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Epigallocatechin gallate inhibits the type III secretion system of Gram-negative enteropathogenic bacteria under model conditions

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One sentence summary: Epigallocatechin gallate inhibits the type III secretion system (T3SS) of enteropathogenic and enterohemorrhagic *Escherichia coli*, *Salmonella enterica* serovar Typhimurium and *Yersinia pseudotuberculosis*.

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ABSTRACT

Epigallocatechin gallate (EGCG), a major polyphenol in green tea, inhibits the type III secretion system (T3SS) of enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC and EHEC, respectively), *Salmonella enterica* serovar Typhimurium, and *Yersinia pseudotuberculosis*. The inhibitory effect causes the inhibition of hemolysis, cell invasion, cell adhesion and apoptosis, which are functions of the type III secretion device. In the case of EPEC, EspB accumulates in the cells. RT-PCR showed that the translation of EspB was not blocked. The transcription of *escN*, which supplies energy for the injection of the effector factor into the host cells, was also not inhibited. EGCG does not suppress the transcription and translation of T3SS constitutive protein in bacterial cells, but it seems to suppress the normal construction or secretion of T3SS. When Luria-Bertani (LB) medium was used to visualize the EGCG-induced inhibition of T3SS, the inhibitory effect disappeared. The inhibition of T3SS was partially canceled when the T3SS inhibitory potency of EGCG was examined by adding yeast extract, which is a component of LB medium, to DMEM. These results suggest that EGCG probably inhibits secretion by suppressing some metabolic mechanisms of T3SS.

Keywords: Epigallocatechin gallate; Type III secretion system; inhibition

INTRODUCTION

The type III secretion system (T3SS) is known to play an important role in disease as a virulence factor and is highly conserved in many Gram-negative pathogenic bacteria, including enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), *Salmonella* spp., *Yersinia* spp., *Shigella* spp., *Pseudomonas aeruginosa*, *Citrobacter rodentium* and *Chlamydia* spp. (Cobum, Sekirov and Finlay 2007). EPEC is the major cause of infant diarrhea in developing countries, and builds up the attaching and effacing (AE) lesion for the initial attachment to the intestinal epithelial cells by using T3SS. *Salmonella* requires T3SS-1 for intracellular invasion, and

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Table 1. Characteristics of pathogenic *E. coli* strains.

	No.	Year	Location	O-type	Gene[a]		
					easA	*stx*	other
EPEC	1	1981	Kenya	O119	+	−	−
	2	1999	Laos	O142	+	−	−
	3	2000	Indonesia	O119	+	−	−
	4	2000	Indonesia	O119	+	−	−
	5	2003	Japan	O128	+	−	−
	6	2003	Japan	O26	+	−	−
EHEC	7	2003	Japan	O111	−	+	−
	8	2003	Japan	O111	−	+	−
	9	2003	Japan	O26	−	+	−
	10	2003	Japan	O26	−	+	−
	11	2003	Japan	O26	−	+	−
	12	2003	Japan	O26	−	+	−

[a]*easA*, EspA; *stx*, Shiga-like toxin; other: *aggR*, EAEC; *ets* and *elt*, ETEC; *ipaH*, EIEC; Toma *et al.* 2003).

Yersinia induces cell invasion and cell death using T3SS. T3SS-deficient strains exhibit greatly reduced pathogenicity (Abe *et al.* 1998), so T3SS is regarded as a novel target for anti-infective drugs.

Green tea is a type of tea made from *Camellia sinensis* leaves and has been widely used as a traditional and private medicine for a variety of diseases (Bao and Peng 2016). Epigallocatechin gallate (EGCG) is the most abundant polyphenol in green tea, exhibiting anti-oxidant, anti-inflammatory and anti-radiation biochemical effects *in vitro* (Nagle, Ferreira and Zhou 2006; Pandey & Rizvi 2009). Here, we investigated whether EGCG can be used as an inhibitor of T3SS.

MATERIALS AND METHODS

Bacterial strains and growth conditions

In this study, we used enteropathogenic *Escherichia coli* (EPEC) strain E248/69 (Levine *et al.* 1978), enterohemorrhagic *E. coli* (EHEC) strain EDL933 (Perna *et al.* 2001), *Yersinia pseudotuberculosis* virulent strain (Gemski *et al.* 1980) and *Salmonella enterica* serovar Typhimurium virulent strain (Gulig and Curtiss 1987). In addition, we also used six EPEC and six EHEC strains, all of which were clinically isolated in our laboratory (Table 1; Toma *et al.* 2003).

