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Role of the *Eikenella corrodens pilA* Locus in Pilus Function and Phase Variation

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The human pathogen *Eikenella corrodens* expresses 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 pilus structure and function in the clinical isolate *E. corrodens* VA1. Earlier work defined the *pilA* locus which includes *pilA1*, *pilA2*, *pilB*, and *hagA*. Both *pilA1* and *pilA2* predict a type IV pilin, whereas *pilB* predicts a putative pilus assembly protein. The role of *hagA* has not been clearly established. That work also confirmed that *pilA1* encodes the major pilus protein in this strain and showed that the phase variation involves a posttranslational event in pilus formation. In this study, the function of the individual genes comprising the *pilA* locus was examined using a recently developed protocol for targeted interposon mutagenesis of S-phase variant VA1-S1. Different *pilA* mutants were compared to S-phase and L-phase variants for several distinct aspects of phase variation and type IV pilus biosynthesis and function. S-phase cells were characterized by surface pili, competence for natural transformation, and twitching motility, whereas L-phase cells lacked these features. Inactivation of *pilA1* yielded a mutant that was phenotypically indistinguishable from L-phase variants, showing that native biosynthesis of the type IV pilus in strain VA1 is dependent on expression of *pilA1* and proper export and assembly of PilA1. Inactivation of *pilA2* yielded a mutant that was phenotypically indistinguishable from S-phase variants, indicating that *pilA2* is not essential for biosynthesis of functionally normal pili. A mutant inactivated for *pilB* was deficient for twitching motility, suggesting a role for PilB in this pilus-related phenomenon. Inactivation of *hagA*, which may encode a tellurite resistance protein, had no effect on pilus structure or function.

Eikenella corrodens is a gram-negative bacterium native to the oral cavity and gastrointestinal tract in humans. This bacterium can also be pathogenic, causing a variety of soft tissue and wound infections (6, 9, 10, 16), endocarditis (4, 9), and other opportunistic infections. *E. corrodens* has also been associated with periodontal diseases (2, 19, 21), although a causal role has not been clearly established. Like several gram-negative pathogens including *Neisseria gonorrhoeae*, *N. meningitidis*, and *Moraxella bovis*, *E. corrodens* exhibits a phase variation that results from altered synthesis of type IV pili and is reflected in colony morphology changes. On solid medium, small (S-phase) corroding and large (L-phase) noncorroding colonies are observed (7, 12, 15, 28). The L-phase variants arise irreversibly from S-phase variants at a frequency much greater than mutation rates. Colony morphology and phase variation correlates with the presence of pili on S-phase variants and the absence of pili on L-phase variants (11, 12). Because type IV pili can be determinants of pathogenesis (1, 5, 18, 29), the molecular basis of phase variation and the related phenomenon of antigenic variation are of considerable interest.

We recently isolated and characterized the *pilA* locus from an S-phase variant of *E. corrodens* strain VA1 (31). The *pilA* locus contains four tandemly arranged genes designated *pilA1*, *pilA2*, *pilB*, and *hagA*. Both *pilA1* and *pilA2* encode a type IV pilin, whereas *pilB* encodes a protein resembling the *Dichelobacter nodosus* FimB fimbrial assembly protein, and *hagA* encodes a putative hemagglutinin. Extensive DNA hybridization analyses indicated that *pilA1* and *pilA2* represent the only type IV pilin genes in this strain. In S-phase and L-phase cells, *pilA1* is expressed as an abundant transcript initiating at an upstream promoter and terminating at a predicted hairpin structure between *pilA1* and *pilA2*, whereas *pilA2* and *pilB* are expressed as a low-abundance readthrough transcript. On the basis of protein and DNA sequence analyses, we determined that the *pilA1*-encoded pilin, designated PilA1, represents the major pilus protein for this strain. In contrast to the *Neisseria* and *Moraxella* species described above, the phase variation exhibited by *E. corrodens* strain VA1 does not involve a genomic recombination or mutagenic event that directly affects expression of the *pilA* locus. Both S-phase and L-phase cells similarly transcribe *pilA1* and synthesize PilA1; however, S-phase cells export and assemble the PilA1 into pili whereas L-phase cells do not (31). The molecular basis of the presumed posttranslational alteration involving PilA1 export and assembly in L-phase variants remains to be determined.

We are examining the role of the strain VA1 *pilA* locus in type IV pilus biosynthesis and the related phenomena of competence for natural transformation and twitching motility. The earlier work established a dominant role for *pilA1* in pilus biosynthesis; however, potential roles in this process for *pilA2*, *pilB*, or *hagA* remained to be defined. In this report, the function of the individual genes comprising the *pilA* locus was

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TABLE 1. Strains and plasmids used

Strain or plasmid	Description[a]	Reference or source
E. corrodens strains		
VA1	Clinical isolate	Laboratory collection
VA1-S1	S-phase variant of VA1	Laboratory collection
VA1-L2	L-phase variant of VA1	Laboratory collection
T18	Derivative of VA1-S1; *pilA1*::Ω	This study
T6	Derivative of VA1-S1; *pilA2*::Ω	This study
T11	Derivative of VA1-S1; *pilB*::Ω	This study
T40	Derivative of VA1-S1; *hagA*::Ω	This study
T99	Derivative of VA1-S1; Δ*pilA1 pilB*::Ω	This study
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 Labs
Plasmids		
pHP45Ω	Source of *aad* gene (Ω cassette); Sp^r	22
pSKS101	Source of *aph* gene; Km^r	27
pUC1819*Eco*RI	Modified pUC18 cloning vector; Ap^r	J. Golden
pUMC315	pUC1819 with 2.0-kbp *Eco*RI fragment from pHP45Ω containing the Ω cassette	This study
pEC114	pGEM3zf(+) containing the 3.9-kbp *Eco*RI fragment from VA1-S1 carrying the *pilA* locus (*pilA1A2B hagA*)	31
pEC207	pEC114 deleted for 299-bp *Hin*dIII fragment (nucleotides 3654–3953)	This study
pEC211	pEC114 with *Bam*HI-digested Ω cassette inserted into *Bcl*I site at nucleotide 1327 in *pilA2*; *pilA2*::Ω	This study
pEC213	pEC114 with *Bam*HI site replacing *Bgl*II site at nucleotide 869 in *pilA1*	This study
pEC216	pEC213 with *Bam*HI-digested Ω cassette inserted into *Bgl*II site at nucleotide 2008 in *pilB*; *pilB*::Ω	This study
pEC218	pEC114 with *Bam*HI site replacing *Bgl*II site at nucleotide 2008 in *pilB*	This study
pEC219	pEC218 with *Bam*HI-digested Ω cassette inserted into *Bgl*II site at nucleotide 869 in *pilA1*; *pilA1*::Ω	This study
pEC230	pEC207 with 1575-bp *Sma*I-*Bgl*II fragment (nucleotides 432-2008) replaced with 1,035-bp PCR product from pEC114; Δ*pilA1*; *pilA2* controlled by $pilA1_p$	This study
pEC232	pEC230 with *Bam*HI-digested Ω cassette inserted into *Bgl*II site at nucleotide 2008 in *pilB*; Δ*pilA1*; *pilA2* controlled by $pilA1_p$; *pilB*::Ω	This study
pEC233	pEC230 with *Bam*HI-digested *aph* gene inserted into *Bgl*II site at nucleotide 2008 in *pilB*; Δ*pilA1*; *pilA2* controlled by $pilA1_p$; *pilB*::*aph*	This study
pEC235	pEC230 with *Sal*I-digested Ω cassette inserted into *Sal*I site at nucleotide 3274 in *hagA*; Δ*pilA1*; *pilA2B* controlled by $pilA1_p$; *hagA*::Ω	This study
pEC237	pEC230 with *Sal*I-digested *aph* gene inserted into *Sal*I site at nucleotide 3274 in *hagA*; Δ*pilA1*; *pilA2B* controlled by $pilA1_p$; *hagA*::*aph*	This study
pEC306	pEC114 with *Sal*I-digested Ω cassette inserted into *Sal*I site at nucleotide 3274 in *hagA*; *hagA*::Ω	This study

