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Eikenella corrodens Phase Variation Involves a Posttranslational Event in Pilus Formation

MARIA T. VILLAR, JENNIFER T. HELBER, BECKY HOOD, MICHAEL R. SCHAEFER,* AND RONA L. HIRSCHBERG†

Division of Molecular Biology and Biochemistry, School of Biological Sciences, University of Missouri—Kansas City, Kansas City, Missouri 64110

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The human pathogen *Eikenella corrodens* synthesizes type IV pili and exhibits a phase variation involving the irreversible transition from piliated to nonpiliated variants. On solid medium, piliated variants form small (S-phase), corroding colonies whereas nonpiliated variants form large (L-phase), noncorroding colonies. We are studying the molecular basis of this phase variation in the clinical isolate *E. corrodens* VA1. A genomic fragment encoding the major type IV pilin was cloned from the S-phase variant of strain VA1. Sequence analysis of the fragment revealed four tandemly arranged potential open reading frames (ORFs), designated *pilA1*, *pilA2*, *pilB*, and *hagA*. Both *pilA1* and *pilA2* predict a type IV pilin. The protein predicted by *pilB* shares sequence identity with the *Dichelobacter nodosus* FimB fimbrial assembly protein. The protein predicted by *hagA* resembles a hemagglutinin. The region containing these four ORFs was designated the *pilA* locus. DNA hybridization and sequence analysis showed that the *pilA* locus of an L-phase variant of strain VA1 was identical to that of the S-phase variant. An abundant *pilA1* transcript initiating upstream of *pilA1* and terminating at a predicted hairpin structure between *pilA1* and *pilA2* was detected by several assays, as was a less abundant read-through transcript encompassing *pilA1*, *pilA2*, and *pilB*. Transcription from the *pilA* locus was nearly indistinguishable between S- and L-phase variants. Electron microscopy and immunochemical analysis showed that S-phase variants synthesize, export, and assemble pilin into pili. In contrast, L-phase variants synthesize pilin but do not export and assemble it into pili. These data suggest that a posttranslational event, possibly involving an alteration in pilin export and assembly, is responsible for phase variation in *E. corrodens*.

Eikenella corrodens is a gram-negative human pathogen (10) that can cause endocarditis (6, 16), a variety of soft tissue and wound infections (10, 15, 20, 30), and other opportunistic infections. This bacterium has also been associated with periodontal diseases (4, 33, 37), although a causal role has not been clearly established. Despite increasing recognition of its role in disease, as reflected in growing numbers of case studies and clinical reports, little is known about the molecular factors that contribute to *E. corrodens* pathogenicity and virulence.

Most strains of *E. corrodens* exhibit an irreversible phase variation that is reflected in colony morphology changes. On solid medium, small (S-phase), so-called corroding, and large (L-phase), so-called noncorroding colonies are observed (11, 23, 28, 46). The L-phase variants arise from S-phase variants at a frequency much greater than mutation rates (24). Colony morphology and phase variation correlates with the presence of pili on S-phase variants and the absence of pili on L-phase variants (22, 23). In terms of piliation, phase variation in *E. corrodens* resembles the phase variation exhibited by pathogens such as *Neisseria gonorrhoeae* (7, 36, 50, 51) and *Moraxella bovis* (18, 34). In the latter species, the phase variation typically involves genomic recombination or mutagenic events that directly affect pilin synthesis or pilus assembly. Given that pili can be determinants of pathogenesis (1, 8, 31, 47), and that modulation of piliation may represent a mechanism to evade host immune response (9, 45, 49), the molecular basis of phase/antigenic variation is of considerable interest.

The pathogens mentioned above, as well as other gram-negative bacterial species, synthesize type IV pili (1, 14, 47). These pili are composed primarily of type IV pilin, a protein which ranges from 150 to 165 amino acids in length. Type IV pilin is synthesized as a precursor form (prepilin) that contains a basic leader sequence of variable length (4 to 25 residues). Following translation, the prepilin is cleaved at an atypical site by a cognate peptidase that simultaneously methylates the resultant amino-terminal amino acid, which is typically a phenylalanine residue. Following processing, the mature pilin is exported to the cell surface by a specific transport mechanism and assembled into pili. Class A type IV pilins from different species share a highly conserved, 30- to 32-residue hydrophobic amino-terminal domain that functions in protein-protein interactions during pilus assembly (47). The remainder of the mature pilin protein is less conserved and contains the major antigenic determinants.

We are examining the molecular basis of the pilus-associated phase variation exhibited by *E. corrodens*. Recently, we characterized the major pilus protein of clinical isolate strain VA1 and confirmed that the observed pili were type IV (27). Although genes encoding type IV pilin have also been cloned from two other *E. corrodens* strains (39, 53), their expression and potential role in phase variation have not been defined. In this report, we document the structure and expression of the major type IV pilin gene in S- and L-phase variants of strain VA1. Analyses of pilin gene expression and pilin processing suggest that a posttranslational event, possibly involving an

* Corresponding author. Mailing address: University of Missouri—Kansas City, School of Biological Sciences, 5100 Rockhill Road, Kansas City, MO 64110. Phone: (816) 235-2573. Fax: (816) 235-5595. E-mail: schaeferm@umkc.edu.

† Present address: Center for Scientific Review, National Institutes of Health, Bethesda, MD 20892.

