Relationship Between the Tat Protein Transport System and Virulence of Enterotoxigenic Escherichia Coli

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Research Article

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Abstract

Background We aimed to investigate the effect of *Tat* deletion on the pathogenicity of enterotoxigenic *Escherichia coli* (ETEC). Changes in bacterial dynamics and virulence after deletion were examined using mobility and animal toxicity tests for ETEC. Further, differential expression of proteins between the gene-deletion and wild-type strains was analyzed using two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to explore the possible mechanisms underlying the effects of this gene on bacterial virulence.

Results After *Tat* knockout, the virulence of ETEC10407 was strongly attenuated, and its invasion in rabbit intestinal mucosa was significantly reduced. Mass spectrometry revealed that 17 differentially expressed proteins involved in physiological functions of bacteria, such as virulence and substance or energy metabolism might affect bacterial pathogenicity.

Conclusions The Tat protein transport system of ETEC is closely related to its virulence.

Background

Many proteins synthesized by bacteria are transported to functional sites through the sec transport system, which requires energy consumption[1]. Studies have shown that most prokaryotes also have a second set of protein transport systems—the twin-arginine translocation system (Tat system)—which is distinct from the Sec system. In 1997, Settles et al.[2] have found that this system is mainly responsible for transporting folded proteins, which did not require ATP hydrolysis, and the N-terminal of the transport substrate contains signal peptides with twin-arginine domain [3]. *Tat* is ubiquitously expressed in most bacterial genomes and plays a vital role in the pathogenicity of many prominent pathogenic bacteria. *Tat* deletion can affect the biological functions of many pathogenic bacteria, including growth, movement, and pathogenicity. In *Vibrio cholerae*, the deletion of *Tat* affected biofilm formation, the invasiveness to HT-29 cells, and the ability to colonize the intestinal tract of mice. In addition, the decrease in the production of cholera toxin was significantly observed [4]. According to other reports, the Tat transport pathway plays an important role in the pathogenesis of *Brucella*, *Pseudomonas aeruginosa*, and other pathogenic bacteria [5–7].

ETEC is an important intestinal *E. coli* that is responsible for infant and traveler diarrhea in developing countries. The severity of diarrhea caused by ETEC ranges from mild to severe diarrhea and has a pathology similar to that of cholera. Colonization ability and enterotoxin expression are important virulence factors, and the influence of the Tat transport system on ETEC virulence has not been reported yet. At present, several studies have employed proteomics to study the functions of the Tat transport system in pathogenic bacteria [8–10]. In this study, we explored changes in physiological functions and virulence of ETEC after *Tat* knockout and investigated the proteins and functions that might be affected by the Tat system using proteomics.

Methods

Materials

Experimental strains The standard ETEC strain 10407 and a strain with the *Tat* knocked out were provided by the State Key Laboratory of Infectious Diseases Control and Prevention, National Institute for Communicable Disease Control and Prevention, Beijing, China.

Reagents and instruments Immobilized pH gradient (IPG) gel strips and protein quantitative kit were purchased from Amersham Pharmacia. The sequencing trypsin and protease inhibitor cocktail tables were purchased from Sigma, USA. PCR was performed using the PTC200 amplification system (MJ Research, USA). PROTEAN IEF CELL and Protean II XL cell (BioRad, USA), respectively were used for isoelectric focusing electrophoresis and vertical electrophoresis. 4700 MALDI-TOF-MS was used for mass spectrometry (Applied Biosystems, USA). Light microscope (Olympus Tokyo, Japan) as used for observing cell morphological changes.

Experimental animals: Male New Zealand white rabbits weighing 2–3 kg were purchased from the Institute of Laboratory Animal Sciences, CAMS & PUMC.

Methodology

Identification of ETEC *Tat* deletion

Tat deletion was identified using PCR with deletion identification primers. The following forward and reverse primers were used for deletion identification: 5'-AAC GTA TAA TGC GGC TTT GTT-3' and 5'-ATA TCA AAC ATC CTG TAC TCC-3', respectively. In addition, morphological changes in the ETEC wild-type and *Tat*-deletion strains were observed under a light microscope.

Bacterial motility test

Sterilized liquid medium containing 0.3% agar was prepared, and an appropriate amount was added into a sterile U-shaped tube. Single colonies were inoculated into the medium for static culture after incubation at 37℃ overnight. The bacterial solution was diluted 1:50 in fresh medium for static culture at 37℃ for 3–4 h. Subsequently, 10 μL of the above bacterial solution was inoculated onto the liquid surfaces at both ends of the U-shaped tube, the tube was incubated at 37℃ for 4–6 h, and the results were observed.