EPEC and EHEC were cultured in Dulbecco's Modified Eagle's Medium (DMEM). *Yersinia pseudotuberculosis* was cultured in LB broth containing 25 mM of oxaloacetic acid and 10 mM of $MgCl_2$. *Salmonella enterica* serovar Typhimurium was cultured in LB broth containing 0.3 M of NaCl.

Type III secretion inhibition assay

Bacteria were cultured in medium supplemented with or without EGCG at 37°C for 5 h with shaking. The culture supernatant was then obtained by centrifugation at 18 800 g for 10 min at 4°C. Proteins that had been secreted into the culture supernatant were precipitated using trichloroacetic acid (final concentration, 10%). The proteins were then subjected to SDS-polyacrylamide gel electrophoresis. After protein staining, the presence or absence of the secreted proteins was examined.

Hemolysis inhibition

A hemolysis inhibition assay was performed as described by Kimura *et al.* (2011) for EPEC and as described by Håkansson *et al.* (1996) for *Yersinia*. Briefly, bacteria were cultured in the medium supplemented with or without EGCG. Sheep erythrocytes were washed with phosphate buffered saline (PBS) and suspended in the same culture medium. Bacterial culture and the erythrocyte suspension (50 μl of each) were mixed in a 96-well round bottomed microtiter plate, and close contact between erythrocytes and bacteria was achieved by centrifugation at 60 g for 10 min. After the plate was incubated at 37°C for 90 min, the plate was centrifuged again. The resulting supernatants were transferred to a new plate, and the OD at 450 nm was measured by using a microplate reader.

Preparation of bacterial cytosol

Bacteria cultured with the extracts were harvested by centrifugation at 20 000 *g* for 10 min. The bacterial pellets were suspended in 2% SDS sample buffer and heated at 100°C for 5 min for the detection of EspB using western blotting.

Cell adherence and invasion assay

HEp-2 cells (2×10^5 cells well^{-1}) were incubated at 37°C in 5% CO_2 for 18 h. Bacteria (EPEC or EHEC) cultured in DMEM supplemented with or without plant extracts were added to the HEp-2 cells at a multiplicity of infection (MOI) of 50 and incubated at 37°C in 5% CO_2 for 2 h. After washing with PBS, the cells were fixed with methanol and stained with Giemsa. The number of adherent bacteria was counted on approximately 100 HEp-2 cells in 10 randomly selected microscopic fields. HEp-2 cell invasion of *Salmonella* was assayed using a method described by Li *et al.* (2013).

Reverse transcription–polymerase chain reaction

To analyse the EspB subtype and the *escN* (cytoplasmic ATPase for protein secretion by T3SS) gene, reverse transcription–polymerase chain reaction (RT-PCR) was performed as previously described (Nakasone *et al.* 2011). Total RNA extracts were prepared from the bacteria using an RNA isolation kit (Rneasy Mini kit; Qiagen, Valencia, CA, USA). 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, Carlsbad, CA, USA). The primer sets for EspB type α (EPEC) (China *et al.* 1999) were B148 (GCCGTTTTTGAGAGCCA) and B151 (TCCCCAGGACAGATGAGAT), while those for EscN (Kyaw *et al.* 2003) were N1 (CGCCTTTTACAAGATAGAAC) and N2 (CATCAAGAATAGAGCGGAC). RT-PCR was performed according to the following protocol: 94°C for 2 min, followed by 20, 25 or 35 cycles of 94°C for 20 s, 55°C for 40 s, and 72°C for 2 min. The PCR products were analysed using gel electrophoresis in 2% agarose.

Statistical analysis

All the experiments were performed two or three times in triplicate. Data were expressed as the mean ± standard deviation (SD). Statistical analyses were performed using unpaired two-tailed Student's *t*-tests. Differences were considered significant at a P value of < 0.05.

