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Pleiotropic Effect of *tatC* Mutation on Metabolism of Pathogen *Yersinia enterocolitica*

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Objective To analyze the impact of depletion of the twin arginine translocation (TAT) system on virulence and physiology of *Yersinia enterocolitica* for a better understanding of its pathogenicity. **Methods** We constructed a Δ*tatC*::Sp^R mutant of *Yersinia enterocolitica* by P1 phage mediated transduction using *Escherichia coli* K-12 Δ*tatC*::Sp^R strain as a donor. **Results** A P1-mediated genetic material transfer was found between the two species of enterobacteria, indicating a great potential of acquisition of antibiotic resistance in emergency of a new threatening pathogen by genetic material exchanges. Periplasmic trimethylamine N-oxidase reductase activity was detected in the wild type *Y. enterocolitica* strain and translocation of this enzyme was completely abolished by the Δ*tatC*::Sp^R mutation. In addition, the Δ*tatC*::Sp^R mutation showed a pleiotropic effect on the metabolism of *Y. enterocolitica*. However, the *tat* mutation did not seem to affect the mobility and virulence of *Y. enterocolitica* under the conditions used. **Conclusion** Unlike other pathogenic bacteria studied, the TAT system of *Y. enterocolitica* might play an important role in the pathogenic process, which is distinct from other pathogens, such as *Pseudomonas aeruginosa* and enterohemorrhagic *E. coli* O157:H7.

Key words: *Yersinia enterocolitica*; TAT system; Mutation

INTRODUCTION

Bacteria put out numerous proteins across the cytoplasm membrane *via* either the Sec machinery or the TAT (also called MTT) system. The TAT system is different from the Sec pathway given its unusual ability to transport folded proteins and even enzyme complexes into the periplasm. Most of the TAT substrates are co-factor-containing enzymes taking part in oxidation-reduction systems involved in energy conservation under anaerobic conditions[1-2]. Recently, increasing evidence indicates that the TAT system plays an important role in the pathogenesis. It was first demonstrated that the TAT system participates in the secretion of two virulence factors *via* the type II secretion system in opportunistic pathogen *Pseudomonas aeruginosa*. This finding has been extended to other virulence factors in this pathogen. The TAT system is essential for the export of phospholipases, proteins involved in pyoverdine-mediated iron-uptake, anaerobic respiration, osmotic stress defense, motility, and biofilm formation. In addition, *tat* deletion mutation of the enterohemorrhagic *E. coli* O157:H7 EDL933 reference strain impaires the secretion of Shiga toxin 1 (Stx1) and abolishes the synthesis of H7 flagellin, which are two major known virulence factors of this pathogen. Similar studies have been performed to characterize the contribution of the Tat pathway to growth, motility, and virulence of the phytopathogen *Agrobacterium tumefaciens*. It has been found that, in addition to its role in secretion of folded cofactor-bound enzymes functioning in alternative respiration, the TAT system of *A. tumefaciens* is an important virulence determinant. Furthermore, this secretion pathway contributes to flagella biogenesis and chemotactic responses but not to sensory perception of plant signals or the assembly of a type

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IV secretion system.

Yersinia enterocolitica is an important pathogen in both humans and animals, which can cause yersiniosis, enteritis, right lower-quadrant abdominal pain mimicking appendicitis, reactive arthritism, and erythema nodosum. Pathogenic *Yersinia* spp have evolved to an exquisite type III secretion system for delivering powerful effectors into cells of the host immune system where they inhibit signaling cascades and block the cells' response to infection[3-6]. In contrast to the type III system, involvement of the TAT system in the pathogenesis of *Yersinia* spp. is unknown. In this study, we constructed a Δ*tatC*::Sp^R mutant by P1-mediated transduction using commensal *E. coli* K12 strain as a donor and the pathogenic *Yersinia enterocolitica* serotype O:8 isolate CMCC52211 as a receptor strain. The resulting *Y. enterocolitica tatC* mutant showed pleiotropic alterations in various metabolism pathways, and translocation of one of the best characterized Tat substrates (the trimethylamine n-oxide reductase) was completely abolished by the mutation. In contrast to *P. aeruginosa* and enterohemorrhagic *E. coli* O157:H7 *tat* mutants, no significant difference was observed in swarm and virulence assays (including guinea cornea test, pestin test) and in LD_{50} BALB/C test between the wild type strain and the *Y. enterocolitica tatC* mutant.

MATERIALS AND METHODS

Reagents and Strains

P1 bacteriphage is a laboratory stock. *E. coli* K12 strain BUDDY (Δ*tatC*::ΩSpecR) has been described elsewhere. *Y. enterocolitica* O:8 isolate CMCC52211 (WA) was kindly donated by Professor En-Shu YU from Fujian Provincial Centre for Diseases Control and Prevention. Methyl viologen dye and trimethylamine N-oxide were purchased from Sigma Company. Bacterial biochemical metabolism was analyzed using Biolog Microstation (USA).

