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SHORT COMMUNICATION

Cloning and Expression of Genes Encoding F107-C and K88-1NT Fimbrial Proteins of Enterotoxigenic *Escherichia coli* from Piglets

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Abstract We cloned two genes coding F107-C and K88-1NT fimbrial subunits from strains *E. coli* C and 1NT isolated from Thua Thien Hue province, Vietnam. The mature peptide of *faeG* gene from strain *E. coli* 1NT (called *faeG*-1NT) is 100 % similarity with *faeG* gene, while the CDS of *fedA* gene from strain C (called *fedA*-C) has a similarity of 97 % with the *fedA* gene. Expression of the *faeG*-1NT and *fedA*-C genes in *E. coli* BL21 Star^{TM} (DE3) produced proteins of ~31 and 22 kDa, respectively. The effect of IPTG concentration on the K88-1NT and F107-C fimbriae production was investigated. The results showed that 0.5 mM IPTG is suitable for higher expression of K88-1NT subunit, while 0.75 mM IPTG strongly stimulated expression of F107-C subunit. The optimal induction time for expression was also examined. Generally, highest expression of K88-1NT subunit occurred after 6 h of induction, while that of F107-C subunit is after 14 h.

Keywords *E. coli* · Fimbriae · *faeG* · *fedA* · F107-C · K88-1NT

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of diarrhea in neonatal and newly weaned piglets [1, 2]. ETEC has the ability to attach to the villi of the intestinal mucosal surface and produce enterotoxin causing fluid hyper-secretion and diarrhea [3]. Porcine ETEC strains produce any of five different adhesins, all of which are fimbriae (also known as pili): K88 (F4), K99 (F5), 987P (F6), F41 (F7) and F18 [1, 4]. Fimbrial adhesins F4 and F18 occur in several antigenic forms. The F4 fimbrial variants include F4ab, F4ac, and F4ad. Variants of F18 include F18ab and F18ac. Adhesins of the F41 type are most often found in association with K99, and their significance in naturally occurring disease is uncertain [2].

F4 fimbriae are encoded by a gene cluster, which contains 10 genes called *faeA–faeJ*, respectively. Among these genes, *faeA* and *faeB* contribute greatly to the regulation of the expression of genes encoding the fimbrial subunits [5]. F4 fimbriae are mainly composed of more than 1,000 copies of FaeG subunit, allowing these bacteria to bind F4 specific receptors on the small intestines to induce diarrhea [6]. FaeG is thus regarded to play a very important role in the ETEC pathogenesis, and many studies have been done to express FaeG in various host systems for the production of a subunit vaccine against diarrhea in piglets. Several reports showed that the recombinant FaeG expressed in *E. coli* could induce specific immune response in mice [7–9].

The genes involved in the biosynthesis of F18 fimbria have been only partially described [10, 11]. The major protein of the F18 fimbria, FedA, is not sufficient for recognizing the F18 receptor [10]. Two additional genes from the *fed* gene cluster, *fedE* and *fedF*, have been described as essential for fimbrial adhesion and fimbrial length [11]. However, until now it can not to evaluate F18 adhesive function in regard to either of the two gene products [12].

In this work, we report the cloning and expression of sequences encoding for subunit F107-C (F18, *fedA* gene) and subunit K88-1NT (F4, *faeG* gene) from ETEC strains isolated in Thua Thien Hue province, Vietnam.

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Table 1 Primers used in PCR of ETEC fimbriae

Gene	Primer[a]	Sequence	References
fedA (CDS)	FedA1	5′-CACCGTGAAAAGACTAGTGTTTATTTC-3′	[10]
	FedA2	5′-CTTGTAAGTAACCGCGTAAGC-3′	
faeG (mature peptide)	FaeGac Forw	5′-CACCATGGCACATGCCTGGATGACTGG-3′	[7]
	FaeGac Rev	5′-TAATAAATTGGCAGCTCATCACG-3′	

[a] To enable directional cloning in the further, the forward primers contain the CACC sequence (adapter) at the 5′ end. These four nucleotides will base pair with the GTGG overhang sequence in pET200/D-TOPO vector (5,741 bp, invitrogen)

The fecal samples from piglets suffering from diarrhea, originating from nonvaccinated herds in the territory of Thua Thien Hue province (Vietnam), were plated onto MacConkey agar medium. More one hundred *E. coli* strains isolated were tested for toxicity based on the method described by Carter [13]. Five of them killed all mice within 24 h and two of five strains were chosen for this study.

