Identification and Characterization of a Fimbrial Gene Cluster of *Edwardsiella tarda* Expressing Mannose-resistant Hemagglutination

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ABSTRACT—We determined the nucleotide sequence of a 8.6 kb DNA region containing *etfA* encoding a putative fimbrial major subunit from chromosomal DNA of *Edwardsiella tarda* KG8401 which expresses mannose-resistant hemagglutination (MRHA). This region contained three novel genes, *etfBCD*, at the downstream region of *etfA*. The deduced amino acid sequences of EtfABCD contained conserved fimbrial domains; fimbrial protein, fimbrial chaperone, fimbrial usher and fimbrial protein, respectively. *Escherichia coli* transformed with the cloned *etf* operon expressed MRHA and fimbriae that reacted with rabbit antiserum against the fimbrial major subunit of *E. tarda*, showing the implication of the fimbriae in the hemagglutination of *E. tarda*.

Key words: *Edwardsiella tarda*, mannose-resistant hemagglutination, fimbriae, gene cluster

Edwardsiella tarda has a wide host range, having been isolated from a variety of animals including fish, birds, mammals and reptiles (White *et al.*, 1973; Owens *et al.*, 1974; van Damme and Vandepitte, 1980; Goldstein *et al.*, 1981). Edwardsiellosis in Japanese flounder *Paralichthys olivaceus*, red sea bream *Pagrus major* and Japanese eel *Anguilla japonica* is characterized by skin lesions and formation of abscesses and granulomas in internal organs such as liver, kidney and spleen (Miyazaki and Kaige, 1985; Rashid *et al.*, 1997) and causes serious damage to their aquaculture industry (Wakabayashi and Egusa, 1973; Nakatsugawa, 1983; Miyazaki and Kaige, 1985; Kusuda and Salati, 1993)

Pathogenicity of *E. tarda* seems to be multifactorial. Several virulence properties and factors have been reported, namely dermonecrotic and lethal toxins (Ullah and Arai, 1983; Suprapto *et al.*, 1996), anti-phagocyte killing (Ainsworth and Chen, 1990; Iida and Wakabayashi, 1993), hemolysins (Janda and Abbott, 1993; Chen *et al.*, 1996; Hirono *et al.*, 1997; Strauss *et al.*, 1997), siderophore (Igarashi *et al.*, 2002), serum resistance and the ability to invade epithelial cells (Janda *et al.*, 1991; Ling *et al.*, 2000). However, very little is known

about the roles of these factors in disease occurrence and the bacterial invasion process, and also the portal of entry of *E. tarda* into hosts has not been shown precisely.

For many pathogenic bacteria, adherence to host is mediated by adhesin existing on the tip of fimbriae (Hoepelman and Tuomanen, 1992; Kuehn *et al.*, 1992; Jones *et al.*, 1995). Although *E. tarda* has fimbriae that exhibit mannose-resistant hemagglutination against guinea pig erythrocytes, hemagglutinin has not been identified (Nowotarska and Mulczyk, 1977). In the previous paper, we identified a gene, *etfA*, encoding a 19.3 kDa protein that was associated with the possession of hemagglutinating activity among *E. tarda* strains (Sakai *et al.*, 2003). The predicted amino acid sequence of EtfA has a significant homology with type-1 fimbrial major subunits of *Serratia marcescens* (Nichols *et al.*, 1990) and *Escherichia coli* O157:H7 (Perna *et al.*, 2001) and long polar fimbrial major subunit of *Salmonella typhimurium* (Bäumler and Heffron, 1995). Type-1 and P fimbriae of *E. coli* are constructed with several thousands copies of the major subunit, forming helical filament, and several minor subunits, and carbohydrate-binding adhesin is located at the distal end of the fimbria (Lindberg *et al.*, 1987; Gong and Makowski, 1992; Kuehn *et al.*, 1992; Jones *et al.*, 1995). Genes encoding fimbrial subunits and accessory proteins participating

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in the formation of fimbriae constitute an operon, and the gene encoding adhesin is located downstream in a fimbrial gene cluster (Boyd and Hartl, 1998).

In this study, we determined the DNA sequence of a fimbrial gene cluster, *etfABCD*, from chromosomal DNA of *E. tarda* and showed that *E. coli* harbored a DNA fragment containing *etfABCD* expressed mannose-resistant hemagglutination against guinea pig erythrocytes.

Materials and Methods

Bacterial strains and growth conditions

E. tarda KG8401 isolated from Japanese eel was cultured at 28°C for 24 h on yeast extract agar, consisted of 1% polypepton, 0.5% Bacto-yeast extract (Difco Laboratories, USA), 0.5% NaCl and 1.5% agar at pH 7.2. *E. coli* JM109 was grown in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989).

Hemagglutination and hemagglutination inhibition test

Hemagglutination test and hemagglutination inhibition test were performed according to Sakai *et al.* (2003). Briefly, a 3% (v/v) formalin-fixed erythrocyte suspension in phosphate-buffered saline containing 1% bovine serum albumin (PBS-BSA) was added to serial twofold dilution of bacterial cells suspended in PBS-BSA at 0.1 g (wet wt) bacteria/mL. Hemagglutination titer was defined as the reciprocal of the highest dilution of a test sample that showed complete hemagglutination. For hemagglutination inhibition test, serial twofold dilution of bacterial cells was prepared in PBS-BSA contained 1% D-mannose or 1% fetuin (Sigma Aldrich, USA).

DNA manipulation

Preparation of chromosomal DNA, restriction endonuclease digestion, ligation, transformation and DNA electrophoresis were performed as described by Sambrook *et al.* (1989). Plasmid DNA was purified using Quantum Prep Plasmid Miniprep Kit (Bio-Rad Laboratories, USA).

Southern hybridization and colony hybridization were performed according to the DIG systems user's guide (Roche Applied Science, Germany). The oligonucleotide probe was end-labeled with digoxigenin (DIG) using DIG Oligonucleotide 3'-End Labeling Kit (Roche Applied Science, Germany).

Nucleotide sequences were determined by the dideoxy chain termination method with BigDye Terminator Cycle Sequencing Kit and ABI 377 DNA sequencer (Applied Biosystems, USA).

Computerized sequence analysis

The nucleotide sequence data was analyzed using the DNASIS program (Hitachi Software, Japan) and the GENETYX sequence analysis program (Software Development, Japan). Analysis of a signal sequence of the deduced aimino acid sequence was performed with the SOSUI program available at the Tokyo University of Agriculture and Technology website (http://sosui. proteome.bio.tuat.ac.jp/sosuimenu0.html). Homology searching was performed using the FASTA program served by Genome Net (Bioinfomatics Center, Institute for Chemical Research, Kyoto University, http://fasta. genome.ad.jp/SIT/FASTA.html). The conserved domain database (CDD) and CD-Search service at the National Center for Biotechnology Information (http:// www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) were used to identify the conserved domains present in protein sequences.