[a] Nucleotide numbers correspond to *pilA* locus sequence deposited in GenBank (accession no. AF079304).

examined using a recently developed protocol for targeted interposon mutagenesis of *E. corrodens*. Analyses of different *pilA* mutants revealed that expression of *pilA1*, but not *pilA2*, is critical for synthesis of the pili responsible for the colony morphology of S-phase variants, competence, and twitching motility. This effort also suggests a role for *pilB* in twitching motility.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains 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. (8). Strain VA1-S1 is an S-phase isolate of strain VA1 that forms S-phase colonies and exhibits a typical frequency of phase variation to L-phase colonies on solid medium (31). Strain VA1-L2 is an L-phase isolate of VA1 that forms only L-phase colonies. *E. corrodens* was cultured aerobically at 35°C on chocolate agar plates (Remel, Lenexa, Kans.) supplemented with 6 ml of a 1.5% agar overlay containing 1% (wt/vol) L-ornithine monohydrochloride and 1% (wt/vol) decarboxylase base Moeller medium (Difco, Detroit, Mich.) to facilitate growth. For selection and maintenance of transformants, streptomycin or kanamycin was added to the overlay to achieve a final antibiotic concentration of 25 μg ml^{-1}.

Escherichia coli strain DH5α was used as the host for cloning vectors. *E. coli* strains were propagated in liquid or on solid Luria-Bertani medium with antibiotics at standard concentrations (26).

DNA methods. Restriction endonucleases and modifying enzymes were purchased from Promega (Madison, Wis.). DNA manipulations including restriction digestion, agarose gel electrophoresis, ligations, PCR amplifications, transformation of *E. coli*, and plasmid minipreparations were performed using established protocols (3, 26). *E. corrodens* genomic DNA was prepared as described for *E. coli* in reference 26 or using kits from Qiagen (Chatsworth, Calif.) or GenoTech (St. Louis, Mo.). For DNA hybridization analysis, digested DNA was transferred to Hybond-N$^+$ (Amersham, Arlington Heights, Ill.) membrane by the method of Reed and Mann (25). DNA probes for the *pilA* locus (3.9-kbp *Eco*RI fragment from pEC114) or *aad* (2.0-kbp *Bam*HI fragment from pHP45Ω) were generated from gel-purified fragments by digoxigenin labeling with a kit from Boehringer (Indianapolis, Ind.). DNA hybridizations were performed at 60°C as described by Sambrook et al. (26).

Construction of mutant *pilA* loci. The plasmids and oligonucleotide primers used in this study are listed in Tables 1 and 2, respectively. All nucleotide numbers referenced below correspond to the *pilA* locus sequence deposited in the GenBank database (accession no. AF079304). A physical map for *pilA* is presented in Fig. 1. Physical maps for the described mutant *pilA* constructs are presented in Fig. 5. Plasmid pEC114 harbors the 3.9-kbp *Eco*RI fragment of strain VA1-S1 genomic DNA encompassing the *pilA* locus (31) and served as the DNA source for all mutant *pilA* constructs. Plasmid pEC207 is a derivative of

TABLE 2. Primers used

Primer[a]	5′→3′ sequence[b]	Position,[c] restriction site
RH-1 (←)	TTCGggAtCcTTAGCAGCACCAGGAGCG	888–915, *Bam*HI site at 892
RH-2 (←)	GGCAACTTGATGGCAAATATCCTAC	1431–1454
RH-3 (→)	GGCACCCAAACCCTTTACAAG	1590–1610
RH-10 (→)	CGGTCAGATCTCTACTTGGACTTGCGC	864–890, *Bgl*II site at 869
RH-7B (←)	GATAGgGATCcGCCTGCATGGAAGGGG	1192–2018, *Bam*HI site at 2008
RH-8 (←)	CCGGTCgGATCcCTACTTGGACTTGC	863–888, *Bam*HI site at 868
RH-9 (←)	GATAGAGATCTGCCTGCATGGAAGG	1994–2018, *Bgl*II site at 2008
RH-18 (→)	AGATCCCgggTGTTTGGCAAGGGGGAT	967–993, *Sma*I site at 971
RH-12(←)	AGCTTctcgaGGCAGAAACTATCTGCCTGC	2128–2158, *Xho*I site at 2133
RH-14 (←)	ATTCActCGaGCTTTTTCGCCAACATCGTC	3299–3328, *Xho*I site at 3318
105-R1 (→)	TGTTATCGCCATTATCGG	531–548
107-F3 (→)	AGAGCAACTCGCTTTACCC	1079–1096
204-F2 (→)	CGGATACGATGTGCATG	2885–2901

[a] Arrows designate forward (→) or reverse (←) primer sequence.
[b] Underscored letters correspond to specified restriction sites; lowercase letters designate introduced base substitutions.
[c] Nucleotide numbers corresponding to *pilA* locus sequence deposited in GenBank (accession no. AF079304).

pEC114 deleted for the 0.3-kbp *Hin*dIII fragment originating downstream of *hagA* (nucleotide 3654) and terminating at the *Hin*dIII site in the multiple cloning region; this deletion eliminates the restriction sites in the multiple cloning region. To facilitate subcloning of the *aad* gene (often referred to as the Ω cassette; confers resistance to the antibiotics streptomycin and spectinomycin), the 2.0-kbp *Eco*RI fragment containing *aad* from pHP45Ω (22) was ligated into vector pUC1819*Eco*RI digested with *Eco*RI. The product, designated pUMC315, provides for excision of *aad* with *Bam*HI, *Sma*I, or *Sal*I.

