RESEARCH LETTER

Detergents enhance EspB secretion from *Escherichia coli* strains harboring the locus for the enterocyte effacement (LEE) gene

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Abstract

The effects of detergents (cholic acid, deoxycholic acid, Triton X-100, and Nonidet P-40) on the secretion of EspB from the locus for enterocyte effacement (LEE) gene-positive *Escherichia coli* strains were examined. Clinical isolates of eight EPEC strains and seven STEC strains were used to detect EspB after they had been cultivated in Luria–Bertani (LB) broth containing one of the detergents. When the bacteria were cultured in LB broth supplemented with one of the detergents, the amount of EspB produced was increased by 2–32-fold depending on the detergent and the strain used. EspB was detected in all strains when they were cultured in LB broth containing all of the detergents. The results obtained in this study can be applied to immunological diagnostic methods for detecting EspB and also to the production of EspB for research purposes.

Introduction

Enteropathogenic *Escherichia coli* (EPEC) is a significant cause of infant diarrhea in developing countries and is often associated with high mortality rates. EPEC attach to the microvilli of enterocytes through their intimin protein, causing an attaching-effacing (A/E) lesion and cell disorders, inducing acute gastroenteritis. The genes responsible for the development of this lesion are clustered on a chromosome and form a pathogenicity island called the locus of enterocyte effacement (LEE) (McDaniel *et al.*, 1995). The LEE of the human EPEC strain E2348/69 was the first to be cloned and sequenced (Elliott *et al.*, 1998). LEE contains genes encoding type III secretion proteins EspA, EspB, and EspD, which are required for attachment and effacement; outer membrane protein intimin, which is required for intimate attachment of EPEC to host cells; and the translocated intimin receptor (Tir) for intimin (Jarvis *et al.*, 1995; Abe *et al.*, 1998). Shiga toxin-producing *E. coli* (STEC) also cause A/E lesions, but their main virulence factor is Shiga toxin.

In research laboratories, EPEC and STEC are defined on the basis of their pathogenic properties, and recently, multiplex PCR has been used (Toma *et al.*, 2003). However, the detection of pathogenic properties is expensive, laborious, and requires expensive apparatus; therefore, they are often defined on the basis of serogrouping, especially in the developing world. Recently, we developed a reversed passive latex agglutination (RPLA) test and a rapid immunochromatographic (IC) test for identifying LEE-positive strains by detecting the pathogenic factor EspB (Lu *et al.*, 2002; Nakasone *et al.*, 2007), which is the most abundantly secreted protein in both pathogens. The RPLA test is more sensitive (detection limit: 1 ng mL^{-1}) than the IC test (detection limit: 4 ng mL^{-1}), but requires overnight incubation. Although Dulbecco's modified Eagle's medium (DMEM) is commonly used to detect EspB from EPEC or STEC, we noticed that some strains grew poorly and sometimes did not grow at all in the medium, even though they were shown to possess the *eae* gene by PCR. Therefore, using DMEM may produce false-negative results due to small amounts of or no EspB being produced. To resolve this problem, a medium in which bacteria can grow and produce EspB is required. If a growth medium that enhances both bacterial growth and EspB production could be created, the sensitivity of the RPLA and/or the IC test for detecting EPEC and/or STEC might be increased. Although various media

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and/or culture conditions have been considered for the enhancement of the proteins secreted by EPEC and STEC (Haigh *et al.*, 1995; Kenny *et al.*, 1997; Beltrametti *et al.*, 1999; Yoh *et al.*, 2003), a medium that works equally well for both pathogens has been identified. Considering the environmental conditions found in the human body, bacterial growth and the secretion of Esp proteins might be affected by bile acid or detergents. In this report, we considered a medium supplemented with various detergents and examined its effects on EspB production. Our results suggested that the detergent-supplemented medium enhanced EspB production in the EPEC and STEC strains and that this new medium is a convenient tool for promoting the expression of EspB.