Construction of Y. enterocolitica ΔtatC::ΩspecR Mutant

E. coli K12 Δ*tatC*::ΩspecR mutant was incubated in 100 mL LB medium to early exponential phase with shaking. Five mL bacteriphage stock was added and incubated overnight. After the concentration of bacteria reached about 1×10^8 CFU, the bacteria were put into a 40℃-41℃ water bath for more 35 min, incubated 37℃ for 1 hour, then 0.5 mL chloral was added, shaked roughly for 5-10 min, centrifuged and filtered to remove bacteria and stored at 4℃.

Screening of Y. enterocolitica Mutation Strain tatC⁻

Y. enterocolitica serotype O:8 adaptor strain WA (Fujian Provincial CDC of China) was cultured in 100 mL liquid medium overnight. When the bacterial concentration reached about 1×10^8 CFU, 5 mL P1 bacterialphages was added and incubated at 37℃ for 20 minutes. The culture was spread on plates with 0.15 mg/mL anti-spectinomycin, and the transductor was picked up and detected with molybdoenzyme.

Detection of Biotype Y. enterocolitics tatC-mutant

Topology detection: To observe if there was a linear array increase in *Yersinia* strains as *E. coli,* original and mutation strains of *Y. enterocolitica* were detected under an optical microscope. First, aerobically grown cells at early exponential phase (optical density at 600 nm[OD_{600}] of 0.4) were fixed in a final concentration of 2.5% glutaraldehyde in 5 mmol/L EDTA. The cells were subsequently harvested, suspended in 50 mmol/L Tris-HCL (pH 8.0), and fixed on a slide. Photographs were taken using a Zeiss Axioplot microscope internal camera.

Characteristics of Biochemical Metabolism

GN2 biochemical metabolization plates of Biolog MicroStationTM System Release 3.50 (Biolog. Inc. USA) was used to detect the biochemical metabolic characteristics of the parent and mutant strains.

Cellular Fractionation and Enzyme Assays

Y. enterocolitica parental strain WA and *tatC* derivative mutant WA-JP1 were incubated at 37℃ in TSB broth for 20 h with shaking. The cells were harvested by centrifugation and washed with 10 mL 50 mmol/L Tris-HCl (pH 8.0). Cells corresponding to one gram bacteria wet weight were resuspended in 5 mL 0.2 mol/L Tris-HCl (pH 8.0), EDTA was added to the final concentration of 1 μm, and isoenzeme was added to the final concentration of 0.1 mg/mL. After incubation on ice for 2.5 min, 10 mL ice-cold water was added. The suspension was incubated on ice for an additional 2.5 min, centrifuged at 13 000 rpm for 20 min, saved as periplasmic fractions and stored at -20℃. Pellet spheroplasts were broken by ultrasonication and centrifuged at 13 000 rpm for 20 min. The supernatant was saved as cytoplasm fractions and stored at -20℃.

Periplasmic and cytoplasmic proteins were resolved by native polyacrylamide (7.5%) gel electrophoresis and trimethylamine n-oxide reductase activity was revealed by activity staining as described elsewhere [Santini, 1998 #813].

Antibiotic Resistance Test

Paper spread method, recommended by WHO, and modified by Kirby-Bauer in 1996, was used in this study. The culture medium used was Mueller-Hinton (MH) agar and the reference strain used was *E. coli* ATCC25922. The test paper was purchased from the Institute of Biological Medicine Identification of China.

Swarm Test

Y. enterocolitica parental wild type and *tatC* mutant strains were tested for their ability to swarm on semisolid agar (0.4%) at 25℃ and 37℃.

Sensitivity Test of Yersinia peptin

Culture medium: Low layer culture medium was prepared with 1 g peptone, 0.5 g yeast powder, 0.5 g NaCl, 2 g agar, 1 g filtered $CaCl_2$, and 1 mL of 10 mol/L glucose. Then 100 mL H_2O was added and mixed well. Seed layer culture medium prepared with 3 g tryptic soy and 0.6 g agar was added in 100 mL distilled water. The medium was autoclaved at 15 pounds for 20 min and then spread on plates as it cooled to about 50℃.

Indicator strain: *Yersinia peptis* strain (Evpar) producing pestin was cultured in blood agar plates before use.

The indicator strain growing in lower layer agar medium was incubated for about 30 h, and exposed to chloroform vapor for 1 min after clear bacterial spots were observed, then 0.5 mL (10^7 CFU/mL) tested bacteria was added into 10 mL melted and chilled seed layer medium and mixed well immediately. The lower layer agar was covered quickly, and incubated at 37℃ for 24 h.

