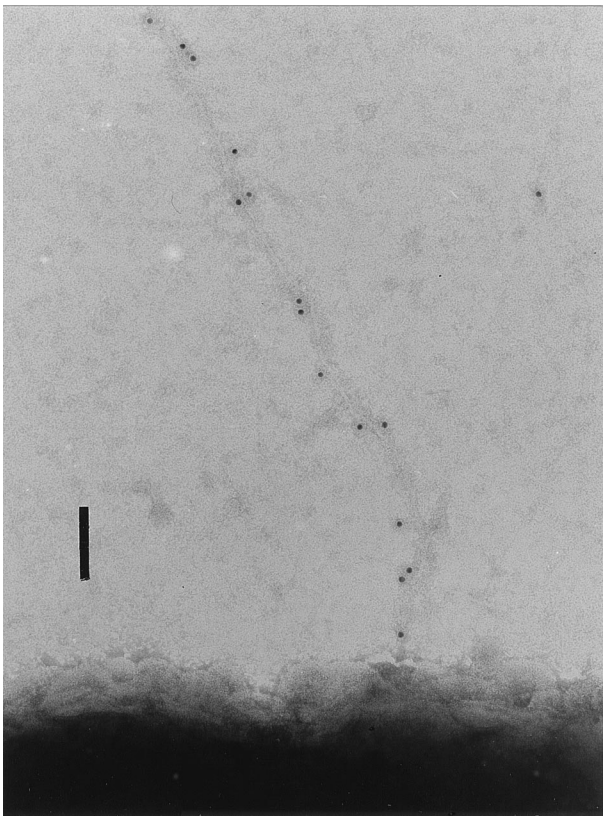

FIG. 6. Immunogold electron micrograph of an *E. corrodens* VA1-2S3 pilus visualized with antipilin serum. Bar = 100 nm.

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