Animal toxicity test

Healthy male rabbits were fasted for 24 h before operation and subjected to ligation of the intestinal segment in accordance with the routine method [16]. Single colonies of ETEC10407 wild-type and *Tat*-deletion strains were inoculated in liquid medium and cultured overnight at 37℃. After ligation of the intestinal segment, 1 mL of the bacterial solution to be tested was injected into each animal using a

sterile syringe. Rabbits were euthanized by embolization of the marginal ear veins at 24 h after operation. Intestinal segments were removed, cleaned, fixed in 40% formalin, embedded in paraffin, sectioned, stained with Hematoxylin and eosin (HE) and were observed for pathology under a light microscope.

Protein 2-DE and mass spectrometry

ETEC wild-type and *Tat*-deletion strain cultures were centrifuged and sonicated. The supernatant was collected by centrifugation to obtain bacterial protein samples, which were quantified using the Bradford method and subjected to 2-DE. Isoelectric focusing was performed in the following manner. Dry IPG gel strips (pH 4–7 and pH 3–10, 17 cm) were loaded in the rehydration buffer containing 600 μg of the sample.(50 V for 12–16 h, 250 V for 30 min, 1000 V for 1 h, and 10000 V for 11–12 h). SDS-PAGE was performed at a constant current of 10 mA for at least 10 h. After electrophoresis, the gel was stained with Coomassie brilliant blue G-250 and destained with 1% acetic acid.

Image information was digitized using a UMAX powerlook 2100XL scan (transmission scan with an optical resolution of 300bpi) and was analyzed using Pdquest (Bio-Rad). Differential proteins were detected and analyzed by mass spectrometry and the NCBI NR database was searched using a GPS workstation.

Results

Identification of *Tat* gene deletion in ETEC 10407

PCR identification

ETEC-specific virulence heat-labile enterotoxin gene was amplified, identified, and sequenced by PCR in the background of the wild-type and *Tat*-deletion strains. The results showed that bands with a molecular weight corresponding to that of the heat-labile enterotoxin gene could be amplified both before and after deletion of *Tat* in ETEC, which indicated that the deletion strain still retained the basic characteristics of the wild-type strain. DNA sequencing confirmed that *Tat* was replaced by the kanamycin resistance gene, as shown in Fig. 1.

Morphological observation

Mutations of the Tat system in *E. coli* prevent bacterial division [11]. In this experiment, single colonies of ETEC10407 wild-type strain and *Tat*-deletion strain were separately smeared and observed after Gram staining. The results revealed significant differences in morphology and arrangement. Compared with those of the wild-type strain, bacteria of the deletion strain were significantly elongated and mostly arranged in series, as shown in Fig. 2.

Bacterial mobility test

A suitable amount of liquid medium was added into the U-shaped tube, and the ETEC10407 wild-type strain and *Tat*-deletion strain were inoculated at opposing ends. After static culturing, compared with the wild-type strain, the deletion strain did not show any significant mobility, as shown in Fig. 3.

Bacterial virulence detection

The ETEC10407 wild-type strain and *Tat*-deletion strain were inoculated into the different intestinal segments of rabbits. The results demonstrated that the ETEC10407 wild-type strain caused significant intestinal effusion, but the deletion strain did not induce any gross changes. See Fig. 4.

Further pathological examination of each intestinal segment revealed that necrosis was relatively severe in the intestinal mucosa colonized by the wild-type strain, with infiltration by a large or moderate number of inflammatory cells. Infiltration with a small number of inflammatory cells was associated with the observation of the basic structure in case of colonization of the intestinal mucosa with the deletion strain. See Fig. 5.

Two-dimensional electrophoresis and mass spectrometry

The electrophoretograms of the whole proteins of ETEC 10407 were analyzed using the image analysis software PDQUEST to identify protein spots with significant differences (differences ≥2 times). This comparison revealed that the differential proteins with spots were mainly upregulated, as shown in Figs. 6 and 7. Two-dimensional electrophoresis revealed 17 proteins to be differentially expressed between the ETEC 10407 wild and deletion strains. We identified these proteins by mass spectrometry, and the results and corresponding information are shown in Table 1.