TABLE 1. Strains and plasmids used

Strain or plasmid	Description	Source or reference
Strains		
E. corrodens		
VA1	Clinical isolate	Laboratory collection
VA1-S1, -S2, and -S3	S-phase variants of VA1	Laboratory collection
VA1-L2	L-phase variant of VA1	Laboratory collection
E. coli		
DH5α	F^- φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44* λ^- *thi-1 gyrA96 relA1*	Bethesda Research Laboratories
BL21(DE3)	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	Novagen
Plasmids		
pGEM3zf(+)	Ap^r cloning and sequencing vector	Promega
pET-22b	Ap^r expression vector	Novagen
pVD203	Carries *pilE1* gene from *N. gonorrhoeae*	7
pEC114	pGEM3zf(+) with 3.9-kbp *Eco*RI fragment from VA1-S1 containing *pilA* locus	This study
pEC203	pBluescript SK (+) with 0.6-kbp *Eco*RI fragment from VA1-S1 containing *pilA1* sequences, used to generate *pilA1*-specific RNA probes	This study
pEC205	pGEM3zf(+) with 0.9-kbp *Pvu*II fragment from VA1-S1 containing mostly *pilA2* sequences, used to generate *pilA2*-specific RNA probes	This study
pEC206	pET-22b with DNA fragment from VA1-S1 encoding residues 26–146 of PilA1	This study

alteration in pilin export and/or assembly, is responsible for phase variation in *E. corrodens*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains and plasmids used in this study are listed in Table 1. *E. corrodens* VA1 is a clinical isolate obtained from the Veterans Administration Medical Center, Kansas City, Mo. (12). Strain VA1 forms both S- and L-phase colonies on solid medium. Strains VA1-S1, VA1-S2, and VA1-S3 are independent S-phase isolates of strain VA1 that form S-phase colonies and exhibit typical frequencies of phase variation to L-phase colonies. Strain VA1-L2 is an L-phase isolate of VA1 that forms only large colonies. *E. corrodens* was cultured aerobically at 35°C on chocolate agar plates purchased from Remel (Lenexa, Kans.).

Escherichia coli DH5α was used as the host for general cloning vectors. *E. coli* BL21(D3) was used as the host for pET-22b-based protein expression vectors. Both strains were propagated in liquid or in solid Luria-Bertani medium with antibiotics at standard concentrations (5).

DNA methods. Restriction endonucleases and modifying enzymes were purchased from Promega (Madison, Wis.). [α-^{32}P]dCTP was purchased from ICN (Costa Mesa, Calif.). [γ-^{32}P]ATP and [^{35}S]dATPαS were purchased from Amersham (Arlington Heights, Ill.). DNA manipulations, including restriction digests, agarose gel electrophoresis, ligations, PCR amplifications, transformation of *E. coli*, and plasmid minipreparations, were performed by established protocols (5, 43). *E. corrodens* genomic DNA was prepared by the procedure described for *E. coli* in reference 43 or with a kit from Qiagen (Chatsworth, Calif.). For DNA hybridization analysis, digested DNA was transferred to a charged nylon membrane (Hybond-N$^+$; Amersham) by the method of Reed and Mann (41). A DNA probe encompassing the *pilA* locus of strain VA1 (3.9-kbp *Eco*RI fragment from plasmid pEC114) was generated from the gel-purified fragment by random-primer labeling with a kit from Promega. DNA hybridizations were performed at 58°C as described by Sambrook et al. (43).

Cloning and sequencing of the *pilA* locus. Total DNA from strain VA1-S1 was partially digested with *Sau*3A, and the digestion products were size fractionated by NaCl gradient centrifugation and purified. To generate a subgenomic library, DNA fragments in the range of 15 to 20 kbp were ligated into the *Bam*HI site of phage λGEM11. The phage library was screened by hybridization to a probe for the *pilE1* gene (1.4-kbp *Sma*I fragment from plasmid pVD203) from *N. gonorrhoeae* MS11 (7), which was kindly provided by M. Koomey. One strongly hybridizing clone was identified, isolated, and designated EP101. Mapping and DNA sequence analyses showed that a terminal 0.6-kbp *Eco*RI-*Xho*I fragment from the EP101 genomic insert contained sequences predicting a type IV pilin-like gene. In a genomic DNA hybridization analysis, the 0.6-kbp fragment identified a single 3.9-kbp *Eco*RI fragment of strain VA1-S1 total DNA. To clone the latter fragment, genomic DNA from strain VA1-S1 was digested with *Eco*RI and size fractionated on an agarose gel. Fragments in the range of 3.9 kbp were eluted from the gel and ligated into the *Eco*RI site of vector pGEM-3Zf(−) (Promega), and the ligation products were used to transform *E. coli* DH5α. The transformants were screened for plasmids containing pilin-like sequences by hybridization against the 0.6-kbp *Eco*RI-*Xho*I fragment from EP101, using a colony screening method (5). Plasmid pEC114, which hybridized to the 0.6-kbp probe and contained the predicted 3.9-kbp insert, was chosen for further analysis.

