Cloning and sequencing of two type 4 (*N*-methylphenylalanine) pilin genes from *Eikenella corrodens*

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Eikenella corrodens is a Gram-negative microaerophilic rod which is gaining recognition as an important human pathogen. We have previously reported the cloning and expression in *Escherichia coli* of a 3.6 kb *Eik. corrodens* genomic DNA fragment which encodes a 31.5 kDa haemagglutinin. Maxicell analysis revealed that this fragment also encodes two proteins of approximately 14 kDa. Nucleotide sequencing of the 2.2 kb fragment upstream of the haemagglutinin gene revealed two open reading frames with strong homology to genes encoding pilin subunit proteins of the type 4 or *N*-methylphenylalanine class. The two pilin genes, *ecpA* and *ecpB*, are complete and are expressed in *E. coli*. Southern analysis of ten additional *Eik. corrodens* strains revealed that all possess fragments homologous to *ecpA*. These data represent the first molecular evidence for pili in *E. corrodens*.

Introduction

Eikenella corrodens is a Gram-negative, microaerophilic rod which colonizes the oral cavity and upper respiratory tract of humans (Henriksen, 1969; Tanner *et al.*, 1979). In recent years, this unusual organism has gained recognition as a opportunistic pathogen (Brooks *et al.*, 1974; DeMello & Leonard, 1979; Stoloff & Gillies, 1986). It has been isolated from abscesses in a variety of sites (Farrington *et al.*, 1983; Decker *et al.*, 1986; Cheng *et al.*, 1988 *a, b*; Hofstad & Horn, 1989) and is now regarded as an important cause of infections resulting from human bite injuries (Schmidt & Herkman, 1983; Stoloff & Gillies, 1986). In addition, *Eik. corrodens* is associated with certain forms of periodontal disease and may play an important role in the extensive bone loss which accompanies this complex process (Tanner *et al.*, 1979; Dzink *et al.*, 1985).

In his initial description of *Eik. corrodens*, Eiken (1958) noted the unusual ability of colonies of this organism to form small depressions or pits in the agar. Indeed, it was this characteristic for which the species

was named. In addition, the colonies were noted to exhibit evidence of peripheral spread under appropriate growth conditions. Darkfield examination of *Eik. corrodens* revealed that while it lacked flagella, it exhibited an unusual form of random movement which is termed twitching motility and which is presumably responsible for spread on agar plates. The earliest descriptions also noted that at a detectable frequency, strains exhibiting all of these characteristics gave rise to variants which could not pit the agar or spread by twitching motility.

Interestingly, many of these characteristics are shared by a number of unrelated organisms, many of which are pathogens, including *Moraxella* spp. (Marrs *et al.*, 1985), *Dichelobacter (Bacteroides) nodosus* (Elleman, 1988) and *Pseudomonas aeruginosa* (Sastri *et al.*, 1985). In these organisms, these microbiological characteristics have been correlated with the expression of pili or fimbriae (Elleman, 1988). More significantly, the presence of these pili has, in the case of *P. aeruginosa*, been definitively linked with the ability to cause disease (Woods *et al.*, 1980; Paranchych & Frost, 1988) and a similar role in pathogenesis has been suspected for the pili of other organisms. The pili of *D. nodosus* (Elleman, 1988), *Moraxella bovis* (Marrs *et al.*, 1985), *Neisseria gonorrhoeae* (Meyer *et al.*, 1984), *N. meningitidis* (Perry *et al.*, 1988), *P. aeruginosa* (Johnson *et al.*, 1986) and *Vibrio cholerae* (Shaw & Taylor, 1990) belong to a family known as the type 4 or *N*-methylphenylalanine ('*N*-Met-

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Phe') class of pili. These pili are homopolymeric structures composed of pilin subunits, each of molecular mass 15–20 kDa. The pilin subunits are synthesized as single polypeptide chains, which typically include an unusually short six- or seven-amino-acid leader sequence at the N-terminus. This leader is proteolytically removed and the resulting N-terminal amino acid methylated, producing the *N*-methylphenylalanine for which these pili are named. In addition, these pilins exhibit a striking conservation of amino acid sequence over the N-terminal 30 amino acids. Genes encoding type 4 pilins have been cloned and sequenced from a variety of these organisms, including *N. gonorrhoeae* (Meyer *et al.*, 1984), *M. bovis* (Marrs *et al.*, 1985), *D. nodosus* (Anderson *et al.*, 1984; Finney *et al.*, 1988) and *P. aeruginosa* (Pasloske *et al.*, 1985). These genes were each expressed in *E. coli*, but in no case were the subunit proteins assembled into pili, leading to speculation that the systems responsible for assembly of type 4 pili differ greatly from those responsible for pilus construction in *E. coli*.