RESULTS AND DISCUSSION

EPEC was cultured in DMEM containing EGCG at different concentrations of EGCG (4–500 μg ml^{-1}). EspB was blocked by about 70% at a concentration of 16 μg ml^{-1} EGCG and by about 80% at a

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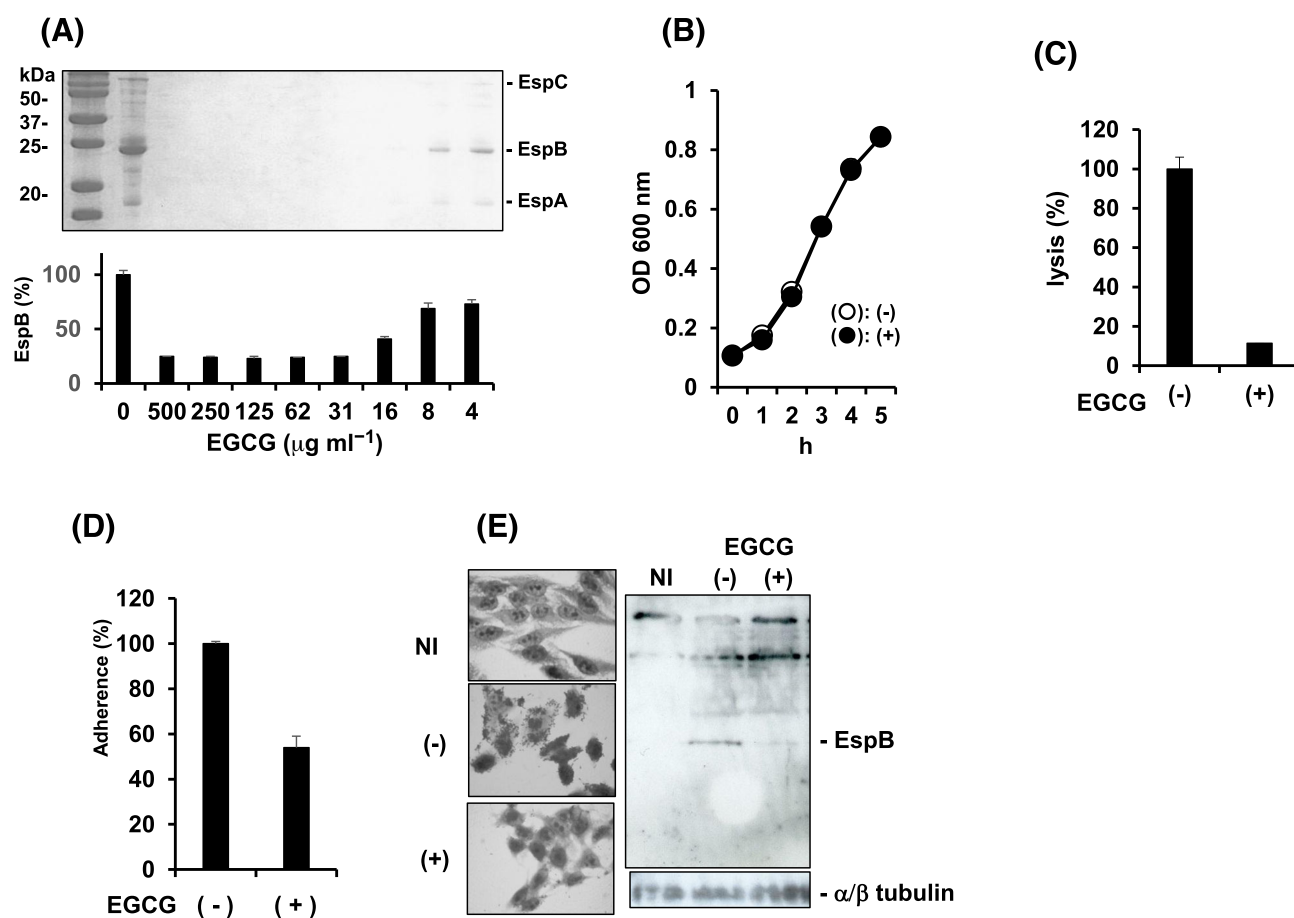