DNA sequence analysis. Double-stranded DNA sequencing templates were isolated and purified with a kit from Promega. Double-stranded sequencing of the 3.9-kbp *Eco*RI fragment of pEC114 was performed by the dideoxynucleotide chain termination method (44), using both manual and automated procedures. Manual sequencing was performed with Sequenase version 2.0 modified T7 DNA polymerase purchased from United States Biochemical (Cleveland, Ohio). Automated sequencing was performed on an Applied Biosystems (Foster City, Calif.) model 377 sequencer with *Ampli*Taq DNA polymerase. Sequencing reactions were primed with M13 universal primers or oligonucleotides synthesized on an Applied Biosystems model 381A oligonucleotide synthesizer. A portion of the *pilA* locus of strain VA1-L2 was sequenced from a PCR amplification product obtained by using primers 105-R3 (5′-GCCAGCTATTGCAGAATA-3′) and 107-R7 (5′-TGCACCACTTCAAACCG-3′), corresponding to sequences determined from pEC114. DNA and protein sequences were analyzed and compared with sequences in the GenBank database by using MacVector (Oxford Molecular Group, Campbell, Calif.) and BLAST (2) sequence analysis programs.

RNA methods. Total RNA was isolated from S- and L-phase variants by using an RNeasy kit (Qiagen). Contaminating DNA was digested with RNase-free DNase, and the RNA was further purified by passage through an RNeasy column. For hybridization analysis, RNA samples were denatured, separated by electrophoresis on 1.2% agarose gels (43), and transferred to Hybond-N$^+$ according to the manufacturer's instructions. The blots were hybridized with radioactive sense or antisense transcripts specific for *pilA1* (generated from pEC203) or *pilA2* (generated from pEC205), using the Riboprobe system (Promega). RNA hybridizations were performed at 42°C as described by Ausubel et al. (5).

Reverse transcriptase PCR. Single-stranded cDNA was synthesized from total RNA with an avian myeloblastosis virus reverse transcriptase system (Promega), using oligo(dT) or random primers. A negative control without reverse transcriptase was performed. An aliquot of the reaction volume was amplified by PCR using *Taq* polymerase (Promega) and the *pilA* locus-specific primers 105-R1 (5′-TGTTATCGCCATTATCGG-3′), 107-R3B (5′-AAATCCCTCAACGCTTGG-3′), 107-F3 (5′-AGAAGCAACTCGCTTTACCC-3′), RH-2 (5′-GGCAACTTGATGGCAAATATCCTAC-3′), 107-R6 (5′-TGTAAAGGGTTTGGGTGCC-3′), RH-3 (5′-GGCACCCAAACCCTTTACAAG-3′), and 107-R7 (5′-TGGACCACTTCAAACCG-3′). The PCR amplification products were transferred to Hybond-N$^+$ membranes and hybridized to antisense RNA probes for *pilA1* and *pilA2* as described above.

Primer extension. cDNA was synthesized with an avian myeloblastosis virus reverse transcriptase primer extension system (Promega) using primer 105-F1 (5′-CAAAATACCGATAATGG-3′; complementary to nucleotides 538 to 555 at the 5′ end of *pilA1*), which was end labeled with [γ-^{32}P]ATP by using T4

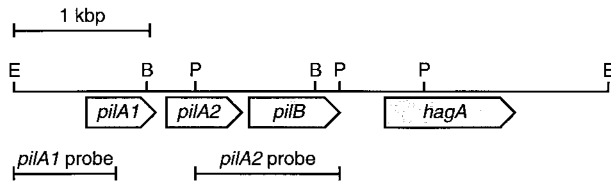

FIG. 1. Physical map of the *pilA* locus for *E. corrodens* VA1-S1. Shaded boxes indicate size and orientation of ORFs as determined by sequence analysis. Flanking and internal restriction sites are shown for enzymes used in cloning and generation of probes. Labeled horizontal bars below map identify regions that correspond to probes used in DNA and RNA hybridization analyses. B, *Bgl*II; E, *Eco*RI; P, *Pvu*II.

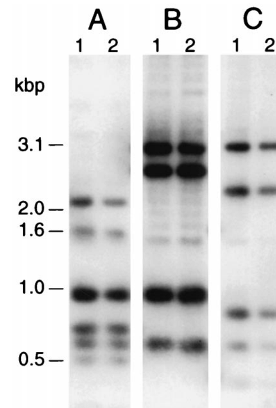

FIG. 2. DNA hybridization analysis of *pilA* locus structure for S- and L-phase variants of *E. corrodens* VA1. Total DNA (4 μg per lane) was isolated from strain VA1-S1 (lanes 1) or VA1-L2 (lanes 2), digested with *Pvu*II and *Bgl*II (A), *Pvu*II (B), or *Pvu*II and *Dra*I (C), and subjected to blot hybridization against a DNA probe for the *pilA* locus of strain VA1-S1. The positions of DNA molecular size standards are shown at the left.

polynucleotide kinase (5). The primer extension products were analyzed against a sequencing ladder on a denaturing polyacrylamide (8% acrylamide) gel.

Cell fractionation. Cell fractionation was performed essentially as described for *E. coli* by Studier et al. (48). Cells from plates were suspended in 0.1 ml of buffer (50 mM Tris [pH 8.0]), vigorously mixed in a vortex mixer for 30 s to shear pili, and then pelleted by centrifugation at 12,000 × *g*. The supernatant (surface fraction) contained proteins, including pilin, that are bound and/or loosely associated with the cell surface. To prepare the soluble and insoluble fractions, the pelleted cells were resuspended in 0.1 ml of buffer, brought to 2 mM EDTA, 0.1 mg of lysozyme per ml, and 0.1% Triton-X100, and incubated for 15 min at 30°C. The suspension was then sonicated (5-s pulse interval) for 1 min on ice and centrifuged at 12,000 × *g*. The resultant supernatant (soluble protein fraction) and pellet (insoluble protein fraction) were isolated for analysis. All protein fractions were mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and stored at −20°C.