Given the cultural characteristics of *Eik. corrodens*, it has long been suspected that this organism too possesses pili. While early microscopic observations by Jackson *et al.* (1971) failed to find pilus-like structures consistently on the facultative *Bacteroides corrodens* (later renamed *Eikenella corrodens*), in more recent studies, Progulske & Holt (1980) noted the presence of fibrils, 4 nm thick, arranged in a peritrichous fashion around the cells. While these fibrils were thought to be pili, there remained some uncertainty as to whether they might represent artifacts of processing. To date, pili have not been purified from *Eik. corrodens*. In the accompanying paper we reported the cloning and sequencing of two haemagglutinin genes from *Eik. corrodens* ATCC 23834 (Rao *et al.*, 1993). Here we report the identification, cloning and sequencing of two type 4 pilin genes from the chromosome of this bacterium.

Methods

Bacterial strains, plasmids, media and growth conditions. *Eikenella corrodens* ATCC 23834 (the type strain) and ATCC 43278 were obtained from the American Type Culture Collection. Strains FDC373, 470 and 1073 are clinical isolates and were obtained from S. S. Socransky, Forsyth Dental Center, Boston, Massachusetts, USA). Strains EC-14, 23, 26, 37, 38 and 50, as described previously (Lacroix & Walker, 1991), were isolated from patients with refractory periodontitis and were obtained from Dr Clay Walker, University of Florida. Identification of strains as *Eik. corrodens* was based on the cell wall fatty acid profile as detected by gas chromatography using the VPI system (Holdeman *et al.*, 1987). *Eik. corrodens* strains were cultured on blood agar plates in an atmosphere of 10% (v/v) CO₂. Broth-grown cells were cultured in BY broth as previously described (Progulske & Holt, 1987). *Escherichia coli* JM109 (Yanisch-Perron *et al.*, 1985), which was used for all cloning experiments, was cultured aerobically on Luria-Bertani (LB) medium. Plasmid pUC19, used for subcloning

Table 1. Bacterial plasmids used in this study

Plasmid	Characteristics†
pUC series*	Ap ^R lacZ ⁺
pVKR201	Ap ^R HA ⁺ ; pUC9 Ω (<i>Hind</i> III 3.6 kb insert from <i>Eik. corrodens</i> 23834)
pVKR202	Ap ^R HA ⁺ ; pUC19 Ω (<i>Hind</i> III 3.6 kb, orientation opposite to that of pVKR201)
pVKR203	Ap ^R HA ⁺ ; pUC18 Ω (<i>Eco</i> RI– <i>Hind</i> III 1.9 kb)
pVKR204	Ap ^R HA ⁺ ; pUC19 Ω (<i>Hind</i> III– <i>Eco</i> RI 1.7 kb)
pVKR205	Ap ^R pUC18 Ω (<i>Kpn</i> I– <i>Hind</i> III 1.5 kb)
pVKR207	Ap ^R HA ⁺ ; pUC19 Ω (<i>Kpn</i> I 0.7 kb)
pVKR208	Ap ^R HA ⁺ ; pUC18 Ω (<i>Hinc</i> II– <i>Hind</i> III 0.7 kb)
pVKR209	Ap ^R HA ⁺ ; pUC18 Ω (<i>Ava</i> I– <i>Hinc</i> II 0.6 kb)

* Characteristics described by Yanisch-Perron *et al.* (1985). All the other plasmids were produced during this work.

† HA⁺, confers haemagglutination activity on *E. coli* JM109. Ω indicates the insert carried (details in parentheses).

experiments, was purchased from Bethesda Research Laboratories. Plasmid pUC18 lacking the deletion mutation (Lobet *et al.*, 1989) was also used for subcloning and was purchased from Pharmacia LKB Biotechnology. Other plasmids are listed in Table 1.

DNA preparation and manipulation. Construction and screening of the genomic library of *Eik. corrodens* ATCC 23834 in *E. coli* is described in the accompanying paper (Rao *et al.*, 1993). Chromosomal DNA used in Southern hybridization experiments was isolated from *Eik. corrodens* strains using standard methods (Sambrook *et al.*, 1989). DNA was further purified by multiple rounds of protease treatment, hexadecyltrimethyl ammonium bromide (CTAB) precipitation phenol/chloroform extraction and ethanol precipitation using conventional techniques (Sambrook *et al.*, 1989). Restriction endonucleases were purchased from Bethesda Research Laboratories or Promega and used according to the manufacturer's instructions.

DNA sequencing. Fragments to be sequenced were cloned in either pUC19 or pUC18. Double-stranded sequencing was carried out by the University of Florida DNA Core Sequencing Facility. Details are given in the accompanying paper (Rao *et al.*, 1993).

Maxicell analysis. This was done as described by Rao *et al.* (1993).