Figure 1. Effect of EGCG on EPEC T3SS. (**A**) Minimum secretion inhibitory concentration of EGCG in EPEC T3SS. EGCG was diluted and added to the medium to obtain the lowest inhibitory concentration of EspB secretion. The degree of secretion was measured using an ELISA at that concentration and was expressed as the relative percentage. (**B**) Growth curve for EPEC in DMEM supplemented with 50 μg ml^{-1} (filled circles) or without (open circles) EGCG. (**C**) Relative percentage of hemolytic activity of bacteria cultured in medium supplemented with or without of 50 μg ml^{-1} EGCG. (−), Bacteria cultured in DMEM supplemented without EGCG; (+), bacteria cultured in DMEM supplemented with EGCG. (**D**) Adhesion of EPEC cultured in medium supplemented with or without EGCG to HEp-2 cells. EPEC was used to infect HEp-2 cells at a multiplicity of infection (MOI) of 50 for 2 h at 37°C in a CO_2 incubator. (**E**) Detection of EspB using western blotting of HEp-2 cytoplasm. NI, non-infection; (−), bacteria cultured in DMEM supplemented without EGCG; (+), bacteria cultured in DMEM supplemented with EGCG.

concentration of 31 μg ml^{-1} (Fig. 1a). The subsequent secretion inhibition experiments were performed using a concentration of 50 μg ml^{-1}. The growth of EPEC was not inhibited at a concentration of 50 μg ml^{-1} EGCG (Fig. 1b). Sheep blood cell lysis caused by T3SS was inhibited by about 80% (Fig. 1c). EPEC adherence to HEp-2 cells was inhibited by about 65% (Fig. 1d). EspB was not detectable in HEp-2 cytoplasm when HEp-2 cells infected with EPEC were cultured in DMEM supplemented with EGCG (Fig. 1e). The same experiment was performed using EHEC, and similar results were obtained (Fig. 2a–c). Since EHEC has almost the same locus of enterocyte effacement (LEE) gene as EPEC, this result seemed reasonable. We also tested the inhibitory effect of EGCG on the expression of EspB by using six EPEC and six EHEC clinically isolated strains (Table 1), and confirmed that EGCG inhibits the secretion of EspB in all the strains (Fig. 2d).

We investigated whether EGCG inhibits *Salmonella* or *Yersinia* T3SS. *Salmonella* Typhimurium T3SS proteins were inhibited at 12 μg ml^{-1} of EGCG (Fig. 3a). EGCG at 25 μg ml^{-1} blocked *Salmonella* invasion into HEp-2 cells by 80% (Fig. 3b). *Yersinia* T3SS proteins were also inhibited by EGCG at 16 μg ml^{-1} of EGCG (Fig. 3c). The hemolytic activity of *Yersinia* by T3SS was reduced by about 40% at 16 μg ml^{-1} of EGCG (Fig. 3d). When HEp-2 cells were infected with *Yersinia* cultured in media with or without EGCG, cleaved caspase 3 was not observed in cells infected with *Yersinia* that were cultured in medium containing EGCG (Fig. 3e).

To examine whether EGCG inhibits the transcription of the *espB* and *escN* genes of EPEC, expression of these genes was examined by using RT-PCR. RT-PCR showed that the *espB* subtype α (98 bp) and *escN* were expressed in the bacteria growing in the medium containing EGCG (Fig. 4a). These results suggest that the EspB protein is produced inside the bacteria. To confirm this hypothesis, we examined the presence of EspB in bacterial cytoplasm using western blotting. Western blotting showed that EspB was present in the cytosol (Fig. 4b). This result suggests that EspB is not secreted extracellularly.

T3SS is known not to be expressed in LB broth unless bicarbonate is added (Abe et al. 2002) or the culturing is performed in the presence of 5% CO_2 (Haigh et al. 1995). These findings suggest that the intracellular metabolic system may be involved in the regulation of expression of T3SS in EPEC and EHEC. EGCG might inhibit T3SS by disturbing the metabolic system of bacteria. To investigate the influence of EGCG on metabolism, EPEC was cultured in LB broth with or without EGCG in a 5% CO_2 incubator. EGCG did not inhibit EspB secretion into the LB medium (Fig. 4c). EPEC T3SS was not blocked even if the amount of EGCG was increased (Fig. 4d). Since LB medium contains tryptone and yeast