Expression of EtfABCD in E. coli JM109

A DNA fragment containing *etfABCD* was PCR amplified with sense primer, 5'-CACTTTCCGCAACC-ATGATC-3' (positions 1574 to 1593 in Fig. 2), and antisense primer, 5'-CTCTCCTTGTCACAATAACGC-3' (positions 7290 to 7310 in Fig. 2). PCR was performed with TaKaRa LA PCR Kit (TaKaRa Bio Inc., Japan) according to the following amplification protocol: after an initial denaturation step (94°C, 2 min), 30 cycles of denaturation (94°C, 30 s), annealing (65°C, 30 s) and extension (72°C, 7 min), followed by a final extension step (72°C, 10 min). The PCR product was ligated into a pGEM-T Easy vector (Promega, USA) and transformed into *E. coli* JM109. Hemagglutinating acitivity and fimbrial formation of cells transformed with recombinant plasmid DNA were then examined.

Immunogold electron microscopy

Bacterial cells were incubated for 1 h at room temperature in rabbit anti-19.3-kDa protein (EtfA) serum (Sakai *et al.*, 2003) diluted to 1:100 in PBS-BSA at 10 mg (wet wt) bacteria/mL. After washed thrice with PBS-BSA, bacterial cells were incubated for 1 h at room temperature in goat anti-rabbit IgG-gold (10 nm; Sigma USA) diluted to 1:100 in PBS-BSA. After washed thrice with 2% ammonium acetate, bacterial cells were negatively stained with 1% sodium phosphotungstate (pH 7.0) and examined under a JEM 100S electron microscope (JEOL, Japan) at 80 kV.

Results and Discussion

Cloning and sequencing of the etf operon

Two oligonucleotide probes were designed based on the nucleotide sequence of a 3.0-kb *Sma* I -*Acc* I fragment containing *etfA* (Sakai *et al.*, 2003), pNUF1 in Fig. 1. Hybridized 2.5-kb *Pst* I and 4.4-kb *EcoR* I -*Sph* I fragments from chromosomal DNA were each subcloned into pUC18, designated pNUF2 and pNUF3 (Fig. 1), respectively, and sequenced. And also, a 1.7-kb *Acc* I fragment hybridized with an oligonucleotide probe designed from the nucleotide sequence of the *EcoR* I -
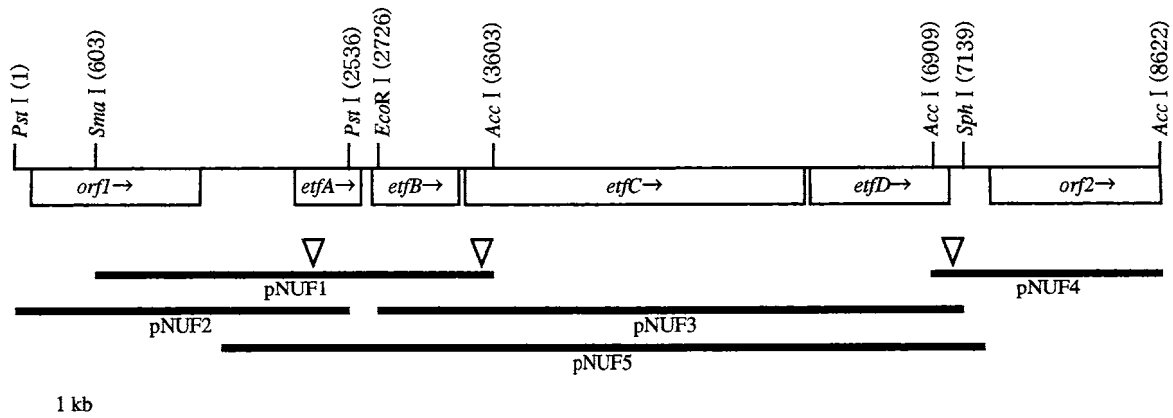
Fig. 1. Restriction and genetic map of the *E. tarda* KG8401 chromosome DNA surrounding *etfA*. Boxes and arrows indicate the open reading frames and the direction of transcription, respectively. The positions of the DNA fragments cloned in 5 recombinant plasmid DNA derivatives (pNUF1, pNUF2, pNUF3, pNUF4 and pNUF5) are given at the bottom of the figure. Triangles indicate the site of the oligonucleotide probes.

Sph I fragment was cloned into pUC18, designated pNUF4 (Fig. 1). The nucleotide sequence of a 8,627-bp DNA region consisted of these overlapping fragments was determined (Fig. 2). This region contained 4 open reading frames, designated *etfABCD*, and a complete open reading frame, designated *orf1*, and a partial open reading frame, designated *orf2*, were found at the sides of the *etf* genes (Figs. 1 and 2). Start and stop codons of the *etf* genes were separated by only a few base pairs. Furthermore, putative ribosome binding sites (Shine and Dalgarno, 1975) were identified at the upstream regions of each gene from *etfA* to *etfD* (Fig. 2). And also, putative promoter sequences located at the upstream regions of *etfA* and *orf2* (Fig. 2). It is suggested that the *etf* genes constitute an operon. The positions of *etfABCD* and sizes of the predicted polypeptides are shown in Table 1 and Fig. 1. All predicted polypeptides were found to contain acceptable signal sequences.

Homology of EtfABCD to other fimbrial proteins

The deduced amino acid sequences of all 4 *etf* genes showed homology to proteins from various bacterial fimbrial systems. EtfABCD revealed homology with type 1 fimbrial major subunit (FimA) of *S. marcescens* (68.9% identity; E value, 4.7e-42) (Nichols *et al.*, 1990), a putative long polar fimbrial chaperone protein (LpfB) of *E. coli* (38.4% identity; E value, 2.7e-26), F1C fimbrial usher protein (FocD) of *E. coli* (39.2% identity; E value, 3.3e-99) (Welch *et al.*, 2002) and a fimbrial protein of *E. coli* (26.3% identity; E value, 2.5) (Hayashi *et al.*, 2001), respectively. The deduced amino acid sequences of Orf1 and Orf2 revealed homology with inosine kinase of *Yersinia pestis* (85.7% identity; E value, 1.5e-155) (Parkhill *et al.*, 2001) and UDP-sugar hydrolase of *Photorhabdus luminescens* subsp. *laumondii* (72.4% identity; E value, 2.7e-135) (Duchaud *et al.*, 2003),

respectively. And also, the domains of fimbrial proteins participating in the formation of fimbriae were shown in the deduced amino acid sequences of mature EtfABCD on the CD-search (Table 2). It is suggested that EtfA, EtfB, EtfC and EtfD function as a fimbrial major subunit, fimbrial chaperone, fimbrial usher and fimbrial subunit, respectively. It is known that long polar fimbriae and F1C fimbriae of *E. coli* are synthesized by chaperone-usher fimbrial biosynthesis system (Soto and Hultgren, 1999). Fimbriae of *E. tarda* may consist of fimbrial subunits, EtfA and EtfD, mediated by fimbrial chaperone, EtfB, and fimbrial usher, EtfC.