Materials and methods

Bacterial strains and growth conditions

E2348/69 (O127:H6) and EDL933 (O157:H7) were used as standard EPEC and STEC strains, respectively. The other strains used in this study were isolated from patients with diarrhea in a variety of countries, as described previously (Lu *et al.*, 2002). The strain of each isolate was determined using a standard biochemical test and the PCR method described by Toma *et al.* (2003). The characteristics of the organisms used in this study are listed in Table 1. To elucidate the optimal concentrations of the detergents for EspB detection, each detergent was serially diluted from 1.5% (w/v) with Luria–Bertani (LB) broth and incubated with the reference strains at 37 °C for 15 h.

Table 1. Serotypes, stx genotypes, and EspB types of the strains used in this study

Strains	Serotype	*stx* type	EspB type
EPEC			
E2348/69	O127:H6	–	α
A2	O111:NT	–	α
A3	O111:H9	–	β
A10	O111:H9	–	β
B6	O111:H46	–	α
D3	O26:H54	–	β
D7	O26:NT	–	α
E6	O128:NT	–	β
E8	O128:NT	–	β
STEC			
EDL933	O157:H7	*stx* 1, 2	γ
A11	O111:H9	*stx* 1	β
B8	O111:NT	*stx* 1, 2	α
C11	O26:H54	*stx* 1	β
C12	O26:H54	*stx* 1	β
D2	O26:H54	*stx* 1	β
D4	O26:NT	*stx* 1	β
D5	O26:NT	*stx* 1	β

NT, nontypable.

EspB preparation and detection

After incubation, the OD at 600 nm was adjusted to 0.7 (*c.* 1×10^8 CFU mL^{-1}) with LB broth. The culture was then centrifuged at 5000 ***g*** for 15 min, and the supernatant proteins were precipitated by the addition of trichloroacetic acid at 10%, as described by Yoh *et al.* (2003). The resultant pellet was resuspended in 50 μL of 1 M Tris-HCl buffer (pH 7.6), and EspB was detected using Western blotting, the RPLA test, or the enzyme-linked immunosorbent assay (ELISA). The RPLA test was carried out as described elsewhere (Lu *et al.*, 2002). For the ELISA assay, 96-well plates were coated with anti-EspB antiserum in phosphate-buffered saline (PBS) at 4 °C overnight and blocked in 1% bovine serum in PBS. The plates were then incubated with 50 μL of culture supernatant from each sample for 1 h at room temperature, before being washed five times in PBS containing 0.1% Tween 20, and then incubated with 50 μL anti-rabbit IgG conjugated to horseradish peroxidase. After a 1-h incubation at room temperature, color was developed using an ELISA POD substrate TMB kit (Nacalai, Japan). Absorbance at 460 nm was detected using an ELISA plate reader. For whole-cell extracts, the bacteria were resuspended in an SDS sampling buffer (2% SDS, 62.5 mM Tris, 10% glycerol; pH 7.5) and boiled for 10 min.

Reverse transcription-PCR (RT-PCR) and type III secretion system mutant

We attempted to detect EspB mRNA using the RT-PCR, and total RNA extracts were prepared from the bacteria using an RNA isolation kit (RNeasy Mini kit; Qiagen, Valencia, CA). RNA samples were subjected to RT-PCR using a pair of primers and an RT-PCR kit (SuperScript III One-Step RT-PCR System; Invitrogen, CA). The primer sets (China *et al.*, 1999) used for the RT-PCR were B148 and B151 for type α (E2348/69) and B148 and B150 for type γ (EDL933), and RT-PCR was performed according to the following protocol: 94 °C for 2 min, followed by 20, 25, or 30 cycles of 94 °C for 20 s, 55 °C for 40 s, and 72 °C for 2 min. The PCR products were analyzed by gel electrophoresis in 2% agarose. An *escN* mutant of EPEC E2348/69, which displays a defective secretion of type III-secreted proteins, was kindly supplied by Prof. Abe.

Detergents

Cholic acid (CA), deoxycholic acid (DOC), Triton X-100 (TX), and Nonidet P40 (P40) were purchased from Nacalai Co. (Tokyo, Japan), and the LB broths supplemented with

each detergent were designated CA–LB, DOC–LB, TX–LB, and P40–LB.