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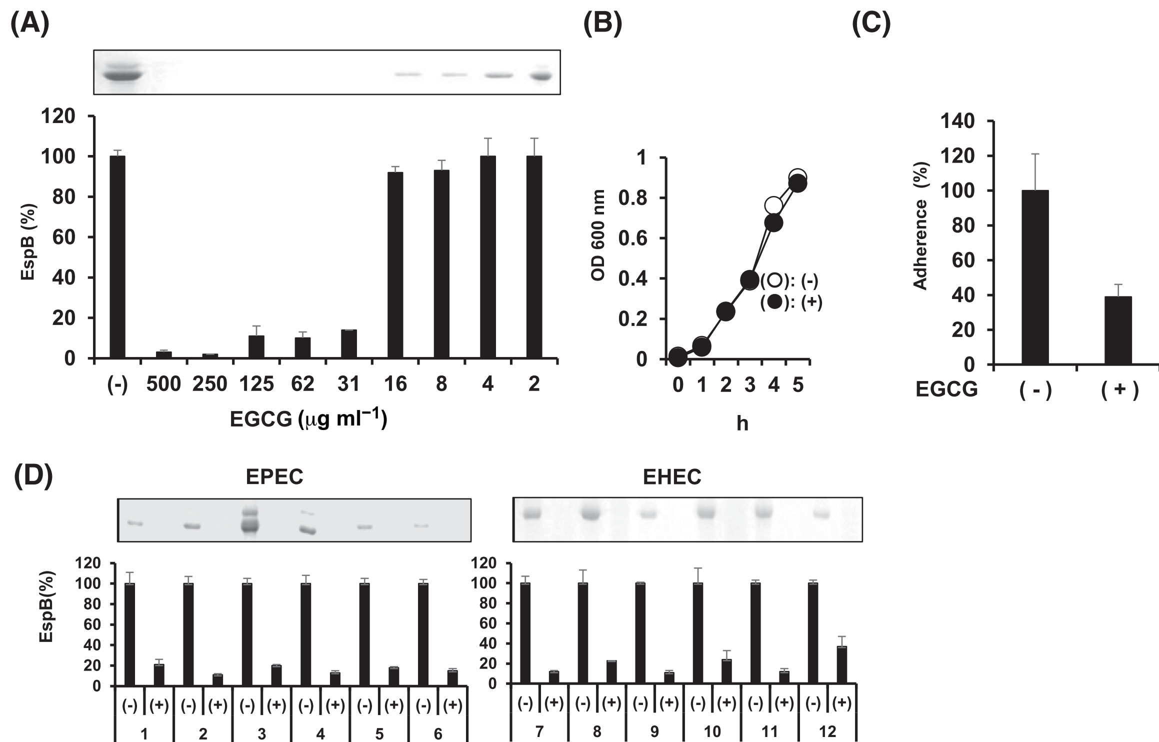

Figure 2. Effect of EGCG on EHEC T3SS. (A) Minimum secretion inhibitory concentration of EGCG in EHEC EspB. The procedure was the same as that described in Fig. 1. (B) Growth curve of EHEC in DMEM supplemented with 50 μg ml^{-1} (filled circles) or without (open circles) of EGCG. (C) Adhesion of EHEC cultured in medium supplemented with or without EGCG to HEp-2 cells. The procedure was the same as that described in Fig. 1. (−), Bacteria cultured in DMEM supplemented without EGCG; (+), bacteria cultured in DMEM supplemented with EGCG. (D) Effect of EGCG on other *E. coli* strains.