Southern hybridization. Procedures were as described by Rao *et al.* (1993), except that after hybridization the membrane was subjected to a wash of low stringency (2 × SSPE, 1%, w/v, SDS) for 60 min at 65 °C followed by a moderate stringency wash (0.5 × SSPE, 1%, w/v, SDS) for 15 min at 65 °C, and positively hybridizing bands were visualized by autoradiography for 12–24 h (rather than 6–12 h) at –70 °C.

Results

Cloning of two pilin genes from *Eik. corrodens*

In the accompanying paper we reported the construction of a genomic clone bank of *Eik. corrodens* ATCC 23834 DNA in *E. coli* JM109, and the identification of two *E. coli* clones with the ability to agglutinate neuraminidase-treated erythrocytes (Rao *et al.*, 1993). As noted previously, clone 1 harboured plasmid pVKR201, which

Fig. 1. Localization of the two pilin genes of pVKR201. A restriction map and the relative positions of various subclones are shown. ORF 1 and 2 represent *ecpA* and *B* respectively. ORF 3 represents the gene for the 31.5 kDa haemagglutinin (see Rao *et al.*, 1993). Large arrows indicate the positions of each gene as well as the direction of transcription. Small arrows denote the direction of transcription from the plasmid *lac* promoter. Sizes of proteins encoded (kDa) are as determined by SDS-PAGE analysis of [³⁵S]methionine-labelled maxicells.

contained a 3.6 kb fragment of DNA from *Eik. corrodens*. A restriction map indicating the position of the open reading frame (ORF) encoding the haemagglutinin is shown in Fig. 1 (ORF 3). Maxicells containing this cloned DNA produced, in addition to the 31.5 kDa protein with haemagglutination activity, two closely migrating proteins with molecular masses of approximately 14 kDa each (see Fig. 4, lane 3, of Rao *et al.*, 1993). We previously presented evidence suggesting that these three proteins are expressed in *E. coli* under the control of a cloned *Eikenella* promoter. Examination of the nucleotide sequence of a 1670 bp fragment containing the haemagglutinin gene allowed the identification of a potential promoter sequence just upstream of the ORF. Since there were no other identifiable ORFs downstream of the haemagglutinin gene, it seemed likely that the two 14 kDa proteins were encoded by genes contained in the 1400 bp upstream of the haemagglutinin gene and that they are expressed under the control of one or more promoters distinct from that controlling expression of the haemagglutinin. Indeed, transformants harbouring pVKR203, which contains a 1.9 kb *EcoRI*–*HindIII* fragment, directed the

production of a 14 kDa protein as visualized by the maxicell technique (Fig. 1).

Nucleotide sequence of two pilin genes from *Eik. corrodens*

The complete nucleotide sequence of the 1.9 kb insert of pVKR205 (see Table 1 for description) was determined. Oligonucleotides were synthesized based on the sequences of the ends of pVKR205 and pVKR204 in order to sequence the joint between these two subclones. In doing so, we identified an additional 82 bp fragment which was deleted during the construction of both pVKR204 and pVKR205 due to the presence of an additional *EcoRI* site which could not be mapped. Fig. 2 shows a portion of the sequence of the coding strand of the insert of pVKR201 (see Fig. 1) extending from the first *KpnI* site to the terminal *HindIII* site. This position includes the complete insert of pVKR205, the 82 bp *EcoRI*–*EcoRI* fragment and a 426 bp portion of pVKR204 which has been described previously (Rao *et al.*, 1993). We have identified two complete ORFs on this fragment. The first, ORF 1, begins at position 1098 and

[illegible]

Fig. 2. Nucleotide sequence of a 2386 bp fragment from pVKR201 containing two pilin genes, *ecpA* (ORF 1) and *ecpB* (ORF 2), from *Eik. corrodens*. The deduced amino acid sequence is shown below the nucleotide sequence. The positions of a potential -10 promoter sequence, an *ntrA*-dependent promoter-like sequence (*ntr1*), and a potential ribosome-binding site S-D 1 preceding *ecpA* are indicated above the sequence. The positions of an *ntrA*-dependent promoter-like sequence and a potential ribosome-binding site preceding *ecpB* are also indicated above the sequence (*ntr2* and S-D 2, respectively). Lines above the sequence indicate the positions of inverted repeats which may function in transcription termination.

extends 477 nucleotides to position 1574. At this position there are two directly consecutive TAA termination codons. ORF 1 encodes a polypeptide of 159 amino acids with a molecular mass of 16.2 kDa as deduced from the nucleotide sequence. ORF 2, which is read in the same direction as ORF 1, but in the -1 frame, begins at position 1652 and extends 477 nucleotides to position 2128. At this point, there is a TGA termination codon. There are two additional stop codons 12 nucleotides downstream, TGA and TAA arranged consecutively, from position 2144 to 2149. This ORF, like the first, encodes a polypeptide of 159 amino acids, with a deduced molecular mass of 17.4 kDa. The third ORF, encoding the previously described haemagglutinin (Rao *et al.*, 1993), begins 122 nucleotides downstream at position 2250 and is read in the same direction but in the $+1$ frame relative to ORF 1.