The *pilA1* gene was interrupted by insertion of *aad* following a minor modification of the *pilA* locus. The 1.14-kbp region encompassing the two internal *Bgl*II sites (nucleotides 869 to 2008) was amplified from pEC114 by PCR with primers RH-10 and RH-7b (substitutes *Bam*HI site for *Bgl*II site at nucleotide 2008). The PCR product was digested with *Bgl*II and *Bam*HI and ligated into pEC114 previously digested with *Bgl*II, effectively replacing the internal 1.14-kpb *Bgl*II fragment (nucleotides 869 to 2008) containing *pilA1A2B* sequences. The resulting plasmid, designated pEC218, harbors an otherwise intact *pilA* locus which lacks the *Bgl*II site in *pilB*. Subsequently, the 2.0-kbp *Bam*HI fragment containing *aad* from pUMC315 was ligated into pEC218 previously digested with *Bgl*II, creating pEC219. The same strategy was used to interrupt *pilB*. In this case, the 1.14-kbp region encompassing the two internal *Bgl*II sites (nucleotides 869 to 2008) was amplified from pEC114 by PCR with primers RH-8 (substitutes *Bam*HI site for *Bgl*II site at nucleotide 869) and RH-9. The PCR product was digested with *Bam*HI and *Bgl*II and ligated into pEC114 previously digested with *Bgl*II as described above. The resulting plasmid, designated pEC213, harbors an otherwise intact *pilA* locus which lacks the *Bgl*II site in *pilA1*. The 2.0-kbp *Bam*HI fragment containing *aad* from pUMC315 was ligated into pEC213 previously digested with *Bgl*II, creating pEC216.

To interrupt *pilA2*, the 2.0-kbp *Bam*HI fragment containing *aad* from pUMC315 was ligated into the unique *Bcl*I site (nucleotide 1327) in pEC114, creating pEC211. Interruption of *hagA* was accomplished similarly by ligating the 2.0-kbp *Sal*I fragment containing *aad* from pUMC315 into the unique *Sal*I site (nucleotide 3274) in pEC114, creating pEC306.

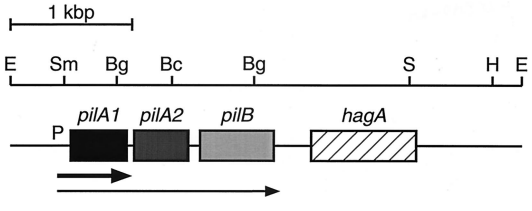

FIG. 1. Physical map of the *pilA* locus for *E. corrodens* VA1-S1. Shaded and hatched boxes indicate sizes and positions of open reading frames as determined by sequence analysis. Arrows below the map designate mapped transcripts originating from the promoter upstream of *pilA1*. Flanking and internal restriction sites are shown for enzymes used in cloning and targeted mutagenesis. Bg, *Bgl*II; Bc, *Bcl*I; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Sm, *Sma*I.

To create a *pilA* locus lacking *pilA1*, pEC207 was digested with *Sma*I and *Bgl*II, and the larger digest product consisting of the original cloning vector and *pilA* flanking regions was gel purified using a kit from Qiagen (Valencia, Calif.). The 1.03-kbp fragment encompassing *pilA2* and most of *pilB* (nucleotides 973 to 2008) was amplified from pEC114 by PCR with primers RH-18 (introduces *Sma*I site upstream of *pilA2* coding region at nucleotide 973) and RH-9. The PCR product was digested with *Sma*I and *Bgl*II and ligated with the purified *Sma*I-*Bgl*II fragment from pEC207. The resulting plasmid, designated pEC230, places *pilA2* and *pilB* under the control of $pilA1_p$. Several selectable derivatives of this plasmid were used in this study. One, designated pEC232, was constructed by ligating the 2.0-kbp *Bam*HI fragment containing *aad* from pUMC315 into the unique *Bgl*II site (nucleotide 2008) in *pilB*. A second, designated pEC233, was constructed by ligating the 2.0-kbp *Bam*HI fragment containing *aph* (confers resistance to the antibiotic kanamycin) from pSKS101 (27) into the same *Bgl*II site. A third, designated pEC235, was constructed by ligating the 2.0-kbp *Sal*I fragment containing *aad* from pUMC315 into the unique *Sal*I site (nucleotide 3274) in *hagA*. A fourth, designated pEC237, was constructed by ligating the 2.0-kbp *Sal*I fragment containing *aph* from pSKS101 into the same site.

Transformation and interposon mutagenesis of *E. corrodens*. The protocol for transformation of *E. corrodens* was based on the procedure developed by Tonjum et al. (30). Cells of strain VA1-S1 were cultured on supplemented chocolate agar as described above. After 48 h, 10 S-phase colonies were harvested and resuspended in 1 ml of medium A (3.7% [wt/vol] brain heart infusion broth [BBL, Cockeysville, Md.], 0.05% [wt/vol] agar, 50 μM $CaCl_2$, 0.2% [wt/vol] bovine serum albumin). For each transformation, a 10-μl aliquot of the resuspended cells was brought to 100 μl with medium A to achieve a cell density of approximately 2.5×10^6 CFU ml^{-1}, and the suspension was provided linearized plasmid DNA (1 μg). Following incubation at 30°C for 45 min, the cells were plated onto supplemented chocolate agar and incubated at 35°C for 8 h. Selection was then applied by transferring the agar to a plate containing 6 ml of brain heart infusion broth supplemented with streptomycin (final concentration, 25 μg ml^{-1}). Transformant colonies were isolated after 72 h and maintained on solid medium. Interposon mutagenesis of a targeted gene by insertion of *aad* via double homologous recombination between the genome of the recipient and the introduced DNA was confirmed for all mutants by PCR with the following primers: *pilA1*, 105-R1 and RH-1; *pilA2*, 107-F3 and RH-2; *pilB*, RH3 and RH12; *hagA*, 204-F2 and RH-14. For some mutants, interposon mutagenesis was also confirmed by DNA hybridization analysis using probes for *pilA* and *aad*. To assay competence for natural transformation, the wild-type and *pilA* mutant strains were subjected to the same protocol using linearized pEC233 (pEC237 for mutant T99) as the introduced DNA and kanamycin (final concentration, 25 μg ml^{-1}) for selection and maintenance of transformants.