Statistics

The results are expressed as the mean ± SD. Differences between two groups were determined using the two-tailed, unpaired Student's *t* test. $P \leq 0.05$ was considered to be significant.

Results

Optimal concentrations of the detergents for EspB secretion

E2348/69 (EPEC) or EDL933 (STEC) was cultured in LB broth supplemented with either 1% or 0.1% detergent at 37 °C for 12 h, and then we examined bacterial growth and EspB production. The bacteria grew as well in each LB broth supplemented with detergent as in LB broth without detergent. EspB was detected in all of the 0.1% detergent–LB cultures by Western blotting, but its concentration varied in 1% detergent–LB (data not shown). To elucidate the optimal detergent concentrations for EspB secretion, the bacteria were cultured in LB broth with various concentrations of detergents (1.5–0.003%), and the numbers of EspB in the culture supernatants were determined. The results obtained from three separate experiments by Western blotting are shown in Fig. 1. The optimal detergent concentrations for both pathogens were estimated as the percentage value that produced the most EspB in both pathogens, and were determined as 0.1% for CA, TX, and P40, and 0.05% for DOC.

Incubation time and RT-PCR

To examine the time course of EspB secretion, the culture supernatant was collected at 2, 6, and 10 h (Fig. 2a). EspB was first detected in TX–LB and P40–LB after 6 h of incubation, and it was detected in all of the media supplemented with detergent, but not in the LB without detergent, at 10 h of incubation. These results suggest that all four detergents used here are useful for detecting EspB production by both pathogens. To determine whether the detergents activate EspB transcription, the expression of EspB mRNA was examined in both strains by RT-PCR during a 6-h culture in LB or detergent–LB. EspB type α (188 bp, EPEC) and type γ (233 bp, STEC) EspB mRNA were detected in LB supplemented with detergent during 25 cycles of PCR (Fig. 2b), whereas the EspB mRNA in the LB without detergent had to be amplified for 30 cycles of PCR. These results indicate that the detergents used in this study induced the expression of EspB. As the detergents were used as membrane protein solubilizing agents, their effects on cellular integrity were examined by culturing the *escN* mutant, which is unable to secrete any known type III secreted protein, in LB broth supplemented with detergent for 10 h. EspB was not detected in the culture supernatant, but was found in whole-cell extracts (Fig. 2c). These results suggested that the detergents enhanced EspB production without causing cell lysis.

Application to other EHEC and EPEC strains

To examine the effects of the detergents on other EPEC and STEC strains, eight EPEC and seven STEC strains that did not produce EspB in DMEM were examined (Fig. 3a). Of the EPEC strains, strain A2 and strain E6 produced EspB in all of the detergent-supplemented LB cultures, but the other strains required CA or DOC for EspB production. Of the STEC strains, strain A11 did not produce in CA–LB, and strain B8 required DOC–LB or TX–LB. Strain D2 produced EspB in CA–LB (Fig. 3a). These results indicate that the EspB of these strains will not be detected when they are cultured in LB broth without the appropriate detergent. Based on this observation, we examined whether EspB was secreted by these strains in LB supplemented with 0.1% CA,