Electron microscopic observation and hemagglutination of E. coli *harbored the* etf *fimbrial operon*

Immunogold-labeling electron microscopy revealed that gold particles attached to the fimbrial structure of *E. coli* JM109 harbored plasmid DNA containing the *etf* operon (pNUF5) (Fig. 3A). On the contrary, no gold particles were observed on the fimbrial structure of *E. coli* JM109 lacking pNUF5 (Fig. 3B).

E. coli JM109 harbored pNUF5 acquired hemagglutinating activity, which was not inhibited by D-mannose but was strongly inhibited by fetuin like *E. tarda* KG8401 (Table 3). It is suggested that fimbriae encoded in the *etf* gene cluster participate in the mannose-resistant hemagglutination of *E. tarda*.

Adherence of pathogenic bacteria to host is considered to be the first step in infection. Although adherence of *E. tarda* on the gill and body surface has been observed in experimentally infected fish (Ling *et al.*, 2001), the factors associated with the adherence were not identified. For many pathogenic bacteria, it has been shown that adherence to host is mediated by fimbriae possessing hemagglutinating activity (Beachey, 1981). In this study, we identified an *etf* gene cluster associated with the formation of fimbriae expressing

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                                                                                                          orf1
  1 CTGCAGCAGCTGGTCGCGGTACCCGCCGCGTAGGATGCCCGCTGACGCGGCCGGGGGGAGTGTGCTATTCTAGCCCGCTCCCTTTTCTTTTTGCCGATAACACAGATGAAATTCCCCGGT 120
                                                                                                            M  K  F  P  G
121 CAACGCAAGTCCAAACACTATTTCCCGGTTAATGCCCGCGATCCGCTGCTGCAAAAGACGCAGCATAATGAAATTGATAAAGCCTACGTCGTGGGTATCGATCAGACGCTGGTGGATATT 240
     Q  R  K  S  K  H  Y  F  P  V  N  A  R  D  P  L  L  Q  K  T  Q  H  N  E  I  D  K  A  Y  V  V  G  I  D  Q  T  L  V  D  I
241 GAGGCCAAGGTGGATGAGGCGTTTATCGCCCGCTACGGCCTGAGCCAAGGGCACTCGCTGGTGATCGAGGATGATGTCGCCGAAGCGCTGTACCAGGAGCTGTGTGACAGTAAGCTGATT 360
     E  A  K  V  D  E  A  F  I  A  R  Y  G  L  S  Q  G  H  S  L  V  I  E  D  D  V  A  E  A  L  Y  Q  E  L  C  D  S  K  L  I
361 ACCCATGAGTTTGCCGGGGGCACGATCGGCAATACCCTGCACAACTACTCCGTGCTGGCCGACGATCGCTCGGTTCTGCTGGGGGTGATGTGCGAAAACGTCAAAATCGGCAGCTATGCC 480
     T  H  E  F  A  G  G  T  I  G  N  T  L  H  N  Y  S  V  L  A  D  D  R  S  V  L  L  G  V  M  C  E  N  V  K  I  G  S  Y  A
481 TACCGCTATCTGTGTAACACCTCAAGCCGCACCGATCTGGACCATTTGCAGGGCGTCGATGGCCCTATCGGGCGCTGTTTTACCCTGATCACCGATAACGGCGAACGCACCTTTGCCATT 600
     Y  R  Y  L  C  N  T  S  S  R  T  D  L  D  H  L  Q  G  V  D  G  P  I  G  R  C  F  T  L  I  T  D  N  G  E  R  T  F  A  I
601 AGCCCGGGCATGATGAACCAGCTGCGCCCGAAAGCATTCCCGAGGCGGTGATCGCCGGTGCCTCGGCGCTGGTGCTGACGGCCTATCTGGTGCGCTGTAAGCCGGGCGAGCCGATGCGC 720
     S  P  G  M  M  N  Q  L  R  P  E  S  I  P  E  A  V  I  A  G  A  S  A  L  V  L  T  A  Y  L  V  R  C  K  P  G  E  P  M  R
721 GATGCGACCATGCAGGCGATCGCCTATGCCAAAAAGCACCACGTTCCGGTGGTGCTGACCCTGGGCACCAAGTATGTGATCGCCGACGATCCGCAGTTCTGGCGCGATTTCCTGCGCGAT 840
     D  A  T  M  Q  A  I  A  Y  A  K  K  H  H  V  P  V  V  L  T  L  G  T  K  Y  V  I  A  D  D  P  Q  F  W  R  D  F  L  R  D
841 CACGTCACCATTGTGGCGATGAACGAAGAAGAGGCTCAGGAGCTGACCGGGCTGTCCGATCCGCTGGCGGCCTCGGACAGGGCGCTGGAGTGGGTGGATCTGGTGCTGTGCACCGCCGGG 960
     H  V  T  I  V  A  M  N  E  E  E  A  Q  E  L  T  G  L  S  D  P  L  A  A  S  D  R  A  L  E  W  V  D  L  V  L  C  T  A  G
961 CCGGTCGGCCTGTATATGGCGGGCTATACCGAAGATGACGGCAAGCGCAAAACCCAGCATCCGCTGCTGCCGGGGGTGATCCCTGAGTTCAACCTGTACGAGTTTAGCCGGGCGATGCGT 1080
     P  V  G  L  Y  M  A  G  Y  T  E  D  D  G  K  R  K  T  Q  H  P  L  L  P  G  V  I  P  E  F  N  L  Y  E  F  S  R  A  M  R
