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Instability of toxin A subunit of AB_5 toxins in the bacterial periplasm caused by deficiency of their cognate B subunits

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ABSTRACT

Shiga toxin (STx) belongs to the AB_5 toxin family and is transiently localized in the periplasm before secretion into the extracellular milieu. While producing outer membrane vesicles (OMVs) containing only A subunit of the toxin (STxA), we created specific STx1B- and STx2B-deficient mutants of *E. coli* O157:H7. Surprisingly, STxA subunit was absent in the OMVs and periplasm of the STxB-deficient mutants. In parallel, the A subunit of heat-labile toxin (LT) of enterotoxigenic *E. coli* (ETEC) was absent in the periplasm of the LT-B-deficient mutant, suggesting that instability of toxin A subunit in the absence of the B subunit is a common phenomenon in the AB_5 bacterial toxins. Moreover, STx2A was barely detectable in the periplasm of *E. coli* JM109 when *stx2A* was overexpressed alone, while it was stably present when *stxB* was co-expressed. Compared with STx2 holotoxin, purified STx2A was degraded rapidly by periplasmic proteases when assessed for in vitro proteolytic susceptibility, suggesting that the B subunit contributes to stability of the toxin A subunit in the periplasm. We propose a novel role for toxin B subunits of AB_5 toxins in protection of the A subunit from proteolysis during holotoxin assembly in the periplasm.

1. Introduction

Shiga toxin (STx) elaborated by *E. coli* O157:H7 is a typical AB_5 exotoxin consisting of an A subunit and five identical B subunits [1–3]. The catalytic A subunit of STx (STxA; ~32 kDa protein) shows a ribosomal RNA (rRNA) *N*-glycosidase activity that specifically cleaves a conserved adenine base of 28S rRNA of the 60S ribosomal subunit, which eventually blocks protein synthesis and causes cell death [4]. The STxB subunit (~7.7 kDa protein) binds specifically to the host cell membrane-anchored globotriaosylceramide (Gb3) receptor [5], thereby mediating subsequent internalization of the toxin [3]. In the absence of STxA, STxB can associate in a ring-like pentameric structure in the periplasm and can be substantially entrapped into OMVs for extracellular secretion [6,7]. In general, STx toxins can be classified into two types: STx1 (also known as VT1 for verotoxin or SLT-I for Shiga-like toxin) and STx2 (also known as VT2 or SLT-II). STx1 is nearly identical to the Shiga toxin of *Shigella dysenteriae* serotype 1, differing only by one amino acid (AA), but shares overall 56% identity with the AA sequence of STx2 types [1,8]. The Sakai strain of *E. coli* O157:H7 is capable of producing both STx1 and STx2, which are encoded by *stx1* and *stx2* operons (*stx1AB* and *stx2AB*), respectively [8]. STxs are believed to play a key role in the pathogenesis of hemolytic uremic syndrome (HUS) [9]. However, effective prevention and therapeutic interventions against the O157 infection and its sequela HUS are yet to be developed.

Heat-labile toxin (LT) of enterotoxigenic *E. coli* (ETEC), the causative agent of traveler's diarrhea, also belongs to the AB_5 toxin family. Like STx, LT, encoded by a two-gene operon (*lt-AB*), is exported in a Sec-dependent manner to the periplasm where holotoxin assembly occurs with one catalytic A subunit (LT-A; ~28 kDa) and a homopentamer of B subunits (LT-B; 11.6 kDa) [10,11]. Extracellular export of LT across the outer membrane (OM) takes place via a specific type II secretion system (T2SS) encoded by a gene cluster comprising *gspC* to *gspM* [11]. However, no specific T2SS has been identified for the extracellular secretion of STx from Shiga toxin-producing *E. coli* (STEC). Thus, OMVs are likely utilized as non-specific extracellular secretion vehicles for exporting STx accumulated in the periplasm of STEC lacking the specialized toxin secretion system [12,13]. Consistently, STx holotoxins and STxB pentamers were known to be largely enclosed within OMVs [7,14]. Although LT is secreted by the specific T2SS, approximately 95% of LT is still associated with the OMV because the extracellular LT is able to bind to sugar residues in lipopolysaccharide (LPS) on the surface of ETEC [15,16].