Genomic DNA of *E. coli* was isolated by phenol extraction method [14]. Genomic DNA was then used as template in PCR amplification with the specific primers which designed to base on the CDS (coding sequence) of the F107-C major fimbrial subunit gene (*fedA*) and the sequence of mature peptide of K88-1NT subunit gene (*faeG*) (Table 1). The PCR component consisted of 80 ng template DNA, 6 µl 2× PCR master mix (Fermentas), and 10 pmol each primer. Distilled water was added to a final volume of 12 µl. After 5 min of genomic DNA denaturation at 95 °C, 35 cycles of 1 min for denaturation at 95 °C, 1 min for annealing at 55 °C and 1 min for polymerization at 72 °C were carried out in the thermocycler (iCycler, BioRad). In the final cycle, the temperature of 72 °C was held for an additional 10 min. PCR products showed two bands were of expected sizes of approximately 830 bp from strain *E. coli* C and 515 bp from strain *E. coli* 1NT. These sizes are similar with the mature peptide of *faeG* gene (accession no. AJ616236) [7] and the CDS of *fedA* gene (accession no. M61713) [10], respectively.

Wang et al. [15] used specific primers to amplify K88 (F4) fimbrial antigen gene from enterotoxigenic *E. coli* strains with the size of 764 bp. Specific primers of variants of K88 such as K88ab, K88ac and K88ad amplified bands with size of about 500 bp.

PCR products were then cloned in pGEM®-T Easy vector (Promega), the ligation component consisted of 50 ng vector, 5 µl buffer, 3 unit T4 DNA ligase, and 25 ng (F107-C) or 40 ng (K88-1NT) PCR product. Distilled water was added to a final volume of 10 µl. The ligation was incubated at 25 °C for 1 h and at 4 °C overnight, then transformed into *E. coli* DH5α by heat-shock method. Recombinant vector was extracted by AccuPrep® Plasmid Mini Extraction Kit (Bioneer) and the PCR products were sequenced by the method of fluorescent dideoxy-terminator on 3130 ABI system (Applied Biosystem). In this study, alignment of both sequences confirmed the identity of the cloned genes, the mature peptide of *faeG* gene from strain 1NT (called *faeG*-1NT) is 100 % similarity with *faeG* gene, while the CDS of *fedA* gene from strain C (called *fedA*-C) has a similarity of 97 % with the *fedA* gene. The changes of *fedA*-C affected the encoded amino acid. These differences were probably due to natural variants amongst *E. coli* strains. Generally, the sequence analysis demonstrated a high similarity of the cloned genes, *fedA*-C and *faeG*-1NT, to the *fedA* and *faeG* genes. The *fedA*-C and *faeG*-1NT sequences were deposited in Genbank with accession number of JX987521 and JX987523, respectively.

Thiry et al. [16] and Verdonck et al. [7] reported the CDS of the *pilin/faeG* gene has 852 bp in length containing the sequence of peptide signal of 60–63 bp and the sequence of mature peptide of 786–789 bp. In our work, the mature peptide sequence of *faeG*-1NT gene is 100 % similarity with that of the *pilin* and *faeG* genes. According to Imberechts et al. [10], the CDS of *fedA* gene was started from nucleotide 160–672 (513 nucleotides in length) containing the signal peptide sequence of 63 nucleotides. In present study, the CDS of *fedA*-C gene has 516 nucleotides also including the peptide signal sequence of 63 nucleotides.

The PCR products (F107-C and K88-1NT) from recombinant pGEM®-T Easy vectors were cut out from 0.8 % agarose gel and purified by Wizard® SV Gel and PCR Clean-Up System Kit (Promega), they were then ligated to a pET200/D-TOPO vector harboring T7 promoter (Invitrogen). The ligation component consisted of 20 ng vector, 1 µl salt solution, and 4 ng (F107-C) or 7 ng (K88-1NT) PCR product. Distilled water was added to a final volume of 6 µl. The ligation was incubated at 25 °C for 60 min. Recombinant vector was then transformed into *E. coli* TOP10 cells according to the manufacturer's instructions (ChampionTM pET200 Directional TOPO® Expression Kit, Invitrogen). The biomass of transformed cells was used to extract vector DNA for the identification of recombination.

Recombinant pET 200/D-TOTO vector was subjected to digestion and PCR. *Sac*I and *Sma*I were chosen for analysis

of *faeG*-1NT and *fedA*-C gene, respectively. Digestion of recombinant vectors with *Sac*I or *Sma*I produced two linearized DNA fragments of 6.5 kb (*faeG*-1NT) or 6.2 kb (*fedA*-C), as expected indicating that the genes were indeed inserted within the vector. PCR analysis showed that genes amplified with the expected sizes of 829 bp (*faeG*-1NT) and 516 bp (*faeA*-C).