Electron microscopy. Negative staining and immunogold electron microscopic examination of whole cells were performed as described elsewhere (14), using a polyclonal antiserum (1:1,000) prepared against pilin purified from strain VA1-S3.

Cell fractionation. Cell fractionation was performed as described by Villar et al. (31). For the wild-type and *pilA* mutant strains, total cellular and surface protein fractions were isolated for analysis. All protein fractions were mixed with

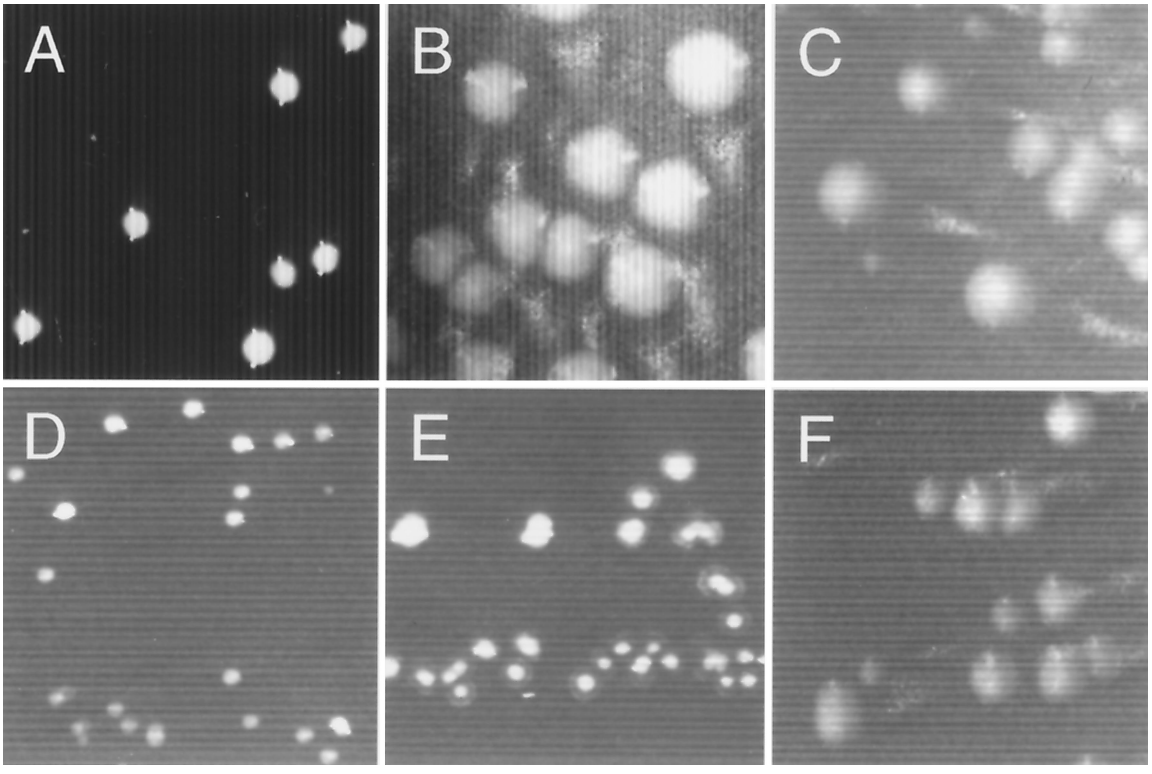

FIG. 2. Colony morphologies of wild-type and *pilA* mutant strains of *E. corrodens*. Cells of strains VA1-S1 (A), VA1-L2 (B), T18 (C), T6 (D), T11 (E), and T99 (F) were cultured on chocolate agar. Magnification = ×12.5.

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and stored at −20°C.

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. (3). The blots were blocked and incubated with a polyclonal antiserum (1:5,000) raised against a truncated PilA1 protein (31). 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.

Twitching motility. Twitching motility by the wild-type and *pilA* mutant strains was assayed by the agar interface method described by McMichael (20) and by analysis of colony morphology. To facilitate microscopic examination of *E. corrodens* colonies in the latter procedure, cells were plated onto a sterilized dialysis membrane affixed to the agar surface of a supplemented chocolate agar plate and cultured as described above. After a 24-h incubation period, the membrane was peeled away from the agar and the colonies were examined under low magnification (×30 and ×50) for the convoluted edge and spreading that are characteristic of twitching motility (11).

RESULTS

Transformation of *E. corrodens*. Earlier attempts by this and other laboratories to transform *E. corrodens* with circular plasmid DNA were not successful. In this work, an effective procedure for transformation of this species using linearized DNA was developed. Using the optimized conditions described above, transformation frequencies ranging from 1×10^{-5} to 3×10^{-5} were typically achieved, yielding 30 to 60 transformants per μg of transforming DNA. For interposon mutagenesis via double homologous recombination between the introduced DNA and the genome of the recipient, a minimum of 0.4 kbp of genomic sequences flanking the selectable marker (*aad* or *aph*) was required. All of the *pilA* mutants described below were generated by this protocol and confirmed for interposon mutagenesis of the targeted gene by DNA hybridization analysis and/or PCR (data not shown).

Phenotypes of wild-type and *pilA* mutant strains. To examine the role of each gene constituting the *pilA* locus, different *pilA* mutants were compared to S-phase variant VA1-S1 and L-phase variant VA1-L2 for colony morphology, the presence of pili, the presence of PilA1 in surface and total cellular protein fractions, competence for natural transformation, and twitching motility. These phenotypes were chosen to represent several distinct aspects of phase variation and type IV pilus biosynthesis and function.