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In our previous reports [7,17], we genetically detoxified the OMVs of O157 *E. coli* with the aim of generating low-endotoxic and STxA-deficient OMVs that could be useful as vaccine vehicles. Next, we tried to produce OMVs of O157 *E. coli* containing STxA uncoupled with STxB by creating an internal deletion in the *stxB* gene. However, we found that the STxA could hardly exist as a free form in the periplasm, which prevents secretion of the free A subunit via OMVs. Collectively, in this study, we have shown that periplasmic expression and subsequent extracellular secretion of STxA-subunit alone is impossible without assistance by STxB. Our findings reveal a novel role for the B subunit in stabilizing the A subunit during AB_5 toxin assembly in the periplasm. A chaperone-like role for the toxin B subunit in the periplasm is thus proposed.

2. Materials and methods

Bacterial strains and plasmids used in this study are listed in Table 1.

2.1. Creation of stx and degP mutants of E. coli O157:H7

E. coli O157:H7 strain Sakai [8] and its derivative Sakai-DM [17] possessing less-endotoxic LPS (due to lack of MsbB myristoylation in lipid A biosynthesis) were used as parental strains for creation of site-specific mutations by using an allelic exchange procedure assisted by the Red recombinase carried in pKD46, as described previously [7,17,18]. In addition to pKD46, plasmid pKD3 was used commonly for construction of mutated alleles containing the chloramphenicol (Cm)-cassette and pCP20 was used for excision of the Cm-cassette from the resulting chromosomal mutant [18]. Transformants were plated in Luria-Bertani (LB) agar, containing the appropriate antibiotics (Cm: 20 μg/ml, ampicillin (Amp): 100 μg/ml), and potential mutants were confirmed by a genomic PCR and DNA sequence analysis.

As *E. coli* O157:H7 Sakai strain possesses two *stx* operons, single Δ*stx1B* or Δ*stx2B* mutant and the combined Δ*stx1B*/Δ*stx2B* double mutant were created with targeting DNA template constructed in pUC18-based recombinant plasmids pSTx1B::Cm and pSTx2B::Cm (Table 1). Construction of those template plasmids (pSTx1B::Cm and pSTx2B::Cm) was started by cloning of intact DNA fragments containing *stx1B* and *stx2B* genes, then an inverse PCR was conducted to remove internal 180-bp DNA fragments from each ORF. The primers for inverse PCR are as follows; the forward primer (Fw1B-Kpn): CTGTCATAAT*GGTACC*GGGATTCAGCGAAG and the reverse primer (Re1B-Sal): TCAGGC*GTCGAC*AGCGCACTTGCTG for *stx1B*, and the forward primer (Fw2B-SacI): CCTGTGAATCA*GAGCTC*CGGATTTGCT and the reverse primer (Re2B-Sal): GCACAATCC*GTCGAC*ATTGCATTAACAG for *stx2B*, respectively. To incorporate the Cm-cassette (from pKD3) into the deleted DNA regions of the two *stxB* ORFs, the inverse PCR products were digested with restriction enzymes recognizing the primer sequence underlined, then the Cm-cassette DNA was inserted into the *stxB* alleles.

Similarly, an inverse PCR strategy was employed for construction of Δ*degP*::Cm allele in pUC18-based pDegP::Cm vector, with a view to creating WT/Δ*stx2B*/Δ*degP*::Cm mutant (Table 1). The inverse PCR was conducted with a primer set; the forward (Deg-SalF): CGGGTGAT-GT*GTCGAC*CTCACTGAAC and the reverse (Deg-KpnR): GACCAAA-CGG*GGTACC*AATCGCTAC. Then, the Cm-cassette was inserted into the *Sal*I-*Kpn*I sites generated by the inverse PCR primers underlined.