Discussion

The Tat protein transport system is present in most bacteria. This system transports fully folded proteins containing a signal peptide with a characteristic conserved sequence (S/T-R-R-x-F-L-K). These proteins are closely related to many bacterial activities [3–7, 12]. In order to investigate the influences of the Tat transport system on ETEC 10407, the biological characteristics and pathogenicity of the ETEC 10407 wild-type strain and *Tat*-deletion strain were compared in this study.

Optical microscopy revealed that the ETEC 10407 *Tat*-deletion strain showed the morphology changed significantly. Similar changes have also been observed in pathogenic bacteria such as *Vibrio cholerae*. The cell division protein FtsZ is an essential factor for bacterial division. In this experiment, the expression of cell division protein FtsZ decreased significantly after *Tat* deletion, which might affect the normal division of bacteria, leading to morphology change, which was one of the typical characteristics associated with Tat transport system blockage.

The bacterial mobility test revealed that the mobility of the *Tat*-deletion strain essentially disappeared. 2-DE and mass spectrometry analyses revealed that flagellin was downregulated in the *Tat*-deletion strain,

which affected the generation of flagella and thus weakened bacterial mobility. Invasiveness is the main condition affecting the virulence of pathogenic bacteria, and the ability of bacteria to adhere, colonize, grow, reproduce and spread are important factors that affect their invasiveness. Not only are flagella an important contributor for bacterial invasion, but they can also be used as an adhesin, which determines the process of bacterial adhesion and colonization. Reid et al. [13] showed that flagella could also directly activate inflammatory signals and enhance the pathogenicity of bacteria, and that the Tat system might significantly reduce the virulence of bacteria by affecting the invasiveness of ETEC. Our rabbit intestine segment ligation test also confirmed that the absence of the Tat system could significantly reduce the virulence of ETEC.

In addition, the level of acid-resistant proteins in the ETEC 10407 *Tat*-deletion strain also significantly decreased. Previous studies have shown that [14, 15] the acid resistance of intestinal pathogenic bacteria is favorable for their invasion of intestinal mucosa through the digestive tract, leading to considerable infection symptoms. In addition, the outer cellular membrane viroporin, cytoplasm ferritin, and other proteins related to energy metabolism (e.g., pyruvate formate-lyase, oleyl-CoA hydratase/isomerase, etc.) were also significantly downregulated in the ETEC *Tat*-deletion strain, although their specific functions require further study.

In summary, the Tat system plays an important role in the pathogenesis of enterotoxigenic *Escherichia coli*, and the mechanism by which the Tat system affects bacterial virulence needs to be further explored. As the Tat protein transport system exists in most bacteria and plant chloroplasts and is not found in mammalian cells, the reduction in bacterial virulence caused by *Tat* kockdown indicates that the Tat system can be used as a potential target for developing antibacterial agents. Further investigation should be directed toward identifying more Tat substrates and defining their roles in ETEC growth and virulence.

Declarations

Ethics approval and consent to participate

All animal procedures were performed in accordance with the protocols approved by animal ethics committee of Chinese Center for Disease Control and Prevention (Beijing, China). This article does not contain any studies with human participants.

Consent for publication

Not applicable

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Competing Interests

The authors declare no conflict of interest.

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Authors' contributions

ZG designed and performed the study, LW, JC and QZ performed the study. All authors have read and approved the manuscript.

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Tables

Table. 1 Mass spectrometry retrieval results and related information of differential proteins

Spot ID	Protein name	Gene	Molecular weight	Isoelectric point
1	Pyruvate-formate lyase	gi|75229892	85288.7	5.69
2	Acid-resistance protein	gi|89110504	12035	5.73
3	Outer membrane porin protein nmpC precursor	gi|33112659	40277.4	4.64
4	Oxygen-insensitive NAD(P)H nitroreductase	gil5800291	23844.2	5.69
5	Cystine transporter subunit	gil16129867	29021.2	6.21
6	Enoyl-CoA hydratase/isomerase	gil91217147	28240.5	8.23
7	Cytoplasmic ferritin	gil15802314	19411.4	4.77
8	Putative formate acetyltransferase	gil15832699	14275.4	5.09
9	Phosphocarrier protein HPr	gil131536	9113.7	5.65
10	Putative detox protein	gil16765785	9836.2	6.06
11	Flagellin [Escherichia coli]	gil33590223	50949.6	4.66
12	Cell division protein FtsZ	gil15799779	40298.6	4.65
13	Outer membrane porin protein nmpC precursor	gi|33112659	40277.4	4.64
14	Deoxyribose-phosphate aldolase	gil15804953	27730.3	5.5
15	A ferritin homolog that binds and protects DNA	gil3660175	18699.7	5.72
16	Cytoplasmic ferritin (an iron storage protein)	gil15802314	19411.4	4.77
17	Putative formate acetyltransferase	gil15832699	14275.4	5.09