Transcription of both ORF 1 and ORF 2 occurs in a direction opposite to that of the vector *lac* promoter as indicated in Fig. 1 and consistent with our finding that expression was independent of orientation of the insert in the vector. ORF 1 is preceded by a strong Shine-Dalgarno sequence (Gold *et al.*, 1981), AGGA, at positions -11 to -14 , while ORF 2 is preceded by a somewhat different potential ribosome-binding site, TGCGG, from positions -9 to -13 . Upon examination of sequences upstream of ORF 1, we identified, from positions 914 to 919, a hexanucleotide, TATAAT, which is identical to the consensus sequence for the Pribnow or TATA box portion of prokaryotic promoters (Rosenberg & Court, 1979). However, we could identify no sequences with similarity to the classical -35 sequence. Interestingly, from positions 949 to 960 is the sequence TTGGCATA GCCG TTGCT, which differs in only two positions from the *ntrA*-dependent promoter consensus sequence, CTGG(C/T)A(C/T)(G/A) NNNN TTGCA (Gussin *et al.*, 1986). A search of the 300 bp immediately upstream of this potential promoter region, however, did not reveal any potential NifA or NtrC binding sites (TG₁₀ACA) as were identified upstream of the *P. aeruginosa* pilin gene and found to be important in the regulation of its expression (Pasloske *et al.*, 1989). Determination of which of these two regions represents the actual promoter will await the completion of primer-extension experiments. We could identify no sequences with strong homology to the *E. coli* promoter consensus sequences immediately upstream of ORF 2. However, from positions 1525 to 1542 is the sequence CTGGTACC GCGA CTGGC, which is somewhat similar to the *ntrA*-dependent promoter consensus cited above, but much less so than that upstream of ORF 1. It is of note that this sequence occurs within the coding sequence of ORF 1. Once again, there were no sequences typical of NifA or NtrC binding sites. Beginning 21 nucleotides down-

stream of the TAA termination codon of ORF 1 at position 1598 and extending to position 1625 is a potential stem-loop structure. Similarly, beginning 21 nucleotides downstream of the TGA termination codon of ORF 2 at position 2152 and extending 30 nucleotides to position 2181 is a second potential stem-loop structure. Each of these may be important in the rho-independent termination of transcription of the respective gene (Rosenberg & Court, 1979).

The deduced amino acid sequences of the proteins encoded by ORFs 1 and 2 were nearly identical over the first 35 amino acids. Moreover, this N-terminal sequence was identical to the N-terminus of the *Moraxella bovis* Q-pilin subunit (Marrs *et al.*, 1985) and strikingly similar to the N-termini of other type 4 pilin subunit proteins. From these data, it was clear that ORFs 1 and 2 encode the pilin subunits of *E. corrodens*. We have named these genes *ecpA* and *ecpB* respectively.

Subcloning of *ecpA* and *ecpB*

As we had the complete nucleotide sequence, it was possible to construct subclones containing each pilin gene. Because of the uncertainty regarding the promoter(s) for these genes, both subclones were constructed such that expression could be under the control of the vector *lac* promoter. The entire ORF of *ecpA* is contained in pVKR209, in which a 600 bp *AvaI*-*EcoRV* fragment extending from position 1041 to 1630 was ligated to the *HincII*-*AvaI* sites of pUC18. This subclone contains little flanking DNA and contains no sequences from the *ecpB* ORF. As noted above, pUC18 was chosen so as to allow transcription from the *lac* promoter. Only 117 nucleotides separate the end of ORF 2 (*ecpB*) and the start of ORF 3, which codes for the haemagglutinin. In this fragment, we could identify no unique restriction sites which would allow convenient subcloning of only *ecpB*, while allowing its insertion in the proper orientation with respect to the plasmid promoter. We therefore ligated a 750 bp *EcoRV*-*KpnI* fragment, extending from position 1630 to 2386 to the *KpnI*-*HincII* sites of pUC19 in order to construct pVKR208. While this construct contains the entire *ecpB* gene with little upstream DNA, it does include 257 nucleotides of downstream DNA, including 140 nucleotides from ORF 3, which encode the N-terminal 46 amino acids of the 31.5 kDa haemagglutinin.

Occurrence of *ecpA* in other strains of *Eik. corrodens*

In order to determine if these pilin genes are present in strains of *Eik. corrodens* other than the type strain, ATCC 23834, we analysed the chromosomal DNA of 10 additional strains of *E. corrodens* using *ecpA* as a probe.