Strain VA1 exhibits an irreversible transition from S-phase to L-phase variants that is reflected in a colony morphology change. This phase transition is demonstrated by strains VA1-S1 and VA1-L2; on solid medium, strain VA1-S1 forms small colonies whereas strain VA1-L2 forms large colonies (compare Fig. 2A and B). As we reported earlier (31), the altered colony morphology of these phase variants correlates with the presence of pili on VA1-S1 cells and the absence of such pili on VA1-L2 cells (compare Fig. 3A and B). The detection of mature PilA1 in the total protein fraction but not the surface protein fraction of VA1-L2 cells (Fig. 4A and B, compare lanes 2 and 3) supports the hypothesis that a post-translational event involving PilA1 export and/or assembly is responsible for the phase variation exhibited by strain VA1. In the assay for competence for natural transformation by linearized pEC233 DNA, strain VA1-S1 yielded the standard frequency of kanamycin-resistant colonies, whereas no resistant

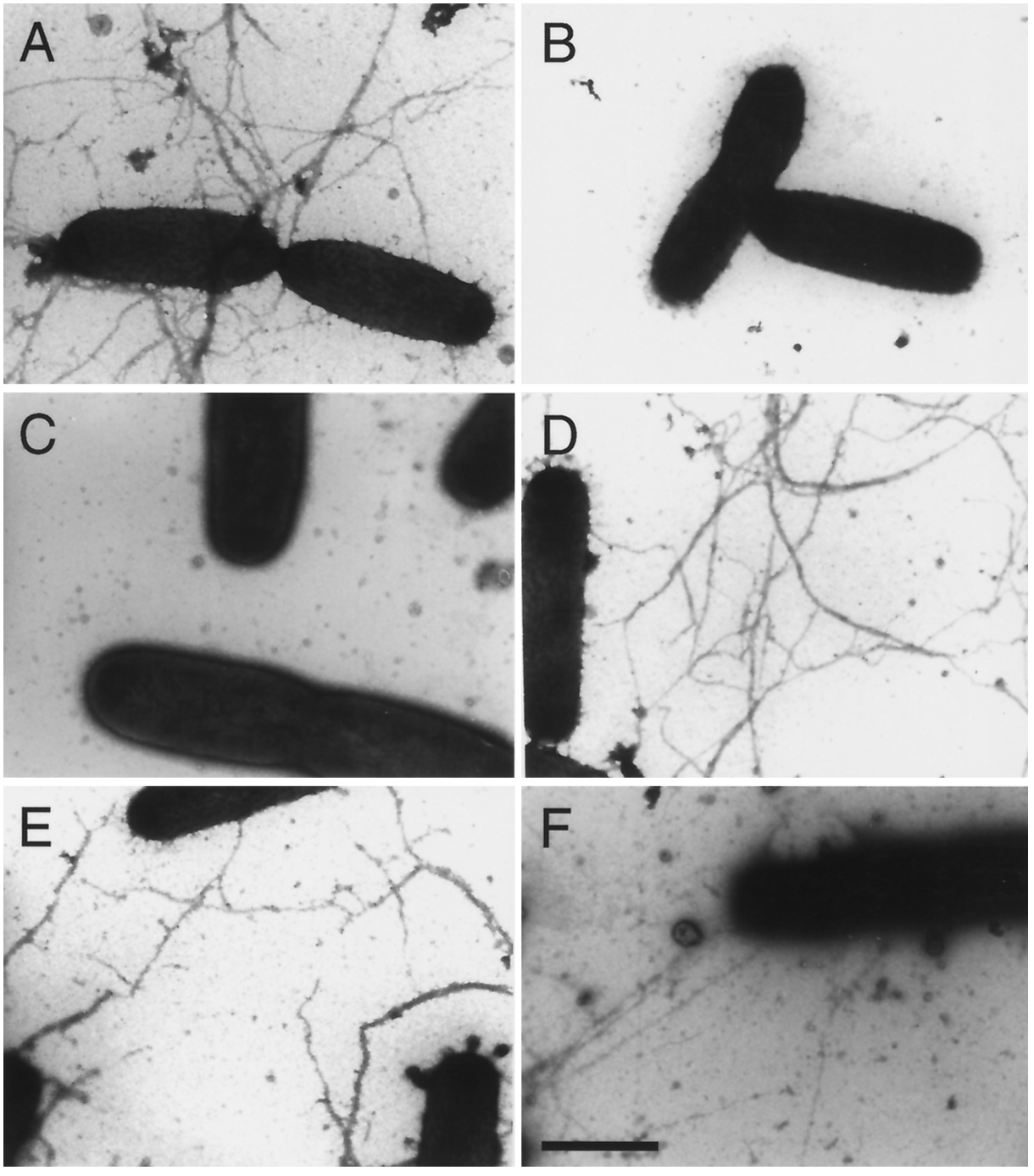

FIG. 3. Differential piliation of wild-type and *pilA* mutant strains of *E. corrodens*. Cells of strains VA1-S1 (A), VA1-L2 (B), T18 (C), T6 (D), T11 (E), and T99 (F) were examined by immunogold electron microscopy. Bar = 100 nm.

colonies were obtained for strain VA1-L2. In addition, two independent assays showed that only strain VA1-S1 is characterized by the phenomenon of twitching motility.

A link between *pilA1* activity and the type IV pilus-associated phenotypes was established with mutant strain T18, in which *pilA1* was inactivated by insertion of *aad* (see Fig. 5). On solid medium, strain T18 formed large colonies that were indistinguishable from those of strain VA1-L2 (compare Fig. 2B and C). Electron microscopic examination showed that like cells of VA1-L2, cells of strain T18 lacked observable pili (compare Fig. 3B and C). In contrast to strain VA1-L2, PilA1 was not detected in the total protein fraction for strain T18 (Fig. 4A, compare lanes 3 and 4). Not surprisingly, strain T18 was not competent for transformation, nor did it exhibit twitching motility (Fig. 5). Thus, strain T18 closely resembles strain VA1-L2, and the collective phenotypes of this mutant suggest that expression of *pilA1* is essential for pilus biosynthesis and related functions.

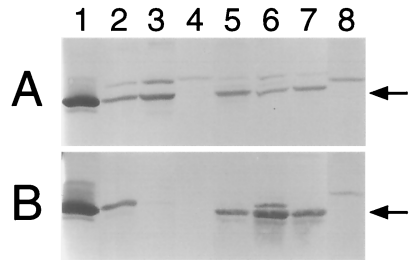

FIG. 4. Localization of PilA1 in wild-type and *pilA* mutant strains of *E. corrodens*. (A) Total protein fraction; (B) surface protein fraction. Purified PilA1 (lane 1) and protein fractions from strains VA1-S1 (lane 2), VA1-L2 (lane 3), T18 (lane 4), T6 (lane 5), T11 (lane 6), T40 (lane 7), and T99 (lane 8) were subjected to immunoblot analysis with a polyclonal antiserum specific for PilA1. Each arrow marks the position of mature pilin.