1081 CGCGCCATGTGCGACGCGCCGTGCCGGATCTACTCGCATATTGCGCCCTATATGGGGGGCCCGGAGAAGATCATGAACACCAACGGCGCCGGAGATGGCGCGCTCTCTGCGCTGCTGCAT 1200
     R  A  M  C  D  A  P  C  R  I  Y  S  H  I  A  P  Y  M  G  G  P  E  K  I  M  N  T  N  G  A  G  D  G  A  L  S  A  L  L  H
1201 GATATCGCGGCCAATGAGTTTCACCGTACCAATGTGCCTAACTCCAGTAAGCATCGAGGTAGCTATCTGACTTACTCCTCCCTGGCGCAGGTGTGCAAAATATGCTAACCGCGTCAGCTAT 1320
     D  I  A  A  N  E  F  H  R  T  N  V  P  N  S  S  K  H  Q  R  S  Y  L  T  Y  S  S  L  A  Q  V  C  K  Y  A  N  R  V  S  Y
1321 CAGGTGCTCAACCAGCACTCGCCGCGTCTGACCCGCGGTCTGCCCGAGCGGGAAGACAGCCTGGAAGAGTCCTACTGGGAGCGCTGAATAGTGTCTCTGCCCGTGGGCAGGTGAGATGAT 1440
     Q  V  L  N  Q  H  S  P  R  L  T  R  G  L  P  E  R  E  D  S  L  E  E  S  Y  W  E  R  *
1441 CTTACGGAGATACGCCGGCGCAGCGCCGGCGTTTTCTTTTTCTCGCGCTCGGCGTTATCTGCGCGGAGTCGGTGGCTAAAGAGGGCGAAAAGGCTAGAAACGATCTCATTTCCCCTAAGCG 1560
1561 GAATACCCCTTTTCACTTTCCGCAACCATGATCAAAGAGCAATTTCTTGATTATTGGTAATGTCATACAAATAAAAAATATGATGTAAAACAATGAATTGATTTCTTTTTTGCATTTGA 1680
1681 TCAAAATCTATTAAATTTAACTATCTTTAAAATTAATCAATTATTTGTTTTTTATCAGTTTTATTGATTTTACTGTTTCTCGTCGGACAATTAATCTTTGCGCAAGTTTTTACATTAATA 1800
1801 AAATAAATATCCTTGCTGTAATATCAGTGAGTGGATATATGCCCTGACAATATATTCAATATTGCGATTCGCCATTGCGCCATATTAATAGCGCAGTGCCAATTTTTGCTATTTTAAATAA 1920
                                                                      -35                          -10
1921 ATCCTTTACCTCGATTGGATTTATGTATTTCGGCATAAATCCATCATTTTATGCGCTGTCGGGAATAAAGATTTTTCTCGTCGGCTCTCTACGCGAAATATATTCGCAGCCTCGTTCCGC 2040
                                        SD                etfA
2041 GCCAACCTAGCGGTCGGATATTTTTAATTGATAAACGGAGTTATGATAATGAAGAAAATTTTACTGCCTGTTATGGCTCTGGCCGCCGCCGCTGTGAGTGGTCAGGCAAGCGCAGCTAAC 2160
                                          M  K  K  I  L  L  P  V  M  A  L  A  A  A  A  V  S  G  Q  A  S  A  N
2161 GGTAAAGTTGAGTTTACGGGTGAAATCGTTAACTCCACCTGCCAGGTAAGCAGCGACTCTCAAAATATCAACGTGTATTTGGGTAAATATCCGACCTCTGCATTTAAAGCGGTTGGCGAT 2280
     G  K  V  E  F  T  G  E  I  V  N  S  T  C  Q  V  S  S  D  S  Q  N  I  N  V  L  G  K  Y  P  T  S  A  F  K  A  V  G  D
2281 AAGTCCGCATCCAAGGCTTTCCAGATCAACCTGGAGCAGTGTGAGCCGGGGAGCTACACCGTACGTTTCGATGGCAATACCGGTTGCCGGTCATCCTGACCTGCTGGCCGTCTCCAGCAGC 2400
     K  S  A  S  K  A  F  Q  I  N  L  E  Q  C  E  P  G  S  Y  T  V  R  F  D  G  N  T  V  A  G  H  P  D  L  L  A  V  S  S
2401 GGTGCCACCGCGGCGGCGAAAGGCGTGGGTATCGAAATTACGGACGTTAACGGCAAGCCGTTCCCGATCGCCGATCAGAGCCAGGGCGATGTGCCCACGGTGACGGTTGTTAGCGATAAG 2520
     G  A  T  A  A  A  K  G  V  G  I  E  I  T  D  V  N  G  K  P  F  P  I  A  D  Q  S  Q  G  D  V  P  T  V  T  V  V  S  D  K
2521 AAGGCCGATATTTAACCTGCAGGCGCGCTATCGCTCTTACGCCAACACGGTTACAGCCGGTCTGGCCAACGCGACCAGCCCGTTCACCATCGAATATAAATAATTATTCGTCGGAATATTC 2640
     K  A  I  F  N  L  Q  A  R  Y  R  S  Y  A  N  T  V  T  A  G  L  A  N  A  T  S  P  F  T  I  E  Y  K  *
2641 GGCCAGGGACGGTCCGTATAGAGGAATAAGATGAAAGAAATCGTTGCCGCCATTATTACTGGTGAGCGCATTTGCTGGCACGGCAGGAATTCAGGTTGATGCTACGCGCGTTATTTATAAC 2760
                              SD            etfB
                                            M  K  R  I  V  A  A  L  L  L  V  S  A  F  A  A  H  A  G  I  Q  V  D  A  T  R  V  I  Y  N
2761 GGCGATGAAAAGTCAGCGTCACTGCCTATCCACAATGACAGCGCGGATGCCTATATGGTGCAGACCTGGCTGGATAAAGGGGATAGCACCAAGGTAGAGAATAAGTTACCGATGGTTGTG 2880
     G  D  E  K  S  A  S  L  P  I  H  N  D  S  A  D  A  Y  M  V  Q  T  W  L  D  K  G  D  S  T  K  V  E  N  K  L  P  M  V  V
2881 GTGCCGCCGATTGTTAAGCTGGATGCGCAAAAATCGGCAATTTTACGCTTCATCTATTCTGGGCAGGGTTTTCCGCAGGATCAGGAAACGCTGCTGTGGGTCAACGTGCAGGAAATTCCG 3000
     V  P  P  I  V  K  L  D  A  Q  K  S  A  I  L  R  F  I  Y  S  G  Q  G  F  P  Q  D  Q  E  T  L  L  W  V  N  V  Q  E  I  P
3001 CCGACGCCCAAGCAAGAAAACGTATTACAGGTTGCGTGCGCACGCGGATTAAGCTGTTTTATCGCCCGAGTTCGCTGCATACCACCCTGGATGAGCAGGTGCATAAGCTGCGCTGACCG 3120