Fig. 3. Occurrence of *ecpA* in other strains of *Eik. corrodens*. Restriction endonuclease digests of genomic DNA from various *Eikenella* strains were probed with 32 P-labelled *ecpA* DNA. Lanes: 1, *E. coli* JM109 chromosomal DNA digested with *Hind*III; 2–12, chromosomal DNA from *Eik. corrodens* strains digested with *Eco*RV (2, ATCC 23834; 3, EC-14; 4, EC-23; 5, EC-26; 6, EC-37; 7, EC-38; 8, EC-50; 9, FDC373; 10, FDC470; 11, FDC1073; 12, ATCC 43278). The sizes of λ DNA fragments (digested with *Hind*III and *Eco*RI) are shown on the left.

Four of these strains, FDC373, FDC470, FDC1073 and ATCC 43278, are well-characterized laboratory strains. The remaining six, EC-14, 23, 26, 37, 38 and 50, are fresh clinical isolates obtained at the University of Florida. As shown in Figure 3, under conditions of moderate stringency, fragments complementary to *ecpA* were present in *Eco*RV digests of genomic DNA from the *Eik. corrodens* type strain, but not in digests of DNA from *E. coli* JM109. Moreover, strongly hybridizing bands of varying size were detected in all 10 of the other *Eik. corrodens* strains tested. In many cases, additional bands were also detected.

Discussion

From a clone bank constructed in *E. coli* JM109 of the chromosomal DNA from *Eik. corrodens* ATCC 23834, we have identified two distinct genes for pilin subunits of the type 4 or N-Met-Phe class. The two genes are located on a 3.6 kb *Hind*III fragment and are separated by only 75 nucleotides. Both appear to be complete genes and have the same orientation. The nucleotide sequences of the two genes are related, with 70% identity over 162 bp with no gaps. However, this similarity is limited to the first 162 nucleotides of each gene, with no significant

relationship observed between the sequences beyond this point or upstream of each gene. This lack of homology is in contrast to the case with *N. gonorrhoeae* strain MS11_{ms}, in which the two *pilE* loci share a 65 bp sequence downstream of each ORF (Meyer *et al.*, 1984). As could be predicted from the nucleotide sequence homology, the amino acid sequences of the two proteins are identical over the N-terminal 29 amino acids. Moreover, over the entire sequence, the two proteins are 42.4% identical and can be aligned with only a single gap. This pattern of identity over the N-terminal 30 amino acids is typical of the type 4 family of pilin subunits, which are all strikingly similar at the N-terminus, with great differences occurring further downstream. These differences occur even between the pilin subunits of a single strain, as is observed here for *Eik. corrodens*.

As could be inferred from the similarities of the amino acid sequences, the two *Eik. corrodens* pilin genes *ecpA* and *ecpB* exhibit strong homology to type 4 pilin genes from a variety of other bacterial species. *ecpA* is most similar to the Q-pilin gene from *Moraxella bovis*, with 61.7% identity over a 632 bp overlap. This homology extends over the entire sequence of each gene, beginning approximately 20 bp upstream of the translation start site and extending to approximately 115 bp downstream of each gene. Interestingly, this region includes the first 35 nucleotides of *ecpB*, although its homologue in *M. bovis* is not part of an ORF. *ecpB* is much less homologous to pilin genes from other bacteria. It is, however, similar to the pilin gene from *P. aeruginosa* strain P1, with 75% identity over a 111 bp overlap (Pasloske *et al.*, 1988). This portion of the gene includes the coding for only the N-terminal 37 amino acids.

A comparison of the proteins encoded by *ecpA* and *B* with other type 4 pilin proteins reveals many interesting features. These two pilins can be aligned with pilins from other bacterial genera as shown in Fig. 4. One common characteristic of this group of proteins is the presence of an unusually short leader sequence, which is cleaved followed by methylation of the terminal phenylalanine. The pilin of *Vibrio cholerae* also undergoes this methylation process, although in this case, the N-terminal residue is methionine instead of phenylalanine (C. Kaufman, personal communication). Although this must be confirmed by isolation of the pilins from *Eikenella* and determination of their N-terminal sequences, it can be assumed that these pilins, which exhibit strong homology to other type 4 pilins, also undergo similar post-translational processing. If true, pilin A possesses a seven-amino-acid leader sequence as do the pilins of *D. nodosus* (Elleman, 1988) and *N. gonorrhoeae* (Meyer *et al.*, 1984), while pilin B possesses a six-amino-acid leader sequence analogous to pilins of