Figures

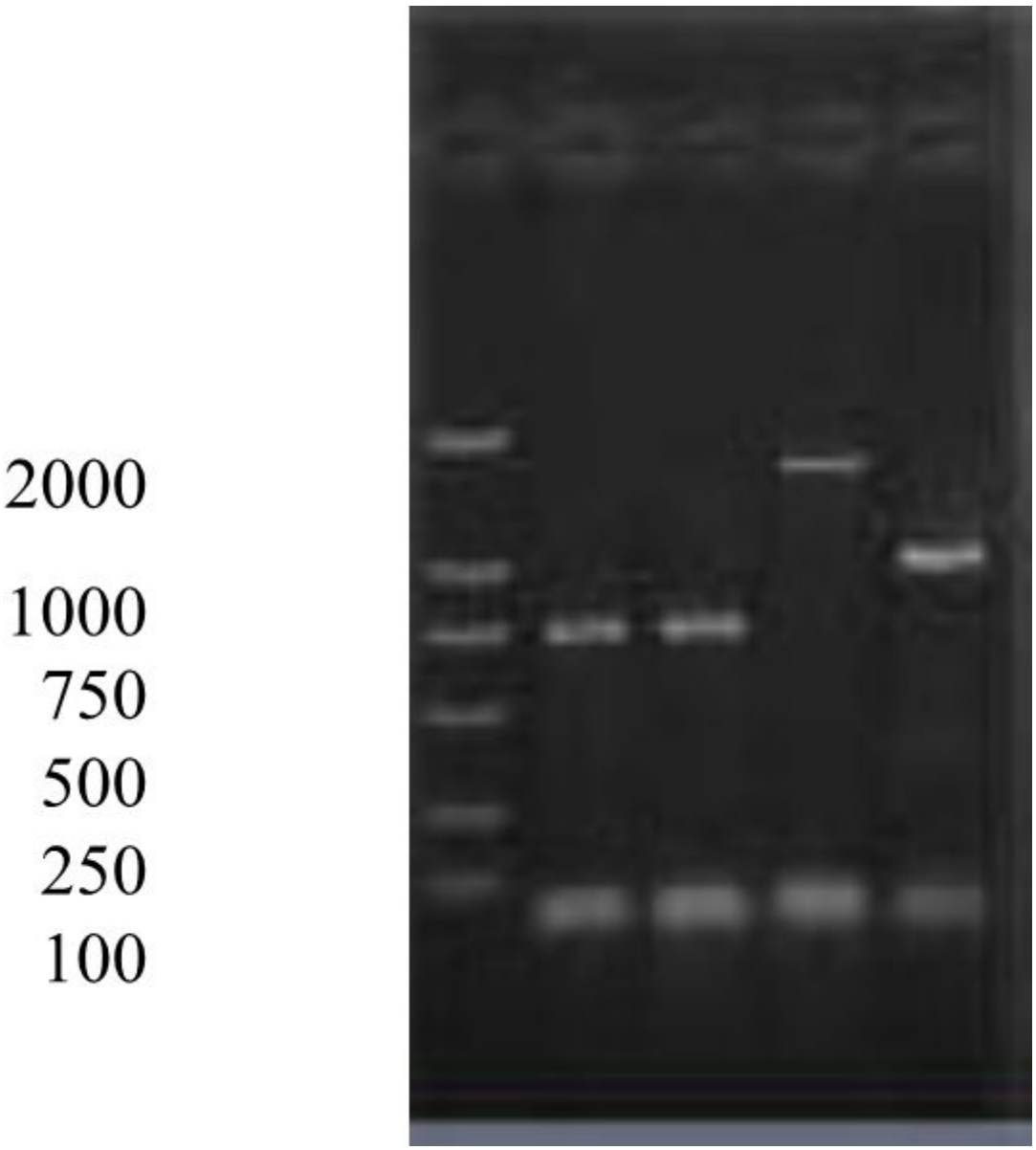

Figure 1

PCR identification of enterotoxigenic *Escherichia coli* (ETEC 10407), the twin-arginine translocation (*Tat*) gene deletion strain

M: molecular weight DL2000; 1-2. Heat-labile enterotoxin gene amplification in wild-type and deletion strains respectively; 3-4. *Tat* amplification in wild and deletion strains, respectively.