The periplasmic fraction was prepared as described by Ames et al. (3). Pelleted cells were resuspended in 0.1 ml of a solution containing 30 mM Tris-Cl (pH 8.0), 20% sucrose, and 1 mM EDTA. After the suspension was shaken for 10 min at room temperature, 0.1 ml of ice-cold 5 mM $MgSO_4$ was added and the suspension was shaken for 10 min at 4°C. The periplasmic fraction was isolated as the supernatant following centrifugation of the suspensions at 12,000 × *g* for 10 min.

Production of PilA1 antisera. The region of *pilA1* encoding residues 26 to 146 of mature PilA1 was amplified from pEC114 by PCR using the following primers to produce flanking *Nco*I and *Xho*I sites: RH-4 (5′-GGCTTCCATGGACTAC ACTGCCCGTGCT-3′) and RH-6 (5′-CTAACTCGAGTTGGCAGCTAGTTG GCAGACGG-3′). The PCR product was digested with *Nco*I and *Xho*I and ligated into expression vector pET-22b. The resulting plasmid, designated pEC206, provides for expression of a truncated PilA1 protein containing the added sequence LEH_6, at the carboxyl terminus. Plasmid pEC206 was used to transform *E. coli* BL21(D3), and the His-tagged recombinant PilA1 protein was expressed, isolated, and purified by Ni-nitrilotriacetic acid (Qiagen) chromatography according to the manufacturer's instructions. A rabbit polyclonal antiserum was raised against the purified PilA1 protein by Atlantic Antibodies (Windham, Maine).

SDS-PAGE and immunoblot analysis. Protein samples were separated by SDS-PAGE on 20% polyacrylamide gels. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (Nitrobind; Micron Separations Inc., Westborough, Mass.) as described by Ausubel et al. (5). The blots were blocked and incubated with the PilA1 antiserum (1:5,000). Bound antibodies were visualized following incubation of the blots with goat anti-rabbit immunoglobulin G (1:5,000) conjugated to alkaline phosphatase (KLC Laboratories, Gaithersburg, Md.) according to the manufacturer's instructions.

Electron microscopy. Negative staining and immunogold electron microscopic examination of whole cells were performed as described (27), using a polyclonal antiserum (1:1,000) prepared against pilin purified from strain VA1-S3.

Nucleotide sequence accession number. The complete DNA sequence of the *pilA* locus has been deposited in the GenBank database under accession no. AF079304.

RESULTS

Sequence analysis of the *pilA* locus. Plasmid pEC114 contains the 3.9-kbp *Eco*RI fragment of strain VA1-S1 genomic DNA that was identified by DNA hybridization against the *pilE1* gene from *N. gonorhoeae* MS11. DNA sequence analysis revealed four potential open reading frames (ORFs) arranged in tandem on the pEC114 insert (Fig. 1). The first ORF (nucleotide positions 484 to 943) and second ORF (nucleotide positions 1013 to 1460) predict proteins of 153 and 149 residues, respectively. A BLAST search of the GenBank database showed that both ORFs predict obvious type IV pilins. On the basis of this identity, we designated the first ORF *pilA1* and the second ORF *pilA2*. These and the subsequent gene designations are consistent with recommended bacterial gene nomenclature (17). The predicted PilA1 and PilA2 proteins respectively contain eight- and six-residue amino-terminal leader sequences upstream of the conserved phenylalanine that is presumed to represent the amino terminus of each mature protein. In comparison, the mature PilA1 and PilA2 proteins share 57% overall sequence identity and 87% sequence identity among their 32 amino-terminal residues. The predicted PilA1 amino-terminal sequence matched the amino-terminal sequence determined earlier for pilin of intact pili isolated from cells of strain VA1-S3 (27), demonstrating that PilA1 is the predominant pili component for this strain.

The third ORF (nucleotide positions 1550 to 2150) immediately downstream of *pilA2* on the pEC114 insert predicts a protein of 200 residues. A BLAST search with the protein predicted by the third ORF identified the *Dichelobacter nodosus* class I FimB protein, which is hypothesized to function in pilus assembly (25). On the basis of this identity, the third ORF was designated *pilB*. Overall, the predicted PilB protein shares 21% sequence identity with FimB from *D. nodosus*.

The fourth ORF (nucleotide positions 2463 to 3324) is located 0.3 kbp downstream of *pilB* and predicts a protein of 287 residues. A BLAST search revealed that the protein predicted by the fourth ORF shared significant sequence identity with the hemagglutinin encoded by the *hae-1* gene of *E. corrodens* ATCC 23834 (40). Given this identity, the fourth ORF was designated *hagA*. The predicted HagA protein shares 90% sequence identity with the *hae-1* gene product. Given the proximity and structure of the *pilA1*, *pilA2*, *pilB*, and *hagA* genes, the corresponding genomic region was designated the *pilA* locus.