<i>ecpA</i>	M K Q V Q K G F T L I E L M I V I A I I G I L A A
<i>ecpB</i>	M Y K Q K G F T L I E L M I V I A I I G I L A A
<i>M. b.</i>	M N A Q K G F T L I E L M I V I A I I G I L A A
<i>D. n. (A)</i>	M K S L Q K G F T L I E L M I V V A I I G I L A A
<i>D. n. (B)</i>	M K S L Q K G F T L I E L M I V V A I I G I L A A
<i>P. a.</i>	M K A Q K G F T L I E L M I V V A I I G I L A A
<i>N. g.</i>	M N T L Q K G F T L I E L M I V I A I I V G I L A A

<i>ecpA</i>	I A L P L Y Q D Y I S K S Q V T R A Y G E M A G T
<i>ecpB</i>	I A L P L Y Q D H M A K A Q I N R V F Y E L G S T
<i>M. b.</i>	I A L P L Y Q D Y I S K S Q T T R V V G E L A A G
<i>D. n. (A)</i>	F A I P A Y N D Y I A R S Q A A E G L T L A D G L
<i>D. n. (B)</i>	I A I P Q Y Q N Y I A R S Q V S R V M S E T I Q M
<i>P. a.</i>	I A I P Q Y Q N Y V A R S E G A S A L A S V N F L
<i>N. g.</i>	V A L P A Y Q D Y T A R A Q V S E A I L L A E G Q

<i>ecpA</i>	K T A I E A A L F E G R T P V L A A T A A A G A A
<i>ecpB</i>	K T A V E S I L A H G G I P T V D P S Q D G V V Q
<i>M. b.</i>	K T A V D A A L F E G K T P K L G K A A N D T E E
<i>D. n. (A)</i>	K V R I S D H L E S G E C K G D A N P A S G S L G
<i>D. n. (B)</i>	R T A I E T C V L D G K E A D K C F I G W T G S N
<i>P. a.</i>	K T T V E E A L S R G W S V K S G T G T E
<i>N. g.</i>	K S A V T E Y Y L N H G K W P E N N T S A G V A S P

<i>ecpA</i>	A T P P N E W V G M L D N P T S N L L S A A T L T
<i>ecpB</i>	N S R R L E F L G L N Q N P N S N L I F T A S V G
<i>M. b.</i>	D I G L T T T G G T A R S N L M S S V N I G
<i>D. n. (A)</i>	N D D K G K Y A L A T I D G D Y N K D A K T A D E
<i>D. n. (B)</i>	L L D G E F T A G T E S T A A A T G Q T G I T I K
<i>P. a.</i>	D A T K K E V P L G V A A D A N K L G T I A L K
<i>N. g.</i>	P S D I K G K Y V K E V E V K N G V V T A T M L S

<i>ecpA</i>	P G A N A G D V T F V G T L G E N A N S S I H G
<i>ecpB</i>	L N S N Q F E R V N A T F G R N A L P Q I Q G
<i>M. b.</i>	G G A F A T G A G T L E A T L G N R A N K D I A G
<i>D. n. (A)</i>	N G C K V V I T Y G G T A G E K I S K L I V G
<i>D. n. (B)</i>	P V A A D D E G N I V A T F G R N A A A A I K P
<i>P. a.</i>	P D P A D G T A D I T L T F T M G G A G P K N K G
<i>N. g.</i>	S G V N N E I K G K K L S L W A R R E N G S V K

<i>ecpA</i>	A T I T L T C T A S G E W T C A V A A G T A T G W
<i>ecpB</i>	A V I S F V R D N Q G Q W T C E I D K S N A A Y C
<i>M. b.</i>	A V I T Q S R D A E G V W T C T I N G S A A P G W
<i>D. n. (A)</i>	K K L V L D Q F V N G S Y K Y N E G E T D L
<i>D. n. (B)</i>	Q T L T W S R S K E G T W T C A T T V
<i>P. a.</i>	K I I T L T R T A A D G L W K C T S D Q D
<i>N. g.</i>	W F C G Q P V T R T D D D T V A D A K D G K E I D

<i>ecpA</i>	K T K F V P S G C N
<i>ecpB</i>	P P K Y T P A T C V T I
<i>M. b.</i>	K S K F V P T G C K E
<i>D. n. (A)</i>	E L K F I P N A V K N
<i>D. n. (B)</i>	E A K F Q P T G C K D G K
<i>P. a.</i>	E Q F I P K G C S R
<i>N. g.</i>	T K K E I D T H L P S T C R D K A S D A K

Fig. 4. Comparison of the two pilins from *Eik. corrodens* with other type 4 pilins. The amino acid sequences of the two pilins from *Eik. corrodens* ATCC 23834 as deduced from the nucleotide sequences of *ecpA* and *B* are aligned with those of pilins from *Moraxella bovis* EPP63 (*M. b.*; Marrs *et al.*, 1985), *Dichelobacter (Bacteroides) nodosus* strain 198 (serotype A) (*D. n. (A)*; Elleman & Hoyne, 1984), *D. nodosus* strain 340 (serotype D) (*D. n. (B)*; Finney *et al.*, 1988), *Pseudomonas aeruginosa* PAK (*P. a.*; Pasloske *et al.*, 1985) and *Neisseria gonorrhoeae* MS11 (*N. g.*; Meyer *et al.*, 1984).