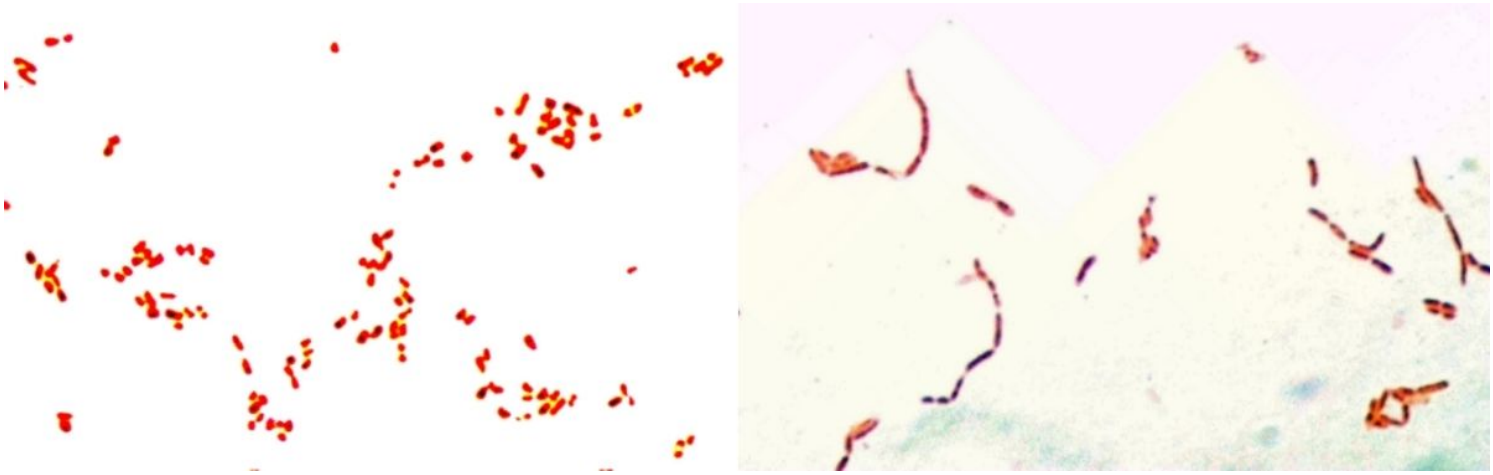

Figure 2

Comparison of morphology by light microscopy before and after the deletion of twin-arginine translocation (*Tat*) in enterotoxigenic *Escherichia coli* (ETEC 10407)

ETEC10407 wild-type strain; 2. *Tat*-deletion strain

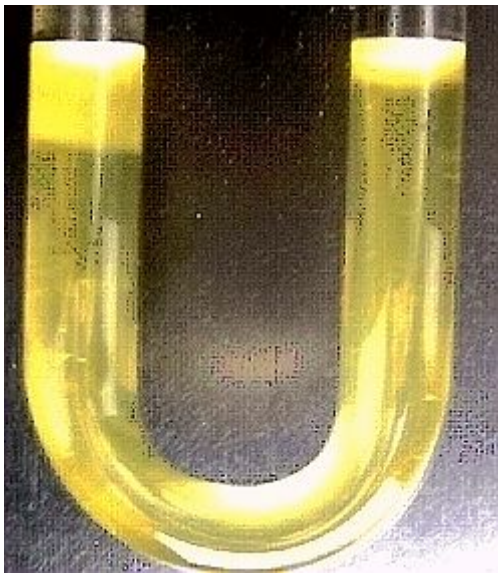

Figure 3

Observation of the mobility of the enterotoxigenic *Escherichia coli* (ETEC 10407) wild-type strain and twin-arginine translocation (*Tat*) deletion strain in the U-shaped tube