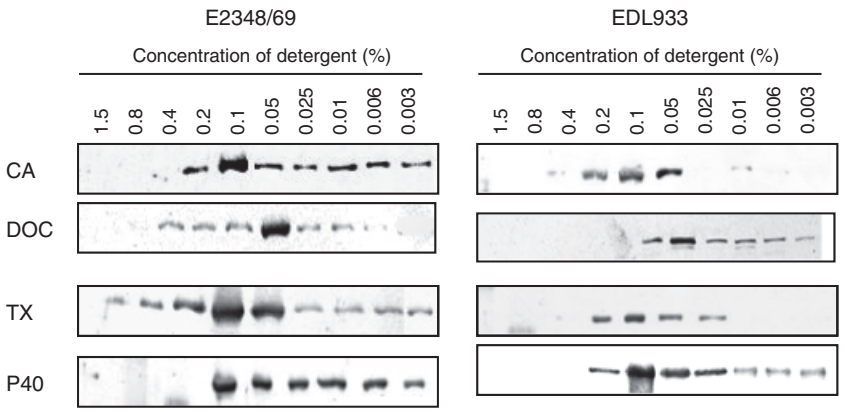

Fig. 1. Western blotting of EspB. The E2348/69 and EDL933 strains were cultured in LB broth supplemented with one of the detergents at 37 °C for 12 h, and then the culture supernatant was obtained by centrifugation. EspB was precipitated using trichloroacetic acid and applied to SDS-PAGE following Western blotting with anti-EspB antibody. The detergent was prepared using twofold serial dilutions with LB broth to yield concentrations ranging from 1.5% (w/v) to 0.003%. CA, cholic acid; DOC, deoxycholic acid; TX, Triton X-100; P40, Nonidet P-40.

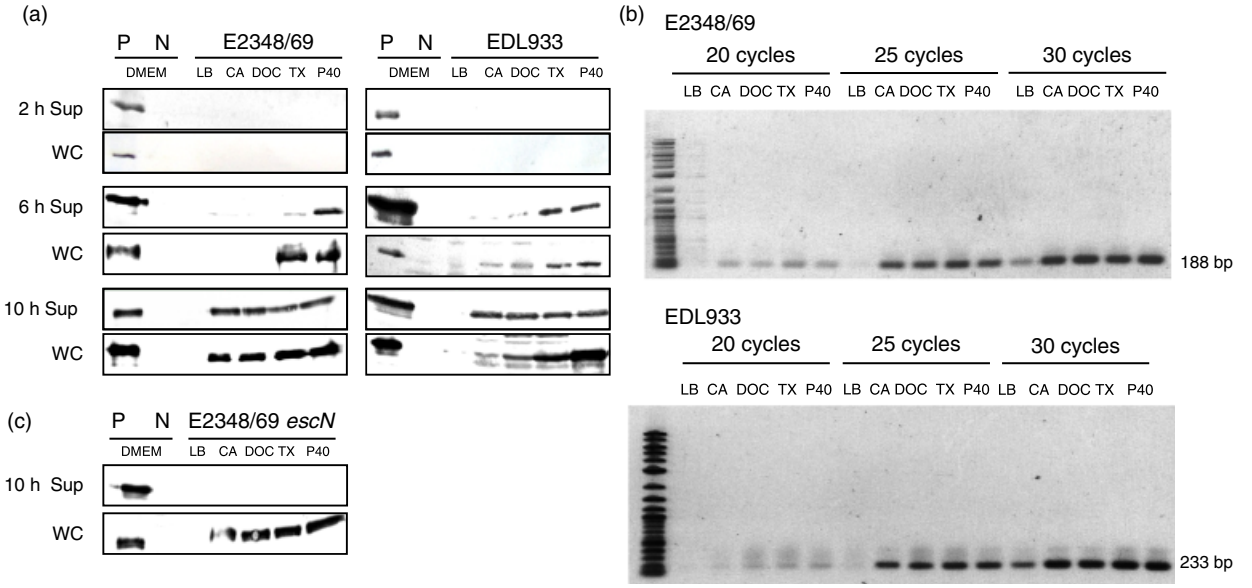

Fig. 2. (a) Time-course detection of EspB produced by E2348/69 or EDL933 in LB broth supplemented with one of the detergents (0.1% CA, 0.01% DOC, 0.1% TX, or 0.1% P40). Samples were taken from each medium at 2, 6, and 10 h, and EspB were prepared from the culture supernatant (Sup) and the whole-cell extracts (WC). P, positive control (E2348/69 or EDL933); N, negative control (*E. coli* K12) cultured in DMEM. (b) Detection of *espB* α (E2348/69) and γ (EDL933) by RT-PCR. Total RNA was extracted from bacteria cultured in LB broth and LB broth containing one of the detergents after 6 h. The RT-PCR products were sampled after 20, 25, and 30 cycles. (c) Western blotting of the *escN* mutant cultured in LB or LB supplemented with detergents. Samples were prepared from the culture supernatant and the cell pellet.