Moraxella spp. (Marrs *et al.*, 1985) and the majority of *P. aeruginosa* pilins (Pasloske *et al.*, 1988). The last three residues of both leaders, Q K G, are identical to those of all of the other pilins as shown in Fig. 4. This intrastain variability in leader sequence length has not been previously reported in type 4 pilins, although different strains of *P. aeruginosa* have been found to have leaders of different lengths (Pasloske *et al.*, 1988). Most of the other pilins possess two cysteine residues which are believed to be in the oxidized state (Rothbard *et al.*,

1984; Elleman, 1988). The class II pilins from *D. nodosus* (serotypes D and H) (Elleman, 1988) as well as one strain of *P. aeruginosa* (K1224) (Pasloske *et al.*, 1988) possess four cysteine residues. Interestingly, both of the *Eikenella* pilins possess only three cysteine residues, all of which are located close to the C-terminus. The cysteine residues at positions 132 and 151 of pilin A are at positions comparable to those in the Q-pilin of *M. bovis* (Marrs *et al.*, 1985). Pilin B, like A, possesses two cysteine residues positioned so as to form a disulphide loop of 18 amino acids, although in this case they are located at positions 131 and 150 of the mature polypeptide. Interestingly, while the third cysteine of pilin A is located outside the potential disulphide loop at position 124, that of pilin B, at position 141, is within the loop. The impact of this difference on pilin and/or pilus structure is unclear.

Experimental data suggested that the pilin genes are expressed in *E. coli* under the control of an *Eikenella* promoter. Examination of sequences upstream of *ecpA* revealed homology to the consensus -10 (TATA box) region of bacterial promoters, but no regions homologous to the -35 sequence (Rosenberg & Court, 1979). Such an arrangement is typical of genes whose expression is regulated and is similar to that observed upstream of the *pilE* transcriptional initiation site in *N. gonorrhoeae* (Meyer *et al.*, 1984). It is interesting to note that of the type 4 pilin genes that have been sequenced thus far, only the *Eikenella* pilin (*ecpA*) and *Neisseria* pilin (*pilE*) possess the TATAAT promoter element. As is the case with the remaining pilin genes, no σ^{70} promoter-like sequences are present upstream of *ecpB*. Since both proteins are produced in *E. coli* harbouring pVKR201, the two genes appear to be transcribed together as a polycistronic message. Evidence for this rests in the fact that transformants harbouring pVKR207, which contains the entire ORF of *ecpB* as well as the 3' half of *ecpA*, do not produce the pilin protein (data not shown). Therefore, the region between *ecpA* and *B* does not contain all of the sequences necessary for expression of *ecpB* in *E. coli*. Johnson *et al.* (1986) determined the transcriptional initiation site of two *P. aeruginosa* pilin genes and identified sequences similar to *ntrA*-dependent promoter sequences. Similar sequences were identified upstream of type 4 pilin genes from other genera as well. In addition, Pasloske *et al.* (1989) identified enhancer sequences upstream of the *P. aeruginosa* pilin gene. It is interesting that both pilin genes from *Eik. corrodens* possess *ntrA*-dependent promoter-like sequences consistent with that observed with other type 4 pilin genes. However, as we were unable to identify the regulatory sequences which function as the targets of either *ntrC* or *nifA*, the functionality of these promoter-like sequences is unclear. While it is likely that in *E. coli*, the σ^{70} -like promoter

upstream of *ecpA* is used, the other promoters may be of greater significance in *Eikenella*. Alternatively, the presence of two different types of promoters may be indicative of a mechanism by which the organism differentially expresses these putative virulence factors under varying environmental conditions.