1. ETEC10407 wild-type strain; 2. ETEC10407 *Tat*-deletion strain

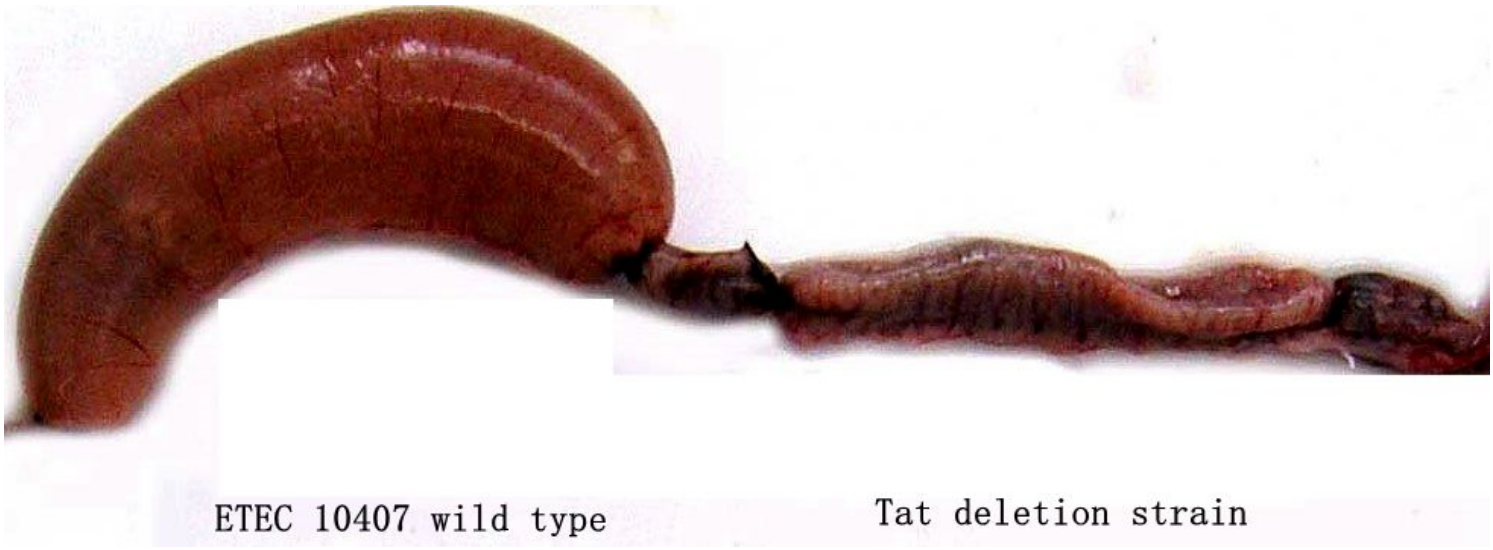

Figure 4

Gross changes in the rabbit intestinal segment ligation test

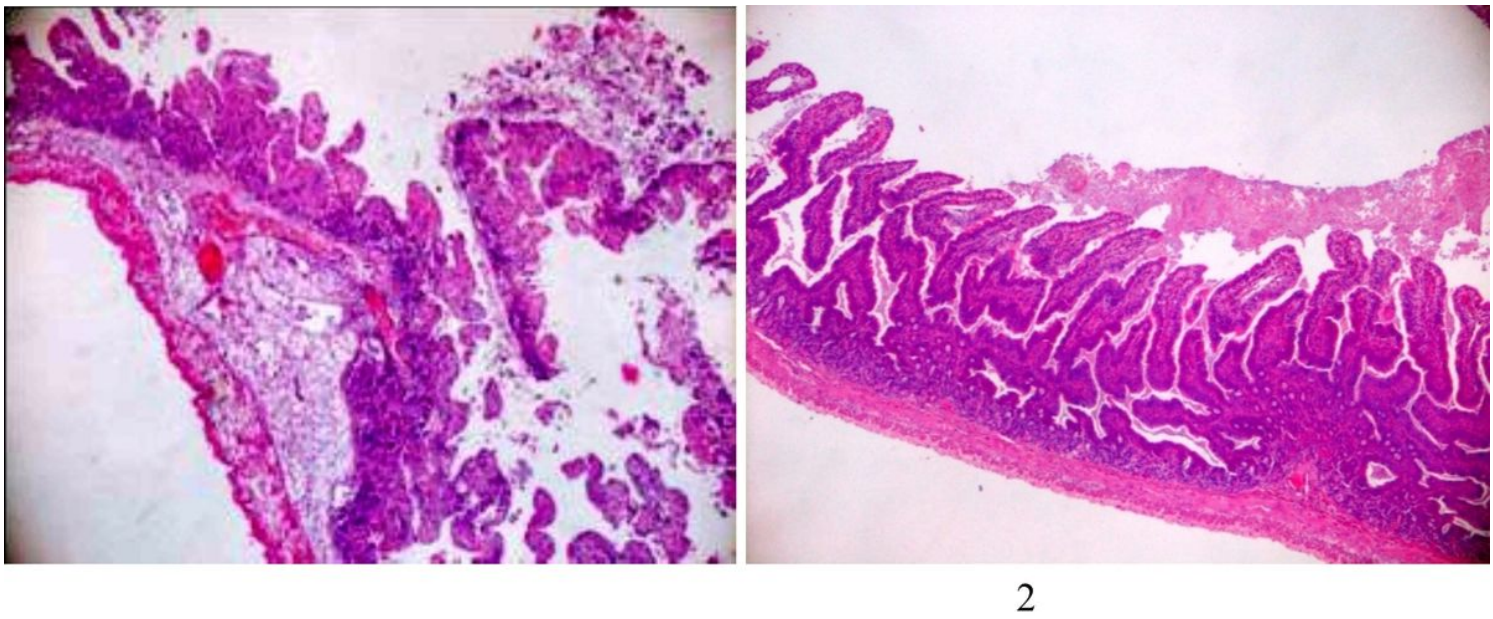

Figure 5

Lesions in the intestinal wall of rabbits colonized by the enterotoxigenic *Escherichia coli* (ETEC 10407) wild-type strain and the