Result Observation

Significant inhibiting ring around the indicator strain was defined as a positive result, and absence of the ring as a negative result.

RESULTS

Construction of Y. enterocolitica tatC Mutant

The essential *tat* genes were found in most bacterial genomes, including pathogens *Yersinia pestis, Helicobacter pylori*, *Vibrio cholerae, Salmonella enterica* subsp. enterica serovar Typhi*, Haemophilus influenzae, Mycobacterium tuberculosis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. In addition, both the *tatABC* operon and the *tatE* gene were highly conserved in the *Y. pestis* genome. The *TatC* of *Y. pestis* was about 80% identical to the *TatC* of *E. coli* at amino acid sequence level. Since the *tat* genes of *Y. enterocolitica* have not been characterized, we constructed a *tatC* mutant by P1-mediated transduction. A P1 lyse was prepared from the *E. coli* K-12 Δ*tatC*::ΩSpecR mutant, and used to infect the *Y. enterocolitica* O:8 isolate CMCC52211. The transductants were selected on spectomycin plates.

One of the typical phenotypes of *E. coli tat* mutants was found to be cytoplasmic accumulation of trimethylamine n-oxide reductase (TorA) activity. However, whether *Yersinia* spp. contains the TorA homologue remains unknow. We used *E. coli* TorA as a query to analyze *Yersinia pestis* isolate CO92, and found three proteins with significant homology scores. The sequence with the highest score (499) encoded the putative biotin sulfoxide reductase with no typical twin-arginine signal peptide. In contrast, the second and third sequences having the scores of 280 and 243, encoded both dimethyl sulfoxide and its reductase which is the TorA counterpart of bacteria such as *Rhodobacter capsulatus* and capable of reducing both trimethylamine n-oxide and dimethyl sulfoxide. In addition, typical twin-arginine motif was found in signal peptides of these two enzymes. Therefore, we detected the cellular location of trimethylamine n-oxide reductase activity to confirm the *tat* mutant phenotype. Interestingly, a single polypeptide showing trimethylamine n-oxide reductase activity was found in periplasm of the wild type *Y. enterocolitica* strain (Fig. 1, lane 1). Since the size of the two putative dimethyl sulfoxide reductases of *Yersinia pestis* isolate CO92 (NP_406791.1 and NP_4064591.1) was very similar (816 and 808 residues, respectively), it is unknown whether the active band found in periplasm of *Y. enterocolitica* resulted from one or two isoeneymes. As observed in *E. coli* strain, the cytoplasmic precursor of TorA counterpart displayed the same mobility as the periplasmic form (Fig. 1, lane 2). Importantly, when spectromycin resistant transductants were analyzed, TorA activity was found only in the cytoplasm, suggesting that *Y. enterocolitica* might possess at least one periplasmic trimethylamine n-oxide reductase and its periplasmic translocation might be dependent of the TAT system. The results of cellular fractionation and activity assays obtained with one of these mutants are shown in Fig. 1.

Another typical phenotype of *E. coli tat* mutants was found to be the chain morphology of the cells due to the misallocation of two amidases (AmiA and AmiC) in the absence of a functional TAT system [Ize, 2003 #1614]. Interestingly, in contrast to the single cell morphology of the wild type strain (Fig. 2A), Δ*tatC*::ΩSpecR derivative showed a long chain structure (Fig. 2B), indicating the defect in cell division.

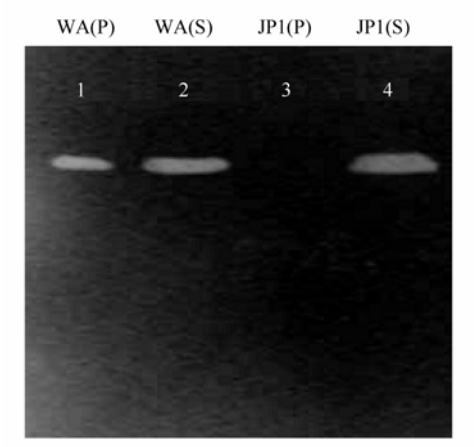

FIG. 1. Results of cellular fractionation and activity assays obtained with one of these mutants. WA (P) indicates pericytoplasm of parent strain, WA (S) cytoplasm of parent strain, JP1 (P) pericytoplasm of *tatC*-strain, JP1 (S) cytoplasm of *tatC*-strain.

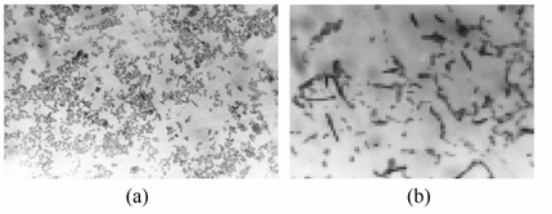

FIG. 2. Typical phenotype of *E. coli tat* mutants in wild type parental strain (A) and Δ*tatC*::ΩSpecR mutant (B).