     P  T  P  K  Q  E  N  V  L  Q  V  A  V  R  T  R  I  K  L  F  Y  R  P  S  S  L  H  T  T  L  D  E  Q  V  H  K  L  R  W  Q
3121 AAAGCGGGTAATCAATTGCTGGCCATCAACGACGGGCCGCTACACATCACCTTTGGCTCTGTACAGATGCAGGGCAGCAACGGCAAACCCATCGCGGTCGATGCCAACATGGTTGACCCC 3240
     K  A  G  N  Q  L  L  A  I  N  D  G  P  L  H  I  T  F  G  S  V  Q  M  Q  G  S  N  G  K  P  I  A  V  D  A  N  M  V  D  P
                                                                                                                      SD
3241 CACAGCCGCCTGGCGATCGCCATTCCGGCGGGGGCTCGCGTCGGCAATACAGTCTCCTTCAGCTACATCAATGACTTTGGAGGTCGGACTGAGGTTAAGGACGCGATAGTACACTGACGG 3360
     H  S  R  L  A  I  A  I  P  A  G  A  R  V  G  N  T  V  S  F  S  Y  I  N  D  F  G  G  R  T  E  V  K  D  A  I  V  H  *
                                etfC
3361 AGAGAAGCGGGATGGCGCAACAACAACGGACAGTATCACTGAAGACAGAGCCGACACGCTGGATGTGCCTGGCGTTTGCCGCCGGTAGCCTGTCCCTGGGGTGCAGCGCATCGGCCTGGG 3480
     M  A  Q  Q  Q  R  T  V  S  L  K  T  E  P  T  R  W  M  C  L  A  F  A  A  G  S  L  S  L  G  C  S  A  S  A  W
3481 CCGAGTTCAACATGTCATTCGTTCACGGCAGCGAAAGCATTCAACGCCGCTCATGCGGTTACCGTGGACAAGCGCTGCCGCCGGAGAGTATCCGTTCGCGATTTACCTGAACAAGCAGC 3600
     A  E  F  N  M  S  F  V  H  G  S  E  N  V  N  A  A  H  A  V  A  E  G  N  A  L  P  P  G  E  Y  P  F  A  I  Y  L  N  K  Q
3601 AGGTCGACAACAAAGTCATCCGCTTCACGGCGAAAGAGGGCGCCGCCGCCCAGCCCTGTCTGACGGTGGAAGATCTGCTTAACTATGGGGTGAAACTGCCCGATACGCTCACGCCGGGCA 3720
     Q  V  D  N  K  V  I  R  F  T  A  K  E  G  A  A  A  Q  P  C  L  T  V  E  D  L  L  N  Y  G  V  K  L  P  D  T  L  T  P  G
3721 GCTGCGTCGACGTGGCCGCCGCCGTCAATGGCGCGACGCTCTCCTTCGATCCGGCGGTGCAGCAGATAGACCTTTCTGTGCCGCAGGTCATGCTGGCGCAGCACGCGCGCGGCGCCATTC 3840
     S  C  V  D  V  A  A  A  V  N  G  A  T  L  S  F  D  P  A  V  Q  Q  I  D  L  S  V  P  Q  V  M  L  A  Q  H  A  R  G  A  I
3841 CGACGACCCTGTACGATCAGGGGATCAACGCCGCCTTTAGCAACTACAGCCTGAACTATAACGTAACAGCCAGCCAGCGCCAGAGCGGCCAAAACAGCGAATATACCTTTCTCTCCCTGA 3960
     P  T  T  L  Y  D  Q  G  I  N  A  A  F  S  N  Y  S  L  N  Y  N  R  N  S  S  Q  R  C  S  G  Q  N  S  E  Y  T  F  L  S  L
3961 ATAACGGTCTTAACCTGTGGAACTGGCGTCTGCGCAATAACATGACCATGAATAAAGTCAGCGGTCAGGCGGCACAGTGGGACAATATCGCGACCTGGGCGGAGACGGATATTGCGTCCT 4080
     N  N  G  L  N  L  W  N  W  R  L  R  N  N  M  T  M  N  K  V  S  G  Q  A  A  Q  W  D  N  I  A  T  W  A  E  T  D  I  A  S
4081 GGCGCAGTCGACTGCAGCTGGGGCAGGCCAGCACCAATAATACCGGTTTTTAATAGCATTCAGTTTCGCGGTGCCCAGCTTTCCAGCGTGGATGACATGCTGCCCGAAAGCCTGCGCGGCT 4200
     W  R  S  R  L  Q  L  G  Q  A  S  T  N  N  T  V  F  N  S  I  Q  F  R  G  A  Q  L  S  S  V  D  D  M  L  P  E  S  L  R  G
4201 ATGCGCCGGTGGTGCGCGGCGTCGCCGCCACCAACGCCCGCGTCGACATTCGTCAGAACGGCTACGTGGTGTACAGCACCAACGTGGCGCCGGGGCCCTTTGAGATCCGCGATATCTATC 4320
     Y  A  P  V  V  R  G  V  A  A  T  N  A  R  V  D  I  R  Q  N  G  Y  V  V  Y  S  T  N  V  A  P  G  P  F  E  I  R  D  I  Y
4321 CGCACTCCAACAGCGGTGACCTGCAGGTAACGGTCAATGAGGCCGATGGTACGCGTAAGCACTTTACCGTTTCTTACTCCTCTATCGCCAACATGCTGCGCGAAGGGATCTGGAACTATC 4440
     P  H  S  N  S  G  D  L  Q  V  T  V  N  E  A  D  G  T  R  K  H  F  T  V  S  Y  S  S  I  A  N  M  L  R  E  G  I  W  N  Y
4441 AGCTGACGGCGGGTCGCTACCAGAACGGCGATACCCGCTATACCCCCTAACTTTATGCAGGGGACGCTGGCGCGCGGCATCGCCTATGGTCTGACGCCCTATGGCGGGCTGATCGTGGCGG 4560
     Q  L  T  A  G  R  Y  Q  N  G  D  T  R  Y  T  P  N  F  M  Q  G  T  L  A  R  G  I  A  Y  G  L  T  P  Y  G  G  L  I  V  A
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4561 ATAACTACCGCGCCGCCGCGCTGGGCGTCGCTAAAAATATGGGGGAATGGGGCGCCATCTCCATCGATGGTTCCTATTCCGATACTACGCTGGCCAACGGCGAGCGCGCCCGCGGGCAGA 4680
     D  N  Y  R  A  A  A  L  G  V  A  K  N  M  G  E  W  G  A  I  S  I  D  G  S  Y  S  D  T  T  L  A  N  G  E  R  A  R  G  Q