twin-arginine translocation (*Tat*)-deletion strain (x4)

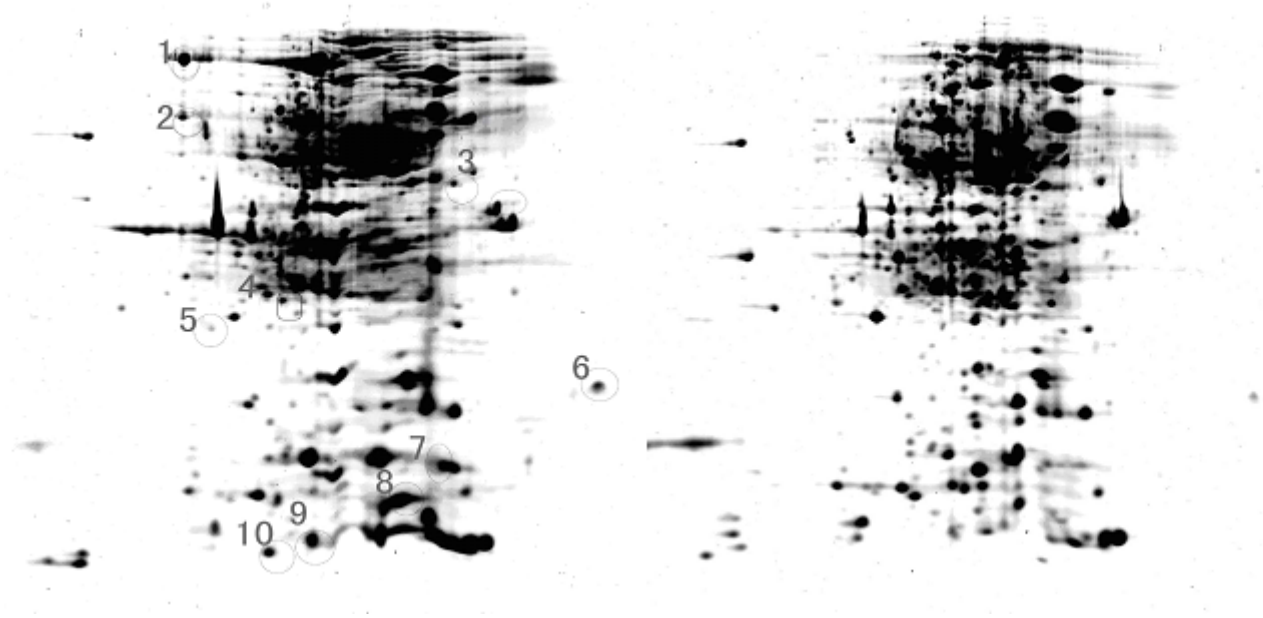

Figure 6

Images of differential proteins in enterotoxigenic *Escherichia coli* (ETEC 10407) before and after twin-arginine translocation (*Tat*) deletion. Electrophoresis was performed with 17 cm pH 3–10 gel strips; Coomassie brilliant blue was used for staining.