Inactivation of *pilA2* or *pilB* resulted in strains that essentially exhibited the phenotypes of strain VA1-S1. By insertion of *aad*, strain T6 was inactivated for *pilA2* whereas strain T11 was inactivated for *pilB* (Fig. 5). Both strains T6 and T11 formed small colonies on solid medium (Fig. 2D and E, respectively) and possessed PilA1-containing pili indistinguishable from those of strain VA1-S1 (compare Fig. 3D and E, respectively, with Fig. 3A). As for strain VA1-S1, mature PilA1 was detected in the total and surface protein fractions for both strains T6 and T11 (Fig. 4, lanes 5 and 6, respectively), and both mutants were competent for transformation by pEC233. However, strain T11 differed from strains VA1-S1 and T6 in that it was deficient for twitching motility, suggesting a possible role for *pilB* in this colony phenomenon. Although *hagA* was not predicted to play a role in pilus biosynthesis, strain T40, which was inactivated for *hagA* by *aad* (Fig. 5), was similarly examined for the pilus-associated phenotypes. Like strain VA1-S1, strain T40 was characterized phenotypically by small colony size (data not shown), PilA1-containing pili (data not shown), detectable PilA1 (Fig. 4, lane 7), competence for natural transformation, and twitching motility.

Strain T99 was generated to examine whether the pilin encoded by *pilA2* could support pilus biosynthesis and the pilus-related phenotypes. The *pilA* locus in strain T99 lacks *pilA1*, contains *pilA2* under the control of $pilA1_p$, and is inactivated for *pilB* by *aad* (Fig. 5). On solid medium, strain T99 formed large colonies (Fig. 2F). Surprisingly, cells of strain T99 were found to possess pili that resembled those of strain VA1-S1 (Fig. 3F). Because the pili were not recognized by the PilA1 antisera (Fig. 3F) and no PilA1 was detected in any protein fraction for the strain (Fig. 4, lane 8), it was assumed that the pili of strain T99 were composed of PilA2. Strain T99 was competent for transformation by pEC237, suggesting that PilA1 is not essential for this process. However, in both assays for twitching motility, colonies of strain T99 did not exhibit any features characteristic of this pilus-associated phenomenon.

DISCUSSION

Genetic manipulation of the gram-negative pathogen *E. corrodens* has been compromised by the lack of an efficient transformation protocol for this species. In an earlier systematics analysis, Tonjum et al. demonstrated that several *E. corrodens* strains were naturally competent for genetic transformation by sheared genomic DNA (30). However, subsequent attempts by this and other laboratories to similarly transform *E. corrodens* with uncut plasmid vectors carrying host genomic sequences were not successful. Rao et al. developed a gene transfer system for *E. corrodens* strain ATCC 23834 that was based on conjugal transfer of a shuttle vector from *E. coli* (23). Although successful, this approach was limited by low frequencies of plasmid transfer and the requirement of a phage-based counterselection to inhibit growth of the donor. In this work, we have demonstrated that *E. corrodens* strain VA1-S1 is naturally competent for transformation by linearized plasmid vectors carrying host genomic sequences. As part of a mutational anal-

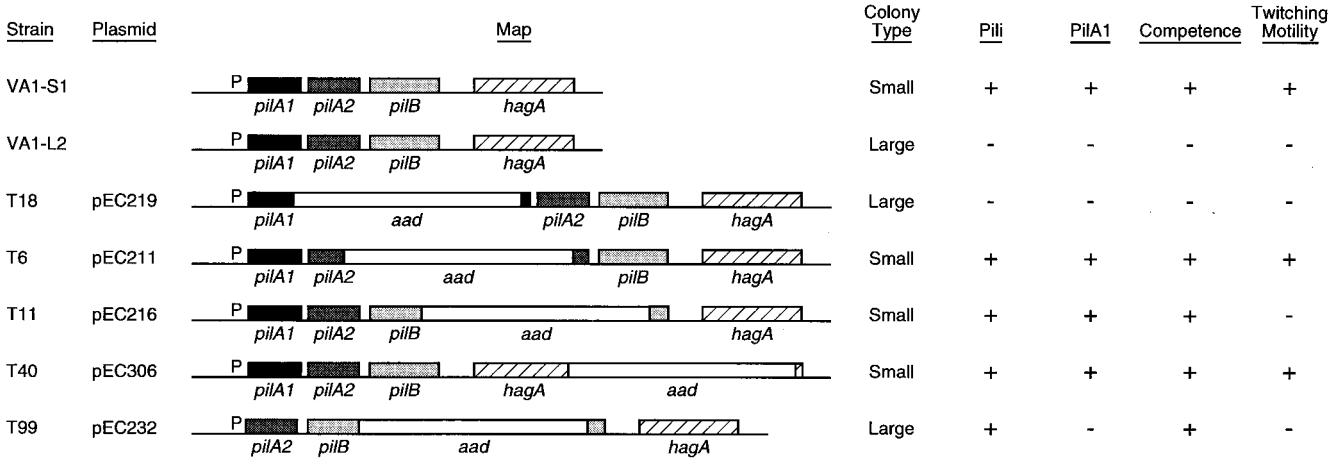

FIG. 5. Physical maps and corresponding phenotypes of examined wild-type and *pilA* mutant strains of *E. corrodens*. Strains VA1-S1 and VA1-L2 are S-phase and L-phase variants, respectively, of the clinical isolate VA1. Mutant strains T18, T6, T11, T40, and T99 were isolated following transformation of strain VA1-S1 with the corresponding plasmid and selecting for double homologous recombinants. Each strain was assayed for colony morphology, surface pili, PilA1, competence for natural transformation, and twitching motility as described in Materials and Methods. Symbols indicate a positive (+) or negative (−) assay result.

ysis of the four genes constituting the *pilA* locus, different plasmids on which the individual genes were interrupted by insertion of the selectable *aad* (or *aph*) marker were used to transform cells of strain VA1-S1. The interrupted *pilA* sequences were stably integrated into the genomic *pilA* locus, presumably via double homologous recombination, with transformation frequencies ranging from 1×10^{-5} to 3×10^{-5}. To our knowledge, this effort represents the first application of both competence for natural transformation and interposon mutagenesis to study gene function in *E. corrodens*, and the results suggest that this organism should be amenable to standard genetic manipulation using these and related procedures.