4681 GCTACCGCTTTCTCTATTCCAAGTCCCTGAACCAGATGGGCACCAACGTGCAGCTGGCAGGCTATCGCTATGCGACGTCGGGCTATTATGACTTTGCCGATGCGGTCGAGGAGCGCAGCC 4800
     S  Y  R  F  L  Y  S  K  S  L  N  Q  M  G  T  N  V  Q  L  A  G  Y  R  Y  A  T  S  G  Y  Y  D  F  A  D  A  V  E  E  R  S

4801 GCTGGCGGCAGGGGATATATGAGAACAACTACTATAATCCGGGCGATGAGCAGACCGGGATCCCCGATCTGGACGATCGCAACCAGCACCGCGTGTATACCTCGCGCTATGCCAACAAGC 4920
     R  W  R  Q  G  I  Y  E  N  N  Y  Y  N  P  G  D  E  Q  T  G  I  P  D  L  D  D  R  N  Q  H  R  V  Y  T  S  R  Y  A  N  K

4921 GCGAGCGCGTGGATGTCTCCATTAACCAGCAGCTGTGGCAGGGGGCGACGCTGTATGCCAACGTCAGTAGCCAGAGCTACTGGGGCGGCGGTGGTCGGGATCGTACCGTCCAGCTGGGCT 5040
     R  E  R  V  D  V  S  I  N  Q  Q  L  W  Q  G  A  T  L  Y  A  N  V  S  S  Q  S  Y  W  G  G  G  G  R  D  R  T  V  Q  L  G

5041 ACAACGACGCCTTTAAGCGCATTAGCTATGGCGTATACCTGCAGGATACGCGCAGCCAGTATGGCTACGCCGATCGCAGCATCAACCTGTCGGTGTCGATTCCGTTGAACTGGGGACGCG 5160
     Y  N  D  A  F  K  R  I  S  Y  G  V  Y  L  Q  D  T  R  S  Q  Y  G  Y  A  D  R  S  I  N  L  S  V  S  I  P  L  N  W  G  R

5161 AAAACCACTCGGCCTCGCTGAACGTCAGCGGAACCCACAGCCGCCAGAGCGGAGATAGCTATAACACCGGTATCAGCGGAACGGCGCTGAGCGATCAGCGGCTGAGCTACGCCCTGAGCA 5280
     E  N  H  S  A  S  L  N  V  S  G  T  H  S  R  Q  S  G  D  S  Y  N  T  G  I  S  G  T  A  L  S  D  Q  R  L  S  Y  A  L  S

5281 CCGGCCATACCGAGTCGGCGGGACAGAGCAGCGCGCTGAACCTGGGCTACCGCGGCGCCTATGGCAACCTGGACAGCAGCTATAGCTACAGCAACAGCTATAACCAGCTGGGGCTGGGGC 5400
     T  G  H  T  E  S  A  G  Q  S  S  A  L  N  L  G  Y  R  G  A  Y  G  N  L  D  S  S  Y  S  Y  S  N  S  Y  N  Q  L  G  L  G

5401 TCTCCGGCGGCGTCTTGCTTCACTCTGGCGGCGTGACGCTGTCGCAGCCGCTGCAGAATACGGTGATTCTGGTCGAAGCGAAGCACGCCAGCGGCGTTCGCCTGGATAACCAGACCGGCG 5520
     L  S  G  G  V  L  L  H  S  G  G  V  T  L  S  Q  P  L  Q  N  T  V  I  L  V  E  A  K  H  A  S  G  V  R  L  D  N  Q  T  G

5521 TGGCCATCGATCCCTTTGGCTATGCGGTCGTCCCCTCAGCGACGCCCATACCGATTCAACGCGGTGGCGCTGCATACGCAGGACTTTGAGCCGGGCCTGGACGTGCCGGTGGCCAGCAAGC 5640
     V  A  I  D  P  F  G  Y  A  V  V  P  S  A  T  P  Y  R  F  N  A  V  A  L  H  T  Q  D  F  E  P  G  L  D  V  P  V  A  S  K

5641 AGGTCGTGCCGACGGAGAAGGCGATCGTGAAGGTGCGCTTCGATACGTTTTACGGCGTGAGTCTGCTGATCCATAGCCACCTCAACGACGGCGGCTATCCGGCGGTCGGCGCCAGCGTCT 5760
     Q  V  V  P  T  E  K  A  I  V  K  V  R  F  D  T  F  Y  G  V  S  L  L  I  H  S  H  L  N  D  G  G  Y  P  A  V  G  A  S  V

5761 TTAACCCGCAGGGGCGTAACAGCGGAACCGTGGGGCTTAACGGCGATCTGTATGTATCCGGGGCACAGCCGGGCGAGACGCTGACCCTGCGCTGGGGCAACCAGCCCGGGCCAGCAGTGCA 5880
     F  N  P  Q  G  R  N  S  G  T  V  G  L  N  G  D  L  Y  V  S  G  A  Q  P  G  E  T  L  T  L  R  W  G  N  Q  P  G  Q  Q  C
                                                                                                   SD      etfD
5881 CGCTGACGCTGCCGTCCGATCTGTCGATGCGGAACACCACGGCCGGCTACCAGGAACTCACTCTACCCTGTCAGACTCAATAGGTGACGGATTAATGAAAAGCCTAAGTATAAAACAGG 6000
     T  L  T  L  P  S  D  L  S  M  R  N  T  T  A  G  Y  Q  E  L  T  L  P  C  Q  T  Q  *                M  K  S  L  S  I  K  Q

6001 CGCTGGCATTCACGCTGCTACTGCTGCTGGGCCTTTGCGTCAGCCGCCAGGCGTCCGCGCTCTCCTGCCGAGAGGGCAGTAATGCAGGGAGCTGGTCGCCCATCGGGAGTATTTATCAGC 6120
     A  L  A  F  T  L  L  L  L  L  G  L  C  V  S  R  Q  A  S  A  L  S  C  R  E  G  S  N  A  G  S  W  S  P  I  G  S  I  Y  Q

6121 GCATCGATATCGGGCGGCCGATTATCCTGTCCGCCGCCGACTTTACCGCCGGTAACCTGATTTGGCGTTCGCAGAACTTTACCACCACCTTTACCTGTTGGGGATAACTGGAATACTGGAA 6240
     R  I  D  I  G  R  P  I  I  L  S  A  A  D  F  T  A  G  N  L  I  W  R  S  Q  N  F  T  T  T  F  T  C  W  D  N  W  N  T  G