Fig. 6a ETEC10407 wild-type strain Fig. 6b ETEC10407 *Tat*-deletion strain

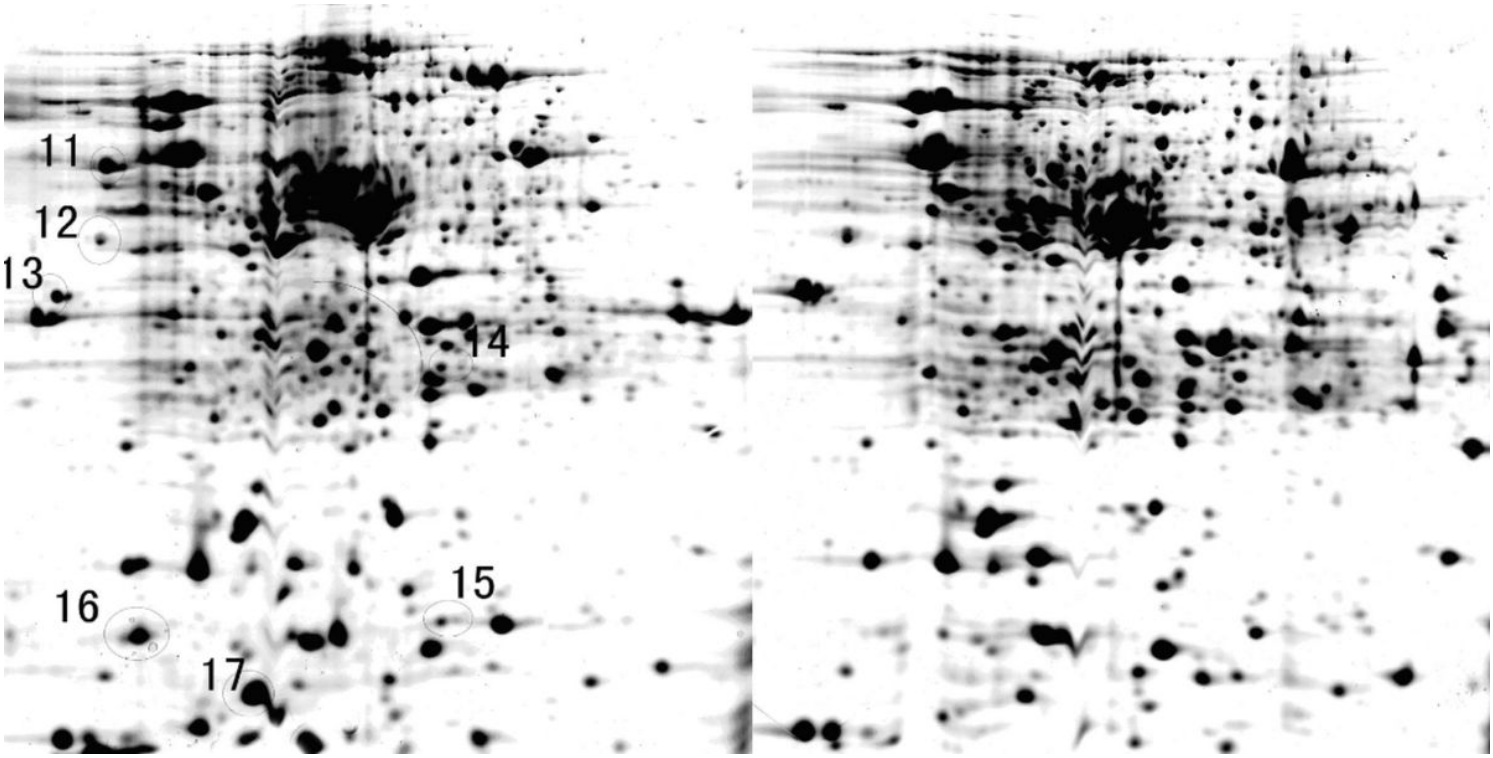

Figure 7

Images of differential proteins in enterotoxigenic *Escherichia coli* (ETEC 10407) before and after twin-arginine translocation (*Tat*) deletion. Electrophoresis was performed with 17 cm pH 3–10 gel strips; Coomassie brilliant blue was used for staining.

Fig. 7a ETEC 10407 *Tat* wild-type strain

Fig. 7b ETEC 10407 *Tat*-deletion strain