***pilA* locus structure in phase variants.** To examine a potential *pilA*-related genomic basis for their differential piliation, the structure of the *pilA* locus in *E. corrodens* S- and L-phase variants was examined by DNA hybridization analysis. Total DNA from strains VA1-S2 and VA1-L2 was digested with *Pvu*II and *Bgl*II, *Pvu*II, or *Pvu*II and *Dra*I and hybridized to the 3.9-kbp pEC114 insert (Fig. 2). For each digest, the hybridization profile obtained for strain VA1-S2 was indistinguishable from that obtained for VA1-L2 (Fig. 2; compare lanes 1 and 2). Identical results for S- and L-phase variants were obtained with

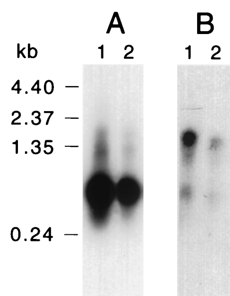

FIG. 3. Expression of *pilA* genes in *E. corrodens* VA1-S1 and VA1-L2. Samples of total RNA (5 μg per lane) from strain VA1-S1 (lanes 1) or VA1-L2 (lanes 2) were subjected to blot hybridization against RNA probes specific for transcripts from *pilA1* (A) or *pilA2* (B). The positions of RNA molecular size standards are shown at the left.

other restriction enzymes. No hybridizing genomic fragments other than those originating from the *pilA1* locus were observed. A similar analysis for other S- and L-phase variants yielded identical results (data not shown). In a related effort, the sequence of a 1.5-kbp region of strain VA1-L2 genomic DNA encompassing *pilA* and *pilB* was determined. When compared, the strain VA1-L2 sequence was found to be identical to that for strain VA1-S1 (data not shown).

Pilin gene expression in phase variants. To characterize differential piliation between S- and L-phase variants at the level of pilin gene expression, transcripts from *pilA1* and *pilA2* in both variants were examined by RNA hybridization analysis. Total RNA isolated from cells of strains VA1-S1 and VA1-L2 was hybridized to RNA probes specific for *pilA1* or *pilA2* (includes *pilB* sequences). Similar hybridization profiles were obtained for both variants: the *pilA1* probe detected an abundant 0.6-kb transcript and a less abundant 1.5-kb transcript (Fig. 3A), while the *pilA2* probe detected a 1.5-kb transcript (Fig. 3B). Transcripts from *pilA1* and *pilA2* were also examined in two additional S-phase variants (strains VA1-S2 and VA1-S3) and two additional L-phase variants (strains VA1-L1 and VA1-L3), yielding identical results (data not shown). The 0.6-kb transcript is predicted to include *pilA1* sequences, whereas the 1.5-kb transcript is predicted to include *pilA1*, *pilA2*, and *pilB* sequences. For both probes, the relative hybridization signal intensity was lower for strain VA1-L2 (Fig. 3; compare lanes 1 and 2). Replicate experiments showed that the *pilA1* and *pilA1A2B* transcript levels in L-phase variants ranged from 43 to 80% of those in S-phase variants. Despite this difference, these results support transcription of the *pilA* locus in strain VA1-L2 and, coupled with pilin localization studies (see below), suggest a posttranscriptional basis for the lack of piliation in L-phase variants.

Transcription of the *pilA* locus was also examined by reverse transcriptase PCR. The reverse transcriptase reactions were carried out with select primer pairs using total RNA from strains VA1-S1 and VA1-L2 as the template. The cDNAs were amplified by PCR, and the products were analyzed by hybridization against the RNA probes specific for *pilA1* or *pilA2*. PCR products corresponding to transcripts encoding *pilA1*, *pilA2*, or *pilB* were equally detected in both phase variants (data not shown). In addition, PCR products corresponding to polycistronic transcripts encompassing *pilA1* and *pilA2*, as well as *pilA1* through *pilB*, were detected in both variants (data not shown). These data further support equivalent transcription of the *pilA* locus in S- and L-phase variants.

The 5′ end of the *pilA1* transcript from strain VA1-S1 was mapped by primer extension. Using a primer complementary to a region in the 5′ end of *pilA1* (nucleotides 538 to 555), we detected two products 200 and 130 nucleotides in length (data not shown). The larger product mapped to a guanine located 117 bases upstream (nucleotide 367) of the putative translation initiation codon, whereas the smaller product mapped to a cytosine located 53 bases upstream (nucleotide 431). Identical products were obtained for strain VA1-L2. An analysis of the DNA sequence in the vicinity of these sites suggests that the larger product reflects the native *pilA1* transcription initiation site: two potential σ^{70} consensus promoter sequences and a potential σ^{54} consensus promoter sequence are located immediately upstream of the identified guanine (Fig. 4). Located further upstream is a 40-bp AT-rich region. A similar analysis of the region between *pilA1* and *pilA2* did not reveal potential promoter sequences; however, a 25-bp region (nucleotides 968 to 993) predicted to form a near-perfect hairpin was detected.