6241 GGGGAGAGCACGCCTACATTTACTGGAACCCGAAGGATGCGTTCGGGAGCCTGCACCGTTCGCTGTCGGTTGGGGTTTCGATCAACGGGATCGACTATGATGCGATTAACCTCAGACAGA 6360
     R  G  E  H  A  Y  I  Y  W  N  P  K  D  A  F  G  S  L  H  R  S  L  S  V  G  V  S  I  N  G  I  D  Y  D  A  I  N  L  R  Q

6361 GCGGCAGCCGGCCCAGTGGCCCCGATCTGGGTGAGGGGACCTCAAACAGCGGCGGCGGCGTCGCCAGACCGCGTACGGTCACCGTCAGCTATTCGGTGTACATCAAGGCCACCGGCATCA 6480
     S  G  S  R  P  S  G  P  D  L  G  E  G  T  S  N  S  G  G  G  V  A  R  P  R  T  V  T  V  S  Y  S  V  Y  I  K  A  T  G  I

6481 CGCCGCCGGCGGGTAACTTCCCGGCGCTGGGGCTGCCGTCGCTGTTTCAAATTGACGGCGTGGGCGGCTTGGACGCGCGCCCCAACGGCAACTTTAACGCCTATATCTCCGGGCTGGATA 6600
     T  P  P  A  G  N  F  P  A  L  G  L  P  S  L  F  Q  I  D  G  V  G  G  L  D  A  R  P  N  G  N  F  N  A  Y  I  S  G  L  D

6601 AGATCCAGGTGATCCACTGCAACCCGCAGATCAGCGTGCTGGCGAATAACGGCAACAGTATCGACTTTGGTACGCTGAGCGCCGCCGGCGCGCGTCCGGGGGTTATCGCTCGCCAGATAC 6720
     K  I  Q  V  I  H  C  N  P  Q  I  S  V  L  A  N  N  G  N  S  I  D  F  G  T  L  S  A  A  G  A  R  P  G  V  I  A  R  Q  I

6721 CGTTCAGCATCAAGGCGACGCTGCCGCGGCGGCGAGTGCGGCGGGCAGCAGCTACAGGCCAGCTTCAGCACCCTGAGGCGGACGTCAGCGATCGATCGCTGATCCTGCCGGATACCAACC 6840
     P  F  S  I  K  A  T  L  R  G  G  E  C  G  G  Q  Q  L  Q  A  S  F  S  T  L  R  P  D  V  S  D  R  S  L  I  L  P  D  T  N

6841 CCGGCGTGGGGATTTTCCTGACGAAGGCGGGCGATACCAGCGCTACGCCGATCCCGCTGCAGACCAATGTCGACTTCGGCGGTCGGCTGCAGGATAAGCAGAATGAGGTGAGCGAGAACT 6960
     P  G  V  G  I  F  L  T  K  A  G  D  T  S  A  T  P  I  P  L  Q  T  N  V  D  F  G  G  R  L  Q  D  K  Q  N  E  V  S  E  N

6961 TTACCGCCAATCTGAAATGGCTGACTAATCGGCCGACGGCGGGGCGCTTCCATGCGACGGCCAACATCGACGTCACCTTTAAATAGCCGGGTGCGCCGCCCCGTGGGTGGGGCGCGAATC 7080
     F  T  A  N  L  K  W  L  T  N  R  P  T  A  G  R  F  H  A  T  A  N  I  D  V  T  F  K  *
                                                                                               -35            -10
7081 CGGGTCACCTCCGCGGCGGCTTTTTGGCCGTCGCCCGCCCGATCCTCTCCCCGTTTTAGCATGCGCTGCATGTAACTCTAGAGTCTTTAATGCATTGTTAGGTGAGTCACGTCACAATAA 7200
                                                                                              SD       orf2
7201 ATGGCTGATTTACAACATAATCACCGCCTGATTCAGGGTAAGATAAAGGGCGTTCGCCGCCGTGCCTCCCCGTGGCTGACATGAGCGGCGCGTTATTGTGACAAGGAGAGAGGGCAATGA 7320
     M
7321 AATTTACCTATCGGGGTGCCGCCTGCGCGATCGCGCTAACGCTGGCGCTGACGCCCAGGCTGGTCGCTGGCCTGGGAAAAAGATAAAACCTATAACCTGACCATTCTGCATACCAACGATC 7440
     K  F  T  Y  R  G  A  A  C  A  I  A  L  T  L  A  L  T  P  G  W  S  L  A  W  E  K  D  K  T  Y  N  L  T  I  L  H  T  N  D

7441 ACCACGGCCACTTCTGGCAGAGCGAGCAGGGCGAATATGGCCTGGCTGCCCAGAAAACGCTGGTGGATCGCCTGCGCGCCGAGATTGCCGCCGAGGGCGGCAGCGTGCTGCTGCTGTCGG 7560
     H  H  G  H  F  W  Q  S  E  Q  G  E  Y  G  L  A  A  Q  K  T  L  V  D  R  L  R  A  E  I  A  A  E  G  G  S  V  L  L  L  S

7561 GCGGCGATATCAATACCGGGGTGCCGGAGTCCGATCTGCAGGATGCCGAGCCCGATTTTCGCGGCATGAACCTGGTGGGCTACGATGCGATGGCCATCGGCAACCACGAGTTCGACAATC 7680
     G  G  D  I  N  T  G  V  P  E  S  D  L  Q  D  A  E  P  D  F  R  G  M  N  L  V  G  Y  D  A  M  A  I  G  N  H  E  F  D  N

7681 CGCTGAGCGTGCTGCGCCAGCAGCAGAAGTGGGCCGATTTCCCGCTGCTGTCGGCCAATATCTATGCGCAGGATAGCGGCAAGCGCCTGTTCCAACCTTATCAGATGTTCGATCGCCAGG 7800
     P  L  S  V  L  R  Q  Q  Q  K  W  A  D  F  P  L  L  S  A  N  I  Y  A  Q  D  S  G  K  R  L  F  Q  P  Y  Q  M  F  D  R  Q

7801 GGATCCGCATTGCGGTGATCGGCCTGACCACCGACGATACGGCCAAGATAGGCAACCCTGAATACCTGAAGAACATCGAGTTCCGCCCCCCGGCGGCGAGGCCAAGGGCGTGGTCGAAC 7920
     G  I  R  I  A  V  I  G  L  T  T  D  D  T  A  K  I  G  N  P  E  Y  L  K  N  I  E  F  R  P  P  A  A  E  A  K  G  V  V  E