TX, P40, and 0.05% DOC. All strains secreted EspB when they were cultured in LB broth supplemented with all four detergents (Fig. 3b). Using a quantitative ELISA assay, the EspB concentrations of the medium were determined (Table 2). The concentration of EspB was increased 10–100-fold in the LB broth supplemented with the detergents.

Discussion

EspB is an appropriate marker for the immunological detection of EPEC and/or STEC because it is the major secreted protein in both pathogens (Lu *et al.*, 2002; Nakasone *et al.*, 2007). Before immunological tests, bacteria are cultured in DMEM to enhance their EspB production; however, some strains neither grow nor produce EspB in DMEM. We attempted to develop a culture medium that promotes the secretion of EspB from the E2348/69 and EDL933 strains without affecting bacterial growth. Although other media and culture conditions reported to date have been used for this purpose, no medium or culture conditions that work for both pathogens have been found. We focused on using detergents as to promote EspB production because the human intestine contains CA and DOC, which might enhance EspB secretion. As shown in this study, the bacteria grew well in LB broth containing each detergent, and EspB secretion was increased in the LB broth containing the detergents compared with that in the LB without the detergents (Table 2). These findings suggested that detergents enhance EspB secretion without affecting bacterial growth.

We predicted that EPEC and STEC would be dependent on the CA and DOC, respectively, because EPEC binds to the small bowel, where CA is abundant, and STEC binds to the large bowel, which contains DOC; however, we could not find a relationship between the effects of the detergents and EspB secretion. Although the precise mechanism of the enhancement of EspB secretion by detergents is unknown, one possibility is that detergents increase membrane permeability, thereby facilitating the leakage of effector proteins without causing bacterial cell death. To confirm this possibility, we examined EspB production using a type III secretion apparatus mutant of EPEC, which is unable to secrete effector proteins. The *escN* mutant (Matsuzawa *et al.*, 2004) did not secrete EspB when it was cultured in LB–detergent, but EspB was localized in its cytoplasm (Fig. 2c). These findings suggested that the detergents did not cause the leakage of cytoplasmic EspB.

The effects of detergents on protein secretion were reported by Pope *et al.* (1995) for *Shigella* spp. (invasion-related proteins), Osawa & Yamai (1996) for *Vibrio parahaemolyticus* (thermostable-directed hemolysin), Malik-Kale *et al.* (2008) for *Campylobacter jejuni* (Cia protein),