The two pilin genes from *Eik. corrodens* described here appear to be complete and are transcribed in the same direction. This type of organization appears to be in contrast to that observed for other type 4 pilin genes. Strains of both *P. aeruginosa* and *D. nodosus* have each been found to contain only one complete pilin gene (Elleman, 1988). *Moraxella* spp. have been found to contain two pilin genes, one of which is non-expressed, as it lacks the N-terminal coding regions and is oriented in a direction opposite to that of the promoter. Switching is believed to occur by an inversion event which brings the non-expressed gene into apposition with the N-terminal coding region and in phase with the promoter (Marrs *et al.*, 1988). *N. gonorrhoeae*, on the other hand, possesses multiple pilin loci, the majority of which are silent as they lack promoter sequences. Laboratory strain MS11_{ms}, which has been characterized extensively (Meyer *et al.*, 1984), also possesses two pilin genes in expression loci. However, in contrast to the case with *Eik. corrodens*, in which the two pilin genes are separated by only 75 nucleotides, the two *pilE* loci of *N. gonorrhoeae* strain MS11_{ms} are separated by approximately 20 kb. Strain MS11_{ms} is apparently unique in that other strains of *N. gonorrhoeae* possess only one copy of *pilE* (Meyer *et al.*, 1990). It is not presently known if both types of pili are expressed by *Eik. corrodens* simultaneously or if some type of switching event similar to that observed for *N. gonorrhoeae* or *M. bovis* occurs here. The fact that colony morphology undergoes spontaneous change at high frequency (corroding to non-corroding transitions) is certainly suggestive of some type of switch mechanism.

Examination of codon frequencies for *ecpA* reveals several interesting features. There is a definite bias against the use of the codons GGG, GGA, AGG, AGA, ATA, CGG, CGA and CTA, which are recognized by minor tRNA species (Grosjean & Fiers, 1982). Of these codons, only CTA appears in *ecpA*. Similarly, of the codons UCA, UCG, ACG and CCC, which appear only rarely in *E. coli* genes which are highly expressed, only CCC is used in *ecpA*. These data are strikingly similar to the codon usage observed for the Q-pilin gene from *M. bovis*, in which both CCC and CTA are utilized, but the remaining rare codons are excluded (Marrs *et al.*, 1985). The codon usage in *ecpB* is, however, very different. Indeed, of the 12 codons listed above, only three, CTA, CGA and TCG, are not used in *ecpB*. This codon usage is in contrast also to that observed for *M. bovis* (Marrs

et al., 1985), *P. aeruginosa* (Sastry *et al.*, 1985), and *D. nodosus* (Elleman & Hoyne, 1984). The significance of these differences in codon usage is unclear, but may once again suggest a means of maintaining expression of pilin genes under different growth conditions. Alternatively, these differences may reflect different origins for the two genes. It is interesting to note that while *ecpA* is homologous at the nucleotide level to the pilin gene from *M. bovis*, *ecpB* is more similar to pilins from *Pseudomonas* spp. Moreover, while the G + C content of *ecpA* is 48.3 mol%, that of *ecpB* is 42.7 mol%. Both are quite different from that determined for the chromosomal DNA of *Eik. corrodens* (56–58 mol%) (Jackson & Goodman, 1984), which suggests that they may have been derived from different sources. It is tempting to theorize that *ecpA* was obtained by *Eik. corrodens* from *M. bovis* or, more likely, from the human pathogen, *Moraxella nonliquefaciens* (Tønnum *et al.*, 1991), while *ecpB* came from *Pseudomonas* spp. Piliation has often been correlated with competence for genetic transformation. Such a mechanism may have been important in the acquisition of additional pilin genes.

Although these genes appear to have been acquired by *Eikenella* relatively recently, they seem nevertheless to be a consistent part of the genome of this organism. DNA fragments homologous to *ecpA* were identified in all 10 of the *Eikenella* strains tested here, despite their diverse origins. As shown in Fig. 3, the *EcoRV* fragments containing *ecpA* are of varying sizes, suggesting a heterogeneity of the DNA surrounding this gene. Although several of the strains possess more than one homologous fragment, only one fragment is observed in the type strain. *EcoRV* was chosen to digest the chromosomal DNA samples since a site for this enzyme is present between the two pilin genes in the type strain. Since the two pilin genes are homologous only at their 5' ends, conditions of much lower stringency than utilized here must be employed to visualize both bands. Multiple homologous fragments are observed in the chromosomal DNA from several of the strains, including EC-14, EC-37, EC-50, FDC470, FDC1073 and ATCC 43278. In some cases, such as with EC-14, EC-37 and EC-50, in which there is one intense and one fainter band, the additional band may represent a second pilin gene more homologous to *ecpA* than is *ecpB*. It is interesting that DNAs from both FDC470 and ATCC 43278 each contain two weakly hybridizing bands. These strains may possess two pilin genes neither of which is closely related to *ecpA*, but nevertheless more so than *ecpB*. Alternatively, unlike the type strain, the pilin genes of these strains may contain an *EcoRV* site within the coding region.

ecpA and *ecpB* are the first pilin genes to be cloned from the genus *Eikenella*, and they represent the first

molecular evidence for the presence of pili in this organism. The fact that fragments homologous to the cloned pilin gene were identified in all of the *Eikenella* strains tested suggests that these proteins may be important in the survival and/or virulence of this bacterium. Our future studies will be directed toward assessing the roles these pili play in the pathogenic process.

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