7921 AGCTGCGGGCCAACGAGAAGCCTGATGTGGTGATCGCCGCGACGCACATGGGTCACTACGACAACGGCGAGCACGGCTCCAACGCCCCGGGCGATGTTGAGATGGCGCGCGCGCTGCCGG 8040
     Q  L  R  A  N  E  K  P  D  V  V  I  A  A  T  H  M  G  H  Y  D  N  G  E  H  G  S  N  A  P  G  D  V  E  M  A  R  A  L  P

8041 CGGGCTACCTCGATATGATCGTTGGCGGTCACTCGCAGGATCCGGTGTGCATGGCACAGGAAAACCGTAAGCAGGCGAACTATGTCCCCGGTACGCCCTGCGCGCCGGATCGCCAGAATG 8160
     A  G  Y  L  D  M  I  V  G  G  H  S  Q  D  P  V  C  M  A  Q  E  N  R  K  Q  A  N  Y  V  P  G  T  P  C  A  P  D  R  Q  N

8161 GGACCTGGATTGTACAGGCCCATGAGTGGGGGAAATATGTCGGGCGGGCCGACTTCACCTTCCGTAACGGGGAGCTGACCCTGCAGCGCTACCAGCTGATCCCGGTGAACCTCAATCGGC 8280
     G  T  W  I  V  Q  A  H  E  W  G  K  Y  V  G  R  A  D  F  T  F  R  N  G  E  L  T  L  Q  R  Y  Q  L  I  P  V  N  L  N  R

8281 AGGAGAAGCAGGCCGACGGTTCGAAGACGCGGATCTACTATACCGAGGCGGATCCCGCAGGATACGGCGATGCTGAAGCTGCTGACGCCGTTCCAGGATCAGGGGCAGGCCGCGCTGGGGG 8400
     Q  E  K  Q  A  D  G  S  K  T  R  I  Y  Y  T  E  A  I  P  Q  D  T  A  M  L  K  L  L  T  P  F  Q  D  Q  G  Q  A  A  L  G

8401 TGAAGATCGGCAGCGTAGAGGGGCGTCTGGAGGGGGATCGCAGCAAGGTTCGCTTTGAGCAGACCAACATGGGGCGCCTGCTGCTGGCGGCGCAGATGGAGCGAACCCATGCCGACCTGG 8520
     V  K  I  G  S  V  E  G  R  L  E  G  D  R  S  K  V  R  F  E  Q  T  N  M  G  R  L  L  L  A  A  Q  M  E  R  T  H  A  D  L

8521 GCGTGATGAGCGGCGGCGGCGTGCGTGACTCGATGGACAGCGGCGATATCAGCTATAAGGATGTGCTTAAGGTGCAGCCGTTCGGCAATACCGTCGCCTATGTCGAC          8627
     G  V  M  S  G  G  G  V  R  D  S  M  D  S  G  D  I  S  Y  K  D  V  L  K  V  Q  P  F  G  N  T  V  A  Y  V  D
```

Fig. 2. Nucleotide sequence of the 8,627-bp DNA fragment encoding *etf* genes. The numbers on the sides indicate the positions of the first and the last nucleotide in each line. The deduced amino acid sequence is shown below the nucleotide sequence. Stop codon is indicated by asterisk. The Shine-Dalgarno motifs (SD) (Shine and Dalgarno, 1975) and −35 and −10 promoter motifs are overscored. A putative signal peptide is dotted line. The DDBJ accession number is AB100170.

Table 1. Size and positions of genes and gene products of the *etf* operon

| ORF	Position (bp)*	Length of polypeptide (amino acids)	Signal sequence (amino acids)	Calculated mass of mature protein
etfA	2089-2622	177	1–22	16,246
etfB	2671-3357	228	1–18	23,162
etfC	3372-5963	863	1–35	90,336
etfD	5976-7046	356	1–26	35,045

* Position in the nucleotide sequence shown in Fig. 1.

Table 2. Protein domain conserved in the deduced amino acid sequences of EtfABCD

Protein	Position (amino acids)	Conserved domain		
		Domain name (CDD* accession no.)	Function	E-value
EtfA	17–169	Fimbrial (981)	Fimbrial protein	4e-30
EtfB	19–221	FimC (12460)	P pilus assembly protein	7e-47
EtfC	38–841	Usher (16770)	Fimbrial usher protein	2e-172
EtfD	228–356	Fimbrial (981)	Fimbrial protein	0.43

* Conserved domain database served by NCBI.
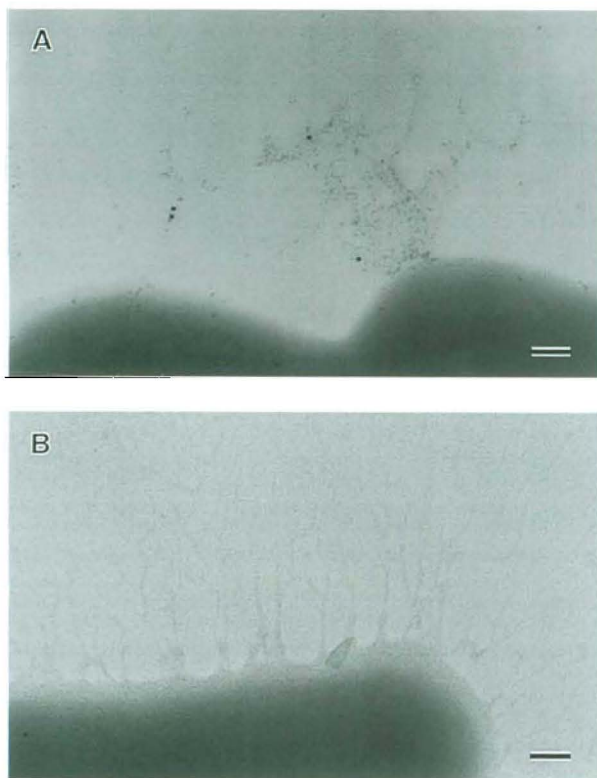
Fig. 3. Immunogold electron micrographs showing *E. coli* (pNUF5) (A) and *E. coli* JM109 (B). Bars; 100 nm.

mannose-resistant hemagglutination. Futher studies are needed to determine the roles of fimbriae in disease occurrence and the bacterial invasion process in piscine edwardsiellosis.

Table 3. Hemagglutinating activity of *E. coli* harbored recombinant plasmid, pNUF5, and *E. tarda* KG8401

Bacteria	Hemagglutination titer (log$_2$) of cells with		
	None	D-Mannose (10 mg/mL)	Fetuin (10 mg/mL)
E. tarda KG8401	13	13	7
E. coli (pNUF5)	9	9	3
E. coli JM109	< 2	NT*	NT

* NT; not tested.

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