Expressions of F107-C and K88-1NT fimbrial subunits were performed in *E. coli* BL21 Star^{TM} (DE3) at 37 °C on LB medium supplemented with 1 % glucose and 50 μg/ml kanamycin. The culture was carried out on a rotation shaker with speed of 200 rpm to an OD_{600} of 0.8. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM for induction. An induction time was extended from 2 to 10 h, and the cells were harvested by centrifugation at 8,000 rpm/4 °C for 1 min. The freezing and thawing at 42 °C was performed with three repeats to break cells and recombinant proteins were recovered by extraction buffer (50 mM K_3PO_4 pH 7.8, 400 mM NaCl, 100 mM KCl, 10 % glycerol and 0.5 % Trixton X-100). Expression levels of fimbrial subunits were assayed by electrophoresis on 12 % (w/v) polyacrylamide gel. The gel was then stained with Coomassie Blue R-250 and image was analysed by Quality One software (ver 4.1, BioRad).

Expressions of the *faeG*-1NT and *fedA*-C genes were expected to produce proteins of ~31 kDa (27 kDa K88-1NT subunit and 3.7 kDa fusion fragment of pET 200/D-TOTO vector) and 22 kDa (18 kDa F07-C subunit and 3.7 kDa fusion fragment of pET 200/D-TOTO vector). For the inclusion body fraction, strong bands of 22 and 31 kDa were obtained in cultures induced with IPTG (Fig. 1). Protein bands of K88-1NT and F107-C subunits in unsoluble fractions are very weak (data not shown). For the uninduced cells, no protein band of expected sizes was observed, suggesting that induction with IPTG is required for expression of *faeG*-1NT and *fedA*-C fimbrial subunit genes.

Imberechts et al. [10] isolated F107 major fimbrial subunit gene (*fedA*) from edema disease strain 107/86 of *E. coli*. The *fedA* gene was sequenced, an open reading frame that codes for a protein with 170 amino acids, including a 21 amino acid signal peptide, was found. The protein without the signal sequence (mature peptide) has a calculated molecular weight of more 16 kDa. Verdonck et al. [7] cloned F4 fimbrial adhesin FaeG monomers from *E. coli* strain GIS26 and expressed in *E. coli* BL21 (DE3) with a molecular weight of about 32 kDa. Hu et al. [9] expressed extracellularly F4 fimbrial adhesin FaeG by recombinant *Lactococcus lactis*. Besides, Huang et al. [17] expressed successfully FaeG, a major subunit of K88ad fimbriae in transgenic tobacco plant with a molecular weight of 27.6 kDa.

For the expression vector with a T7 lac promoter, final IPTG concentration should be optimized because of its great contribution to recombinant protein expression and serious harm to cell growth. We investigated the effect of IPTG at different concentrations on the K88-1NT and F107-C fimbriae production. IPTG concentrations from 0.05 to 2 mM used to induce expression of K88-1NT and F107-C subunits after 5 h. Comparison of intensity of the bands in SDS-PAGE showed that 0.5 mM IPTG is suitable for higher expression of K88-1NT subunit, while 0.75 mM IPTG strongly stimulated expression of F107-C subunit.

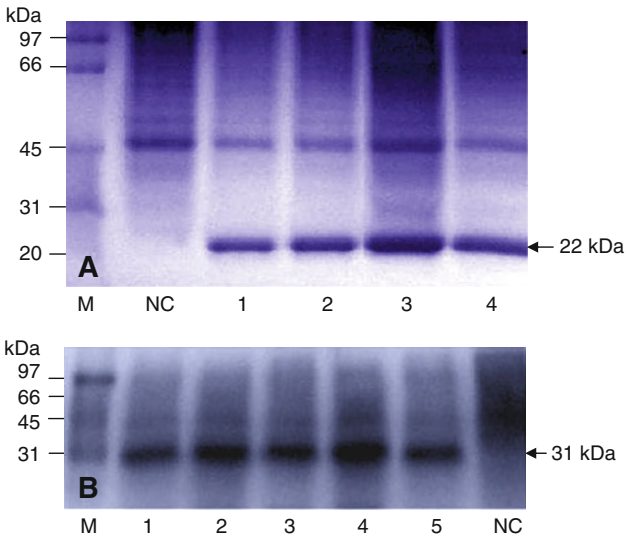

Fig. 1 Expression of F107-C (**a**) and K88-1NT (**b**) fimbrial subunits in *E. coli* BL21 (DE3) cells were induced by 0.5 mM IPTG. *M* Protein weight marker (97–14.4 kDa), *NC* non-induced *E. coli* cells, *1–4* expression of F107-C subunit after 2–8 h of induction respectively, *1–5* expression of K88-1NT subunit after 2–10 h of induction respectively

After adding the IPTG into the medium, the target protein begins to be synthesized and induction time is necessary for recombinant protein production. In this work, the optimal induction time for expression was examined by analyzing samples taken in every 2 h, from 2 to 16 h, after induction with 0.5 mM IPTG (K88-1NT) and 0.75 mM IPTG (F107-C-PT2) at 25 °C. The results showed that highest expression of K88-1NT subunit occurred after 6 h of induction, while that of F107-C subunit is after 14 h.

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