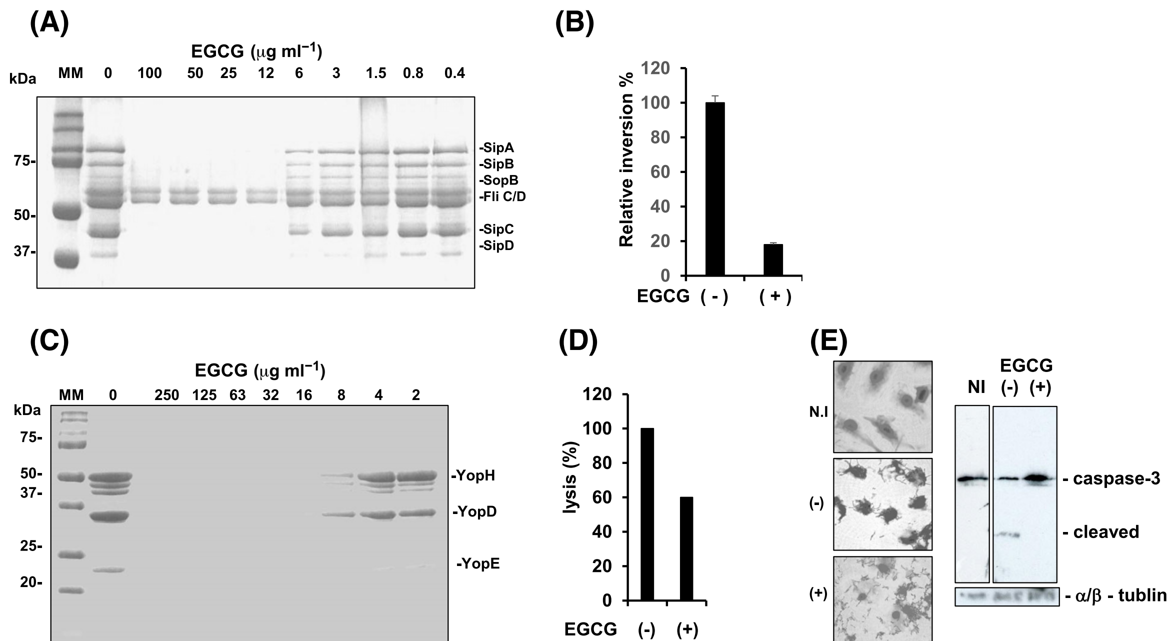

Figure 3. Effect of EGCG on *Salmonella* T3SS. (A) Minimum secretion inhibitory concentration of EGCG in *Salmonella* T3SS. The procedure was the same as that described in Fig. 1a. (B) HEp2 cell invasion of *Salmonella* Typhimurium. HEp2 was infected with *Salmonella* Typhimurium at an MOI of 50 for 2 h; extracellular bacteria were killed by gentamicin, and the intracellular bacteria were counted. The number of invaded bacteria was expressed as the relative percentage. (C) Minimum secretion inhibitory concentration of EGCG in *Y. pseudotuberculosis* T3SS. The procedure was the same as that described in Fig. 1a. (D) Relative percentage of hemolytic activity of bacteria cultured in medium supplemented with or without of 50 μg ml^{-1} EGCG. (−), Bacteria cultured in medium supplemented without EGCG; (+), bacteria cultured in medium supplemented with EGCG. (E) Cleavage of caspase 3 induced by macrophage infection of *Y. pseudotuberculosis*. *Yersinia* was infected with mouse bone marrow-derived macrophages at an MOI of 100 for 2 h, and the activity of caspase 3 in the macrophages was examined using western blotting. NI, non-infection; (−), bacteria cultured in medium supplemented without EGCG; (+), bacteria cultured in medium supplemented with EGCG.

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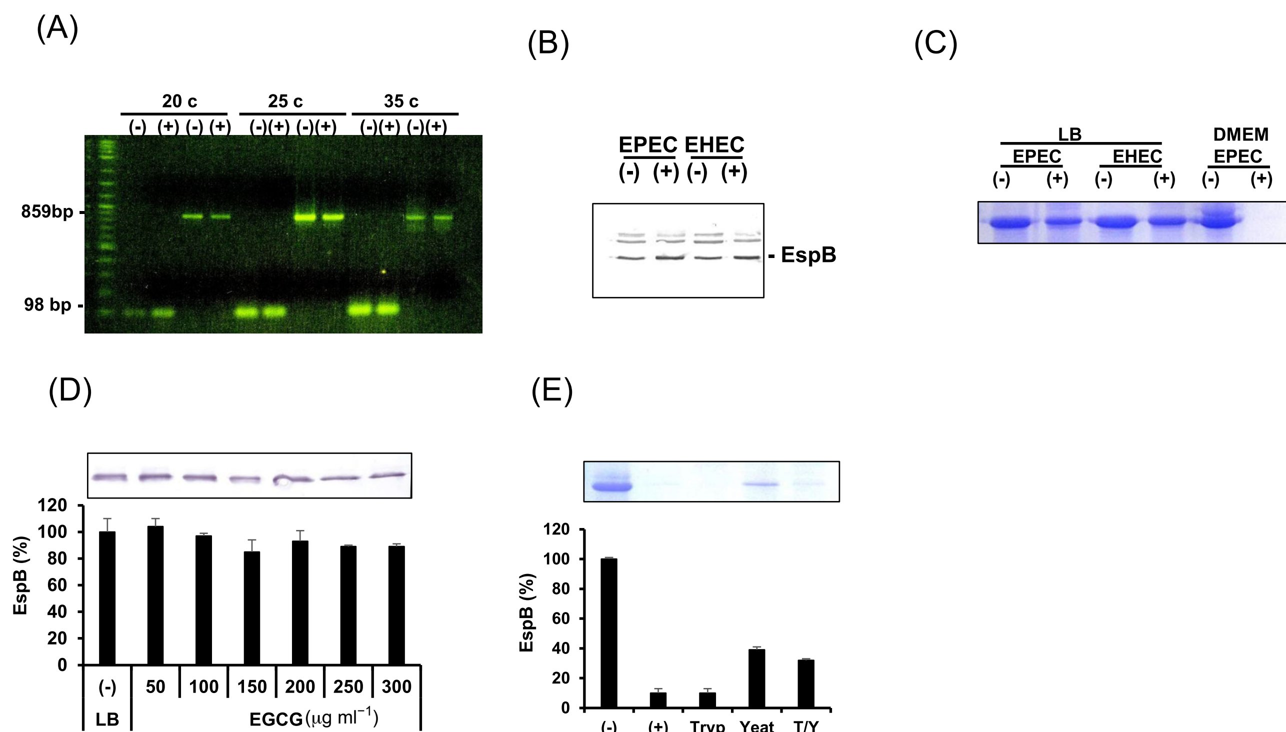