Pilin localization in phase variants. The experiments described above indicated that both S- and L-phase variants express intact *pilA1* transcripts. To define a cellular basis for the lack of piliation among L-phase variants, we examined the synthesis and localization of PilA1 in both S- and L-phase variants. Surface, soluble, and insoluble protein fractions were isolated from strains VA1-S1 and VA1-L2 and analyzed for PilA1 content by using a PilA1-specific antiserum. The strain VA1-S1 surface fraction, which should include pilin from pili and pilin loosely associated with the cell surface, contained a considerable amount of mature PilA1 (Fig. 5, lane 1). In contrast, no PilA1 was detected in the surface fraction of strain VA1-L2 (lane 2). Comparable amounts of mature PilA1 were detected in the soluble fraction of both strains (compare lanes 3 and 4). Similarly, the insoluble fraction of both strains contained comparable amounts of mature PilA1 (compare lanes 5 and 6). In addition to mature pilin, the insoluble fraction of both strains contained a unidentified 19-kDa immunoreactive protein (lanes 5 and 6). Control assays with different *pilA1* and *pilA2* mutants indicated that the 19-kDa immunoreactive species is ubiquitous to the insoluble fraction and is not a form of pilin. No PilA1 was detected in the periplasmic fraction of

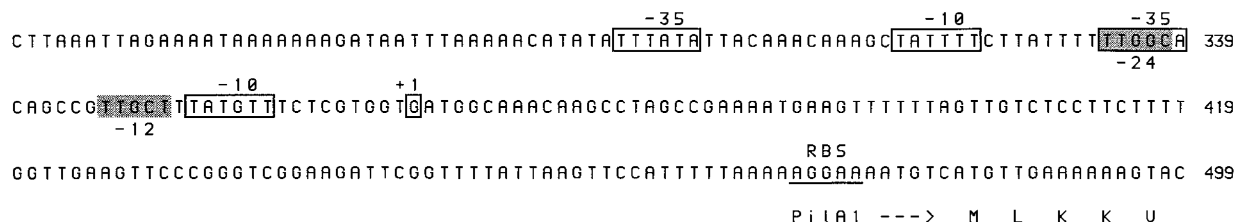

FIG. 4. Nucleotide sequence of the *E. corrodens* VA1-S1 *pilA1* promoter region. The putative *pilA1* transcription initiation site (+1) determined by primer extension analysis is boxed, as are potential σ^{70} promoter sequences (−35 and −10). Potential σ^{54} promoter sequences (−24 and −12) are shaded. A putative ribosome binding site (RBS) is underlined. Numbers to the right correspond to the sequence deposited in the GenBank database (accession no. AF079304).

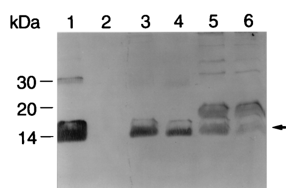

FIG. 5. Localization of PilA1 in S- and L-phase variants of *E. corrodens* VA1. Surface (lanes 1 and 2), soluble (lanes 3 and 4), and insoluble (lanes 5 and 6) protein fractions from strains VA1-S1 (lanes 1, 3, and 5) and VA1-L2 (lanes 2, 4, and 6) were subjected to immunoblot analysis with a polyclonal antiserum specific for PilA1. The arrow marks the position of mature pilin. The positions of protein molecular weight standards are shown at the left.

either strain VA1-S1 or strain VA1-L2 (data not shown). Collectively, these data suggest that the lack of piliation in L-phase variants is due to a posttranslational event, possibly involving PilA1 export and/or pilus assembly.

Piliation of phase variants. Previous reports have described differential piliation among S- and L-phase variants of *E. corrodens* (22, 23). However, this phenomenon was not well documented, and subsequent studies have questioned the presence of pili on *E. corrodens* (32, 38). To document their differential piliation, cells of strains VA1-S3 and VA1-L2 were examined by immunogold electron microscopy using a polyclonal antiserum raised against pilin purified from strain VA1-S1 (27). In an analysis of over 200 strain VA1 cells, we detected one or more immunoreactive pili on 47% of the strain VA1-S3 cells but on none of the strain VA1-L2 cells. Furthermore, no immunoreactive material was observed on the cell surface of either strain. A representative electron micrograph for strains VA1-S3 and VA1-S2 is presented in Fig. 6. In a similar analysis of over 200 *E. corrodens* ATCC 23834 cells, immunoreactive pili were detected on all of the S-phase cells and none of the L-phase cells.

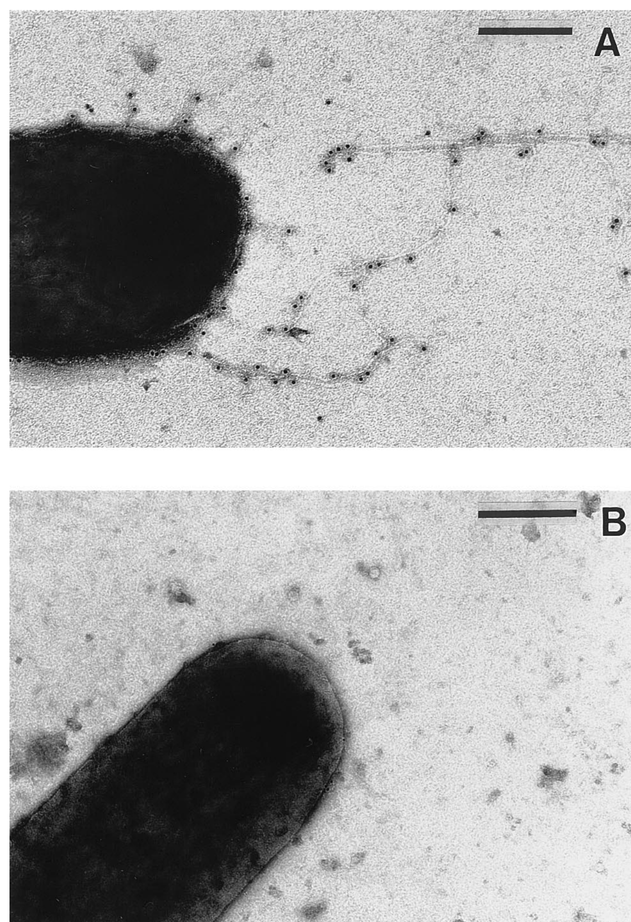

FIG. 6. Differential piliation of S- and L-phase variants of *E. corrodens* VA1. (A) Immunogold electron micrograph of a strain VA1-S3 cell; (B) equivalent micrograph of a strain VA1-L2 cell. Bars = 200 nm.