2.2. Creation of Δlt-B::Cm mutant of ETEC H10407

An allelic exchange approach was employed to make a mutation in the chromosome of ETEC H10407 carrying pKD46, by using the mutated allele constructed in the template plasmid. Construction of the Δ*lt-B*::Cm allele was started by cloning of DNA fragment (~834-bp) for the region of *lt-AB* operon. Then, an inverse PCR was carried out with primers of LTB-BamF (CTATCATATAC*GGATCC*GATGGCAGG) and LTB-SalR (GAGAGGATA*GTCGAC*GCCGTAAATAA) to delete internal 120-bp DNA and to generate the restriction enzyme sites incorporated in the primers underlined. The Cm-cassette DNA digested with *Sal*I-*Bam*HI at both ends was then inserted into the deleted region of the *lt-B* allele. Subsequently, the Δ*lt-B*::Cm allele was introduced into ETEC H10407 carrying pKD46 by electroporation to create the Δ*lt-B*::Cm mutant of ETEC H10407, which was designated ETEC/dLT-B (Table 1).

2.3. Cloning and expression of stx2 genes

For expressions of *stx2A* and *stx2AB* operon DNA in JM109, DNA fragments encoding *stx2A* and *stx2AB* operon were cloned into pBAD24 digested with *Kpn*I and *Sph*I, respectively. High-fidelity PCR was carried

Table 1
Bacterial strains and plasmids used in this study.

Strains/plasmids	Property	Source/Refs.
E. coli O157:H7 strain Sakai	Wild type	[8]
Sakai-DM	Δ*msbB1*/*msbB2* mutant of wild type Sakai strain	This study
DM/Δ*stx1B*	Δ*stx1B* mutant of Sakai-DM	This study
DM/Δ*stx1B*/*stx2B*	Δ*stx1B*/*stx2B*::Cm mutant of Sakai-DM	This study
DM/Δ*stx2A*::Cm	Δ*stx2A*::Cm mutant of Sakai-DM	[7]
WT/Δ*stx1B*	Δ*stx1B* mutant of Sakai wild type (WT)	This study
WT/Δ*stx2B*	Δ*stx2B* mutant of Sakai-WT	This study
WT/Δ*stx2B*/Δ*degP*	Δ*degP*::Cm mutant of Sakai-WT/Δ*stx2B*	This study
ETEC H10407	A reference strain of enterotoxigenic *E. coli* (LT^+)	Lab collection
ETEC/dLT-B	Δ*lt-B*::Cm mutant of ETEC H10407	This study
E. coli JM109	*endA1 gyrA96 thi hsdR17 supE44 relA1 traD36* Δ(*lac-proAB*)/F′(*proAB lacI*^{q}*Z* M15)	Lab collection
pKD46	A t^S-plasmid encoding arabinose-inducible λ-Red recombinase (Amp^R)	[18]
pKD3	A template plasmid carrying the FRT-Cm-FRT cassette (Cm^R)	[18]
pCP20	A t^S-plasmid encoding the FLP recombinase (Amp^R/Cm^R)	[18]
pSTx1B::Cm	pUC18-based recombinant plasmid carrying the Δ*stx1B*::Cm allele (Cm^R/Amp^R)	This study
pSTx2B::Cm	pUC18-based recombinant plasmid carrying the Δ*stx2B*::Cm allele (Cm^R/Amp^R)	This study
pDegP::Cm	pUC18-based recombinant plasmid carrying the Δ*degP*::Cm allele (Cm^R/Amp^R)	This study
pUC18	General cloning vector (Amp^R)	Lab collection
pACYC184	General cloning vector (Cm^R), p15A (*rep*) origin	Lab collection
pAY-*stx1B*	pACYC184-based recombinant plasmid carrying the *stx1B* gene (Cm^R)	This study
pAY-*stx2B*	pACYC184-based recombinant plasmid carrying the *stx2B* gene (Cm^R)	This study
pBAD24	An arabinose-inducible expression vector (Amp^R), pBR322 (pMB1 *rep*) origin	[23]
pBAD-*stx2A*	pBAD24-based recombinant plasmid carrying the *stx2A* gene (Amp^R)	This study
pBAD-*stx2AB*	pBAD24-based recombinant plasmid carrying the *stx2AB* operon (Amp^R)	This study
pBAD-*stx2A1*	pBAD24-based recombinant plasmid carrying the *stx2A1* allele (Amp^R)	This study