Figure 4. Properties of EspB secretion during T3SS inhibition by EGCG. (**A**) Reverse transcription–polymerase chain reaction (RT-PCR) of EspB and escN of EPEC. RT-PCR was performed as described in 'Materials and methods'. (**B**) Western blotting of cytoplasmic EspB. EPEC cultured in medium supplemented with or without EGCG was harvested by centrifugation. After washing the cells three times with PBS, the cells were dissolved in 2% SDS solution and heated at 100°C for 5 min. EspB in the bacterial cytoplasm was then examined using western blotting. (**C**) Effect of LB medium on T3SS inhibitory action by EGCG. EPEC or EHEC was cultured in LB medium with or without EGCG at 37°C for 12 h in a 5% CO_2 incubator. (**D**) Minimum secretion inhibitory concentration of EGCG in EPEC EspB. (**E**) Effect of EGCG on T3SS inhibition when tryptone or yeast extract or both were added to DMEM. Tryptone or yeast was added at concentrations of 1% and 0.5%, respectively.

extract, tryptone or yeast or both were added to DMEM supplemented with EGCG, and EspB secretion was examined. As shown in Fig. 4e, EspB secretion was restored by the addition of yeast extract, although the degree of restoration was not large (about 40%). Thus, it seems unlikely that the yeast extract component in the LB medium inhibits the function of EGCG, since the LB medium is used to express T3SS in *Yersinia* or *Salmonella* and its expression is inhibited by EGCG. Alternatively, the mechanism by which EGCG inhibits T3SS may differ among these bacteria.

EGCG is known to possess various types of biological activities, such as antiproliferative (Jochmann, Baumann and Stangl 2008), antimutagenic (Gupta, Saha and Giri 2002), antioxidant (Forester SC and Lambert 2011), antibacterial (Ben Lagha, Haas and Grenier 2017), antiviral (Butler and Wu 2011), antimetabolic syndrome (Thielecke F and Boschmann 2009) and chemopreventive effects (Gerhauser 2013; Singh, Shankar and Srivastava 2011). Among these functions, various mechanisms have been proposed. For example, EGCG regulates signal transduction pathways (JAK/STAT, MAPK and NF-kB), induces tumor suppressor activities (p53, p21, p16 and Rb), and modulates function of a variety of receptors (laminin receptor and androgen receptor) in eukaryotic cells. EGCG may therefore have an inhibitory effect on the bacterial T3SS in a similar manner. Further investigation will be required to reveal the target molecule of the EGCG on the T3SS machinery.

Our result suggests that EGCG or green tea may be used as a medicine or special beverage to prevent the infection of enteropathogenic bacteria that have the T3SS as a virulent factor, such as EPEC, EHEC, *Yersinia* spp. and *Salmonella* spp. However, as our result also suggest, it will be important for there to be optimal conditions in the intestine for EGCG or green tea to inhibit the bacterial T3SS.

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