Taken together, these results suggest that the upstream and downstream regions of the *tatC* gene were highly conserved to allow replacement of the *Y. enterocolitica tatC* gene by *E. coli* K-12 Δ*tatC*::ΩSpecR. In addition, the basic function of TAT system was well conserved in these two enterobacteria.

Impact of tatC Mutation on Y. enterocolitica Metabolism

To obtain general information about the physiological consequence of *tatC* mutation, we compared the activities of some key enzymes in the mutant and the wild type strain by the Biolog Station. Decrease was observed in mono-methylsuccinate, formic acid, glucuronamide, alaninamide, and glycyl-L-glutamic acid. In addition, the mutant strain completely lost its capacity of metabolizing bromo succinic acid, suggestive of the characteristic change in biochemistry metablism. Tests showed that some substrates metabolized slower in *Yersinia enterocolitica* TatC-mutant strain than in its parent strain. These substrates were mono-methylsuccinate, formic acid, glucuronamide, alaninamide, glycyl-L-glutamic acid. In addition, the mutant strain could not metablize bromo succinic acid.

We analyzed the antibiotic resistance of *tatC* mutant. As a wild type strain, this mutant remained sensitive to ampicillin, chloramphenicol, trimethoprim, tobramycin, nitrofurantoin, fosfomycin, fleroxacin, cefoperazole, piperacillin, enoxacin, cephalothin, streptomycin, and ofloxacin.

Influence of tatC Mutation on Virulence of Y. enterocolitica

To assess the role of TAT system in the pathogenesis of *Y. enterocolitica*, we compared the virulence of *tatC* mutant with its parental wild type strain. When bacterial motility, one of the traits linked to virulence was analyzed by semisolid swarm assays, no significant difference was found between the wild type strain and *tatC* mutant (data not shown). In addition, both the parental and mutant strains were positive for guinea pig cornea test, indicating that the mutation could not invade the epithelia of *Y. enterocolitica*. The sensitivity to Yersiniabactin, a receptor of pestin, was found to be related to the utilization of Fe in bacteria.

DISCUSSION

TAT system is a new protein transit system in bacteria. Its protein substrate, a completely folded signal tider, has a specific twin-arginine structure with two linked arginines conserved[7-9]. Because the Tat substrate is an important component of energy metabolism respiratory chain, we compared the biochemical metabolism of the parent and mutant strains, showing that the mutant has a significant linear form and there is a difference in biochemical metabolism between the two strains. The mutant strain occurred 3 h later than the parent strain during the metabolism of mono-methylsuccinate, formic acid, glucuronamide, alaninamide, and glycyl-L-glutamic acid. In addition, the mutant strain could not metabolize bromosuccinic acid. It was reported that *Yersinia enterocolitica* strains show a more linear form after mutation as *E. coli*[9]. However, different phenomena were found in *Vibrio cholerae* in our study. No in crease in linear form was found in *Vibrio cholerae*, but chanhes in characteristics of some other colonies were observed. For instance, the mutant has lost the original smoothness and thickness, showing positive agglutination to anti-sera of a rough biotype[10]. These findings indicate that TAT system has different effects on different bacteria. *Yersinia enterocolitica* is widespread in China; biotype 0:3 is common and has a different genetic background[11]. Further study is required to show if TAT system has different effects on different strains and/or biotype of

Yersinia enterocolitica.

During metabolism of bacteria, nutriment is absorbed mainly depending on the filter enzyme attached onto the cell membrane, which transfers some metabolic substances, such as amino acids and fatty acids, to cytoplasm. Like other enzymes, this enzyme is classified as a unique and relatively specific enzyme. The difference is that this enzyme has a cross-membrane helix area to fix it on the cytoplasm. We think that biochemical changes in the mutant strain are related to the secretion block of this enzyme or the secretion of other proteins influencing its functions.

TatC transit substrates are located in cytoplasm. Some studies with *E. coli* TAT system showed that some substrates are related to cell division, anti-penetrability, and antibiotic resistance. It is interesting that Professor Ochsner from Harverd University found that TAT system is related to pathogenesis in his study of Bacilli involving nosocomial infection.

In conclusion, *Yersinia enterocolitica* Tat system after mutation has no significant change in invasion, mobility, antibiotic sensitivity, and LD_{50}. No change has been found in pathogenesis of the mutant strain, but the difference in biochemistry and topology shows that the Tat system is an important factor for the survival of bacteria. Because of the close relationship between metabolism and pathogenesis of the bacteria, further study is needed to provide more evidence for it.

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