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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 man nose-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, man nose-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). Edwardsielliosis in Japanese flounder Paralichthys olivaceus, red sea bream Pgrus 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; lida 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...
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 mannosace-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 amino 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 (Bioinformatics Center, Institute for Chemical Research, Kyoto University, http://fasa.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 EtflABCD in E. coli JM109**

A DNA fragment containing etfABCD was PCR amplified with sense primer, 5'-CCTTTTCCGCAACC-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 (55°C, 30 s) and extension (72°C, 30 s), 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 activity 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 (EtA) 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 -
SphⅠfragment 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 *Phototrophicus luminescens* subs. *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 adherence.
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 *eft* operon

<table>
<thead>
<tr>
<th>ORF</th>
<th>Position (bp)*</th>
<th>Length of polypeptide (amino acids)</th>
<th>Signal sequence (amino acids)</th>
<th>Calculated mass of mature protein</th>
</tr>
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<tbody>
<tr>
<td><em>eftA</em></td>
<td>2089-2622</td>
<td>177</td>
<td>1-22</td>
<td>16,246</td>
</tr>
<tr>
<td><em>eftB</em></td>
<td>2671-3357</td>
<td>228</td>
<td>1-18</td>
<td>23,162</td>
</tr>
<tr>
<td><em>eftC</em></td>
<td>3372-5963</td>
<td>863</td>
<td>1-35</td>
<td>90,336</td>
</tr>
<tr>
<td><em>eftD</em></td>
<td>5976-7046</td>
<td>356</td>
<td>1-26</td>
<td>35,045</td>
</tr>
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</table>

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

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

<table>
<thead>
<tr>
<th>Protein</th>
<th>Domain name (amino acids)</th>
<th>Function</th>
<th>E-value</th>
</tr>
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<td><em>EtfA</em></td>
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<td>Fimbrial protein</td>
<td>4e-30</td>
</tr>
<tr>
<td><em>EtfB</em></td>
<td>FimC (12460)</td>
<td>P pilus assembly protein</td>
<td>7e-47</td>
</tr>
<tr>
<td><em>EtfC</em></td>
<td>Usher (16770)</td>
<td>Fimbrial usher protein</td>
<td>2e-172</td>
</tr>
<tr>
<td><em>EtfD</em></td>
<td>Fimbrial (981)</td>
<td>Fimbrial protein</td>
<td>0.43</td>
</tr>
</tbody>
</table>

* Conserved domain database served by NCBI.

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

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Hemagglutination titer (log2) of cells with</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>D-Mannose (10 mg/mL)</td>
<td>Fetuin (10 mg/mL)</td>
</tr>
<tr>
<td><em>E. tarda</em> KG8401</td>
<td>13</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td><em>E. coli</em> (pNUF5)</td>
<td>9</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td><em>E. coli</em> JM109</td>
<td>&lt;2</td>
<td>NT*</td>
<td>NT</td>
</tr>
</tbody>
</table>

* NT; not tested.

References