DISCUSSION

The gram-negative pathogen *E. corrodens* elaborates type IV pili and exhibits a phase variation involving an irreversible transition from piliated to nonpiliated cells. To initiate an investigation into the molecular basis of this transition event, we have cloned and characterized the *pilA* locus from *E. corrodens* VA1. The *pilA* locus includes four putative genes arranged in tandem. The first two genes, designated *pilA1* and *pilA2*, each encode a type IV pilin. The third gene, designated *pilB*, encodes a potential pilus assembly protein, whereas the fourth gene, designated *hagA*, encodes a putative hemagglutinin. In terms of the pilin and hemagglutinin genes, the strain VA1 *pilA* locus structurally resembles the similar locus described for *E. corrodens* ATCC 23834 (39) (see below). However, the strain VA1 *pilA* locus differs from the strain ATCC 23834 locus by the presence of *pilB*. Given that DNA hybridization analyses of genomic DNA using heterologous and homologous probes failed to detect additional hybridizing fragments, *pilA1* and *pilA2* most likely represent the only type IV pilin genes present in strain VA1.

This study showed that S- and L-phase variants of *E. corrodens* are identical with respect to *pilA1* and *pilA2* structure and sequence. This differs from what is most commonly observed for the best-characterized bacterial pathogens exhibiting a similar phase and/or antigenic variation that involves expression of type IV pilin. Among these are *N. gonorrhoeae* and *N. meningitidis*, which possess one or two pilin (*pilE*) genes and a variable number of silent partial pilin (*pilS*) genes. For these strains, phase variation can be achieved by spontaneous mutations in *pilE*, which results in nonpiliated cells (7, 50, 51). In addition, irreversible intragenic recombination events involving the *pilE* and *pilS* genes result in the synthesis of structurally altered pilins, giving rise to antigenic variation (19, 36, 45, 52). Another example is *M. bovis*, in which two different pilin genes (*fimL* and *fimQ*) are alternatively expressed by means of a DNA inversion that links one or the other gene to a single common promoter (18, 34). Thus, the most common phase and/or antigenic variation exhibited by *N. gonorrhoeae*, *N. meningitidis*, and *M. bovis* results from a genomic mutation or recombination events directly involving type IV pilin genes. In comparison, the phase variation exhibited by *E. corrodens* is unique in that it does not involve a pilin gene-associated mutation or genomic recombination event.

The RNA hybridization analysis indicated that transcripts from *pilA1*, but not *pilA2*, were abundant in both S- and L-phase variants of strain VA1, consistent with previous work showing that PilA1 was the major pilus protein for this strain (27). The 5′-end mapping and sequence analyses suggest that the abundant *pilA1* transcript originates from a σ^{70}-type promoter located 200 bp upstream of the putative PilA1 translation initiation codon. If correct, the 0.6-kb *pilA1* transcript terminates at the *pilA1-pilA2* intergenic region, the sequence of which predicts a potential hairpin structure. A low-abundance 1.5-kbp transcript that hybridized to probes for both

pilA1 and *pilA2* was also detected in the S- and L-phase variants. This larger transcript is most likely the product of transcription from the *pilA1* promoter through the *pilA1-pilA2* intergenic region, giving rise to a *pilA1A2B* polycistronic message. Presumably, the predicted hairpin structure between *pilA1* and *pilA2* represents the terminator component of a transcription attenuation mechanism. Such an attenuation mechanism would provide for controlled expression of *pilA1*, *pilA2*, and *pilB* as required for pilus formation and resembles a similar mechanism that has been proposed for *D. nodosus* (25).

The detection of transcripts from *pilA1* and *pilA2* in the L-phase variant of strain VA1 was somewhat unexpected. In the absence of a detectable pilin gene-associated recombination or mutagenesis event, it was hypothesized that the phase variation exhibited by strain VA1 might be achieved through differential expression of *pilA1* or *pilA2*. The abundance of both the 0.6-kb *pilA1* transcript and 1.5-kb *pilA1A2B* transcript was consistently lower in L-phase variants; however, this difference was deemed insufficient to account for their lack of piliation. We suspect that the decreased level of the *pilA1* and *pilA1A2B* transcripts is related to factors associated with extraction of RNA from the morphologically distinct L-phase variants, as opposed to factors associated with transcription. Collectively, the *pilA1* and *pilA2* structure and transcription data support a posttranscriptional basis for the nonpiliated phenotype of the L-phase variants.