Earlier work in our laboratory showed that for *E. corrodens* strain VA1, colony morphology and phase variation correlates with the presence of type IV pili on S-phase variants and the absence of such pili on L-phase variants. In this study, S-phase variant strain VA1-S1 and L-phase variant strain VA1-L2 were analyzed for competence for natural transformation and twitching motility, both of which have been associated with the expression of type IV pili in certain gram-negative bacteria. Not surprisingly, the piliated S-phase variant exhibited both competence and twitching motility whereas the nonpiliated L-phase variant exhibited neither, indicating that intact type IV pili are required for both processes in *E. corrodens*. This observation parallels the tight association between type IV pili and competence for natural transformation and twitching motility exhibited by *N. gonorrhoeae* (17). In contrast to *N. gonorrhoeae* and other type IV piliated pathogens, very little is known about the structure and function of the *E. corrodens* type IV pilus. However, recent work in our laboratory with S- and L-phase variants of strain VA1 indicates that the type IV pilus is essential for adherence to and cytotoxicity of human epithelial cells, suggesting that *E. corrodens* shares similar determinants of pilus structure and function with the better-characterized pathogens.

The type IV pilin gene *pilA1* of strain VA1 plays a major role in pilus biosynthesis. Inactivation of *pilA1* in S-phase variant strain VA1-S1 yielded mutant strain T18, which is phenotypically indistinguishable from L-phase variant strain VA1-L2; both strains T18 and VA1-L2 grow as large colonies and both lack pili, competence, and twitching motility. By design, interruption of *pilA1* with *aad* would also have the polar effect of abolishing expression of *pilA2* and *pilB* due to the intrinsic terminator encoded by the cassette (22). However, independent inactivation of *pilA2* (strain T6) or *pilB* (strain T11) did not affect pilus formation, demonstrating that the T18 phenotype is dependent on the loss of *pilA1* activity. The colony and piliation phenotypes of strain T18 corroborate earlier work showing that PilA1 is the major pilus protein for strain VA1. Recently we showed that both S-phase and L-phase cells transcribe *pilA1* and synthesize PilA1; however, S-phase cells export and assemble the PilA1 into pili whereas L-phase cells do not, resulting in nonpiliated cells that grow as large colonies. Thus, despite the presence of the second type IV pilin gene *pilA2*, native biosynthesis of the type IV pilus in strain VA1 is dependent on expression of *pilA1* and proper export and assembly of PilA1.

The type IV pilin gene *pilA2* of strain VA1 does not play a major role in pilus biosynthesis. This was demonstrated by strain T6, which is inactivated for *pilA2* and is phenotypically indistinguishable from S-phase variant strain VA1-S1, indicating that expression of *pilA2* is not essential for biosynthesis of functionally normal pili. Earlier work showed that in both S- and L-phase variants of strain VA1, *pilA1* is represented by an abundant transcript that terminates between *pilA1* and *pilA2*, whereas *pilA2* is represented by a much less abundant readthrough transcript encompassing *pilA1*, *pilA2*, and *pilB*. Whether pilin PilA2 is a minor pilus component in strain VA1-S1 is not known. In this study we showed that enhanced expression of *pilA2* in a *pilA1* null mutant background provided for synthesis of pili, presumably composed of PilA2, that share some features of the native pilus forms. This was demonstrated by mutant strain T99, in which *pilA1* was deleted from the *pilA* locus in a manner that placed *pilA2* adjacent to the native *pilA1* promoter. Cells of strain T99 possessed pili indistinguishable from those of strain VA1-S1 and were naturally competent, suggesting that PilA2 is sufficient for synthesis of a functional pilus. Interestingly, strain T99 grew as large colonies, suggesting that the large-colony morphology of L-phase variants is due not to their lack of pili but rather to their lack of pili composed of PilA1. The structural features of PilA1 specific to the small-colony morphology of S-phase variants remain to be determined.

The *pilB* gene of the *pilA* locus is not essential for pili biosynthesis but may play a role in twitching motility by strain VA1. Inactivation of *pilB* in strain VA1-S1 yielded strain T11, which in these analyses was phenotypically indistinguishable from the parental strain except that it did not exhibit detectable twitching motility. A deficiency in twitching motility was also exhibited by strain T99, which possesses pili composed of PilA2 and is inactivated for *pilB*. Phenotypically, strain T11 resembles characterized *pilT* mutants of *N. gonorrhoeae* (34), *Pseudomonas aeruginosa* (33), and *Myxococcus xanthus* (35), which are piliated but lack twitching motility. Several lines of evidence suggest that *pilT* encodes a motor protein involved in pilus retraction (32). However, the predicted PilB protein does not show significant sequence identity to any reported PilT homologs. Instead, PilB shows greatest, albeit limited, sequence identity to the *D. nodosus* class I FimB protein, which is hypothesized to function in pilus assembly (13). Given that pilus biosynthesis was not noticeably impaired in strain T11, we favor the hypothesis that PilB represents a pilus structural component that provides for retraction of the filament, possibly by a mechanism that includes a PilT homolog.

The *hagA* gene of the *pilA* locus is not involved in pilus structure or function. In this study, inactivation of *hagA* yielded a strain that was phenotypically indistinguishable from the parental strain VA1-S1, which was not surprising given that *hagA* was thought to encode a hemagglutinin. The hemagglutinin gene designation for *hagA* was originally based on a BLAST analysis showing that the predicted HagA protein showed greater than 90% sequence identity to the protein predicted by the *hae-1* gene of *E. corrodens* strain ATCC 23834 (31); correction of a presumed error in the deposited *hae-1* sequence would render the two proteins nearly identical. The *hae-1* gene product has been characterized as a hemagglutinin capable of inducing agglutination of neuraminidase-treated erythrocytes (24). However, a recent BLAST analysis suggests that the homologous *hagA* and *hae-1* genes actually encode a tellurite resistance protein, bringing into question the earlier

hemagglutinin gene designation for *hae-1*. Whether *hagA* encodes a hemagglutinin or a tellurite resistance protein would not seem to affect the results of this study but clearly needs to be resolved.