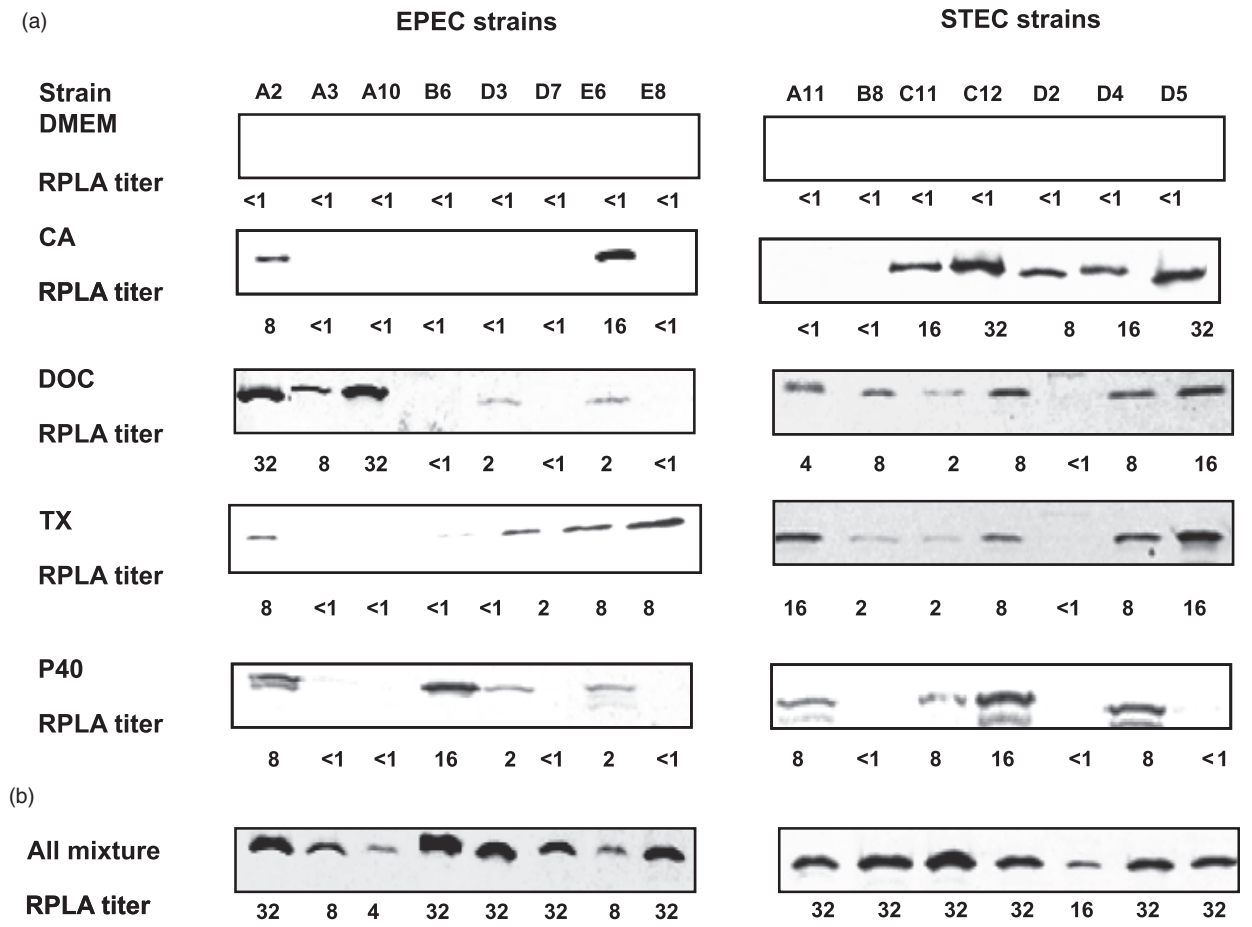

Fig. 3. Western blotting and RPLA of EspB. EPEC and STEC strains were cultured in LB broth with or without detergent. Each sample was prepared as described in Fig. 1. The RPLA titer indicates the highest dilution yielding positive results. The data shown are the mean of three experiments. (a) Concentrations of 0.1%, 0.01%, 0.1%, and 0.1% were used for CA, DOC, TX, and P40, respectively. (b) All four detergents were mixed together.

Table 2. EspB concentrations in EPEC and STEC culture supernatant

	EspB (ng mL^{-1})	
Strains	DMEM	LB+detergents
EPEC		
A2	1.5 ± 1.0	140 ± 13.0
A3	1.3 ± 1.0	18.6 ± 2.0
A10	1.2 ± 0.2	10.8 ± 1.8
B6	0.6 ± 0.3	181 ± 32.0
D3	0.3 ± 0.1	155 ± 5.1
D7	1.3 ± 0.3	188 ± 7.3
E6	0.6 ± 0.1	15.2 ± 5.6
E8	0.8 ± 0.2	194 ± 6.5
STEC		
A11	1.2 ± 0.6	121 ± 9.0
E8	1.6 ± 0.7	173 ± 1.7
C11	0.6 ± 0.1	114 ± 18.0
C12	1.9 ± 1.0	125 ± 10.0
D2	0.3 ± 0.1	35.1 ± 14.0
D4	0.5 ± 0.4	102 ± 15.0
D5	0.8 ± 0.3	148 ± 13.0

Values are shown as the mean ± SD of three independent determinations according to a quantitative ELISA assay.

and Hung & Mekalanos (2005) for *Vibrio cholerae* (cholera toxin). Hung and Mekalanos speculated that bile acids in the inner membrane of *V. cholerae* interact with the transmembrane domain of the transcriptional regulator of cholera toxin (ToxR). The detergents used in this study may interact with the type III secretion system because EspB is secreted by this apparatus. However, as the regulation of this system is complex (Spears *et al.*, 2006), a genetic approach to studying the relationship between EspB secretion and the effects of detergents is required to clarify the mechanism behind their effects.

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