The pilin localization studies revealed that L-phase variants are not compromised in pilin biosynthesis but that they differ from S-phase variants with respect to the fate of synthesized pilin. For both S- and L-phase variants, similar levels of mature PilA1 were detected in the insoluble protein fraction. Because this fraction includes the cytoplasmic membrane and associated components, the presence of mature PilA1 is consistent with studies of *Pseudomonas aeruginosa* and other species showing that initial processing of type IV pilins (leader sequence cleavage and amino-terminal methylation) is accomplished on the cytoplasmic surface of the cytoplasmic membrane by membrane-associated cognate prepilin peptidases (47). Moreover, the similarity in pilin composition of the insoluble protein fractions suggests that initial processing of PilA1 is not significantly altered in the L-phase variants. The S- and L-phase variants were also indistinguishable in the PilA1 composition of their soluble protein fractions, as similar levels of mature PilA1 were detected for both. In contrast to these results is the PilA1 composition of the surface protein fraction: whereas mature PilA1 was readily detected in the S-phase variant surface protein fraction, none was detected in the corresponding fraction from the L-phase variant. This lack of pilin in the L-phase variant surface fraction is consistent with the immunogold electron microscopic analysis showing that only S-phase variants possessed intact pili. On the basis of these data, we conclude that an altered pilin posttranslational event, possibly involving one or more steps in pilin export and/or assembly, is responsible for the lack of piliation associated with the L-phase variants of *E. corrodens* VA1.

The specific stage or event in export or assembly of pili that might be affected in the L-phase variants is not known. In general, the pathway for processing, export, and assembly of type IV pilins is not well defined. Perhaps the best-characterized type IV pilus biosynthetic pathway is that of *P. aeruginosa*, for which more than 22 genes involved in pilin expression and/or pilus assembly and function have been identified by transposon tagging (35). More than half of these genes appear to be directly involved in pilus assembly and function and are thought to represent a subset of a general system for the formation of surface-associated protein complexes (26). Of particular interest is the observation that mutations in the individual genes in this subset result in the lack of piliation. Homologs to many of the putative pilus assembly genes have been identified in other type IV piliated bacteria, supporting a common mechanism for processing, export, and assembly of type IV pili. Presumably, *E. corrodens* shares this mechanism; if this is so, events that affect one or more of the corresponding genes might be involved in the transition from S- to L-phase variants. Experiments to examine this possibility are in progress.

A precedence for phase variation mediated by a type IV pilin-associated posttranslational event has been established for *N. gonorrhoeae* MS11. This strain, like most examined *N. gonorrhoeae* strains, contains two unlinked copies of the *pilC* gene (not a *P. aeruginosa pilC* homolog), designated *pilC1* and *pilC2*, which encode a protein involved in pilus assembly (29). Only *pilC2* is expressed in piliated MS11 cells due to a translational frameshift in *pilC1*. Spontaneous phase variation of MS11 cells is achieved by frameshift mutations in a run of G residues within the region of *pilC2* encoding the signal peptide of PilC, abolishing translation of the protein. Pilin synthesis is maintained in the absence of PilC, but no pili are assembled. In this regard, the strain MS11 *pilC2* mutants and the L-phase variants of *E. corrodens* VA1 are phenotypically indistinguishable. Whether *E. corrodens* VA1 possesses a homolog to *pilC* from *N. gonorrhoeae* remains to be examined; however, we note that thus far, PilC appears to be unique to *N. gonorrhoeae* (47). A significant difference between the *pilC2*-based phase variation in *N. gonorrhoeae* MS11 and the phase variation in *E. corrodens* VA1 is that the former is reversible. Piliated revertants to strain MS11 *pilC2* mutants are readily obtainable; the reversion involves mutations in *pilC1* or *pilC2* that result in translation of PilC. In contrast, no L- to S-phase revertants of *E. corrodens* VA1 have been observed. Given these data, we predict that the putative posttranslational basis of phase variation in *E. corrodens* VA1 differs mechanistically from *pilC2*-based phase variation in *N. gonorrhoeae*.

Genes encoding type IV pilins have been cloned from two other *E. corrodens* strains, ATCC 23834 (39) and ATCC 31745 (53). A feature common to these two strains and strain VA1 is the presence of two tandemly arranged type IV pilin genes separated by 70 to 80 bp. The strain ATCC 23834 pilin genes are designated *ecpA* and *ecpB* (39), whereas the strain 31745 pilin genes are designated *ecpC* and *ecpD* (53). For the strain VA1 pilin genes *pilA1* and *pilA2*, we adopted the designations of the *P. aeruginosa* pilin gene system (1), which is consistent with recommendations for bacterial gene nomenclature (17). The six predicted pilins share 80 to 97% sequence identity at their amino termini (first 32 residues of the mature protein), which is typical for all examined type IV pilins (47). Overall, the six predicted pilins share 35 to 43% sequence identity, which is consistent with the reported decreased sequence conservation within the carboxyl regions of type IV pilins (47). In general, the two pilins from a given strain share greater overall sequence identity with each other than with pilins from another strain, suggesting that for each strain, the pilin gene pairs arose by a duplication event. The G+C contents of *pilA1* and *pilA2* (47.9 and 40.1%, respectively) significantly differ from the genomic G+C contents (53 to 58%) reported for several different *E. corrodens* strains (13, 28, 42), suggesting that the pilin gene(s) may have been acquired by horizontal transfer. Since these are characteristics of a variety of virulence genes acquired by intra- and interspecies gene transfer in bacterial pathogens (21, 54), the *pilA* locus may represent an acquired region encoding pathogenicity-associated proteins.

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