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REFERENCES

1. **Alm, R. A., and J. S. Mattick.** 1997. Genes involved in the biogenesis and function of type-4 fimbriae in *Pseudomonas aeruginosa*. Gene **192:**89–98.
2. **Ashimoto, A., C. Chen, I. Bakler, and J. Slots.** 1998. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontal lesions. Oral Microbiol. Immunol. **11:**226–273.
3. **Ausubel, F. M., R. Brent, R. E. Klingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1989. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
4. **Berbari, E. F., F. R. I. Cockerill, and J. M. Steckelberg.** 1998. Infective endocarditis due to unusual or fastidious microorganisms. Mayo Clin. Proc. **72:**532–542.
5. **Bieber, D., S. W. Ramer, C. Y. Wu, W. J. Murray, T. Tobe, R. Fernandez, and G. K. Schoolnik.** 1998. Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic *Escherichia coli*. Science **280:**2114–2118.
6. **Chen, C. K., and M. E. Wilson.** 1992. *Eikenella corrodens* in human oral and non-oral infections: a review. J. Periodontol. **63:**941–953.
7. **Chen, C. K., and M. E. Wilson.** 1990. Outer membrane protein and lipopolysaccharide heterogeneity among *Eikenella corrodens* isolates. J. Infect. Dis. **162:**664–671.
8. **Cobb, C. M., J. T. Helber, and R. Hirschberg.** 1994. Scanning electron microscopy of *Eikenella corrodens* colony morphology variants. J. Periodontal Res. **29:**410–417.
9. **Decker, M. D., B. S. Graham, E. B. Hunter, and S. M. Liebowitz.** 1986. Endocarditis and infections of intravascular devices due to *Eikenella corrodens*. Am. J. Med. Sci. **292:**209–212.
10. **Griego, R. D., T. Rosen, I. F. Orengo, and J. E. Wolf.** 1995. Dog, cat, and human bites: a review. J. Am. Acad. Dermatol. **33:**1019–1029.
11. **Henrichsen, J.** 1975. The occurrence of twitching motility among gram-negative bacteria. Acta Pathol. Microbiol. Scand. Sect. B **83:**171–178.
12. **Henrichsen, J., and J. Blom.** 1975. Examination of fimbriation of some gram-negative rods with and without twitching and gliding motility. Acta Pathol. Microbiol. Scand. Sect. B **83:**161–170.
13. **Hobbs, M., B. P. Dalrymple, P. T. Cox, S. P. Livingstone, S. F. Delaney, and J. S. Mattick.** 1991. Organization of the fimbrial gene region of *Bacteroides nodosus*: class I and class II strains. Mol. Microbiol. **5:**543–560.
14. **Hood, B. L., and R. Hirschberg.** 1995. Purification and characterization of *Eikenella corrodens* type IV pilin. Infect. Immun. **63:**3693–3696.
15. **Jackson, F. L., and Y. Goodman.** 1984. *Eikenella*, p. 591–597. *In* N. R. Krieg (ed.), Bergey's manual of systematic bacteriology, 9th ed., vol. 1. Williams & Wilkins, Baltimore, Md.
16. **Kentos, A., P. De Vuyst, M. J. Stuelens, F. Jacobs, P. de Francquen, B. Delaere, P. Demaeyer, and J. P. Thys.** 1995. Lung abscess due to *Eikenella corrodens*: three cases and review. Eur. J. Clin. Microbiol. Infect. Dis. **14:**146–148.
17. **Koomey, M.** 1998. Competence for natural transformation in *Neisseria gonorrhoeae*: a model system for studies of horizontal gene transfer. APMIS Suppl. **84:**56–61.
18. **Krogfelt, K. A.** 1991. Bacterial adhesion: genetics, biogenesis, and role in pathogenesis of fimbrial adhesins of *Escherichia coli*. Rev. Infect. Dis. **13:**721–735.
19. **Lowenguth, R. A., I. Chin, J. G. Caton, C. M. Cobb, C. L. Drisko, W. J. Killoy, B. S. Michalowicz, B. L. Pihlstrom, and J. M. Goodson.** 1995. Evaluation of periodontal treatments using controlled-release tetracycline fibers: microbiological response. J. Periodontol. **66:**700–707.
20. **McMichael, J. C.** 1992. Bacterial differentiation within *Moraxella bovis* colonies growing at the interface of the agar medium with the Petri dish. J. Gen. Microbiol. **138:**2687–2695.
21. **Page, R. C.** 1986. Current understanding of the aetiology and progression of periodontal disease. Int. Dent. J. **36:**153–161.
22. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
23. **Rao, V. K., J. A. Whitlock, and A. Progulske-Fox.** 1993. Development of a genetic system for *Eikenella corrodens*: transfer of plasmids pFM739 and pLES2. Plasmid **30:**289–295.
24. **Rao, V. K., J. A. Whitlock, and A. Proguske-Fox.** 1993. Cloning, characterization and sequencing of two haemagglutinin genes from *Eikenella corrodens*. J. Gen. Microbiol. **139:**639–650.
25. **Reed, K. C., and D. A. Mann.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. **13:**7207–7221.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Shapira, S. K., J. Chou, F. V. Richaud, and M. J. Casadaban.** 1983. New versatile plasmid vectors for expression of hybrid proteins coded by a cloned gene fused to *lacZ* gene sequences encoding an enzymatically active carboxy-terminal portion of beta-galactosidase. Gene **25:**71–82.
28. **Shiozu, I., J. Shiozu, I. Takazoe, and K. Okuda.** 1992. Corroding characteristics of *Eikenella corrodens*. Bull. Tokyo Dent. Coll. **33:**1–6.
29. **Strom, M. S., and S. Lory.** 1993. Structure-function and biogenesis of the type IV pili. Annu. Rev. Microbiol. **47:**565–596.
30. **Tonjum, T., N. Hagen, and K. Bovre.** 1985. Identification of *Eikenella corrodens* and *Cardiobacterium hominis* by genetic transformation. Acta Pathol. Microbiol. Immunol. Scand. Sect. B **93:**389–394.
31. **Villar, M. T., J. T. Helber, B. Hood, M. R. Schaefer, and R. L. Hirschberg.** 1999. *Eikenella corrodens* phase variation involves a posttranslational event in pilus formation. J. Bacteriol. **181:**4154–4160.
32. **Wall, D., and D. Kaiser.** 1999. Type IV pili and cell motility. Mol. Microbiol. **32:**1–10.
33. **Whitchurch, C. B., M. Hobbs, S. P. Livingston, V. Krishnapillai, and J. S. Mattick.** 1991. Characterisation of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialised protein export system widespread in eubacteria. Gene **101:**33–44.
34. **Wolfgang, M., P. Lauer, H. S. Park, L. Brossay, J. Hebert, and M. Koomey.** 1998. PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated *Neisseria gonorrhoeae*. Mol. Microbiol. **29:**321–330.
35. **Wu, S. S., J. Wu, and D. Kaiser.** 1997. The *Myxococcus xanthus pilT* locus is required for social gliding motility although pili are still produced. Mol. Microbiol. **23:**109–121.