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Atypical *Shigella boydii* 13 encodes virulence factors seen in attaching and effacing *Escherichia coli*

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Abstract

Enterohemorrhagic *E. coli* (EHEC) is a foodborne pathogen that causes watery diarrhea and hemorrhagic colitis. In this study, we identified StcE, a secreted zinc metalloprotease that contributes to intimate adherence of EHEC to host cells, in culture supernatants of atypical *Shigella boydii* 13 (*Shigella* B13) strains. Further examination of the *Shigella* B13 strains revealed that this cluster of pathogens does not invade but forms pedestals on HEp-2 cells similar to EHEC and enteropathogenic *E. coli*. This study also demonstrates that atypical *Shigella* B13 strains are more closely related to attaching and effacing *E. coli* and that their evolution recapitulates the progression from ancestral *E. coli* to EHEC.

Keywords

StcE; *E. coli* O157:H7; attaching and effacing lesions; *Shigella boydii* 13

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) cause diarrheal disease that ranges from watery diarrhea to hemorrhagic colitis. Virulence factors of EHEC include the chromosomally-encoded Shiga toxin and the locus of enterocyte effacement (LEE). LEE is a 35 kb pathogenicity island that confers the attaching and effacing phenotype to both EHEC and enteropathogenic *E. coli* (EPEC), wherein intimate adherence of the bacteria to host cells induces formation of actin-rich pedestals beneath the bacteria. The majority of the clinical EHEC disease in United States is caused by serotype O157:H7 (Manning *et al.*, 2007), which carries a 92 kb virulence plasmid, pO157, that encodes many potential virulence factors, including *stcE* (Burland *et al.*, 1998).

The *stcE* gene is encoded on the large virulence plasmids of *E. coli* O157:H7, O157:H-, ON:H7, and O55:H7 (Lathem *et al.*, 2003). In all cases, *stcE* is found linked to *etpD*, which encodes the subunit of the type II secretion apparatus responsible for the secretion of StcE protein (Lathem *et al.*, 2002). StcE is a 96 kDa zinc metalloprotease that cleaves specific O-linked glycoproteins and contributes to the intimate adherence of *E. coli* O157:H7 to HEp-2 cell surfaces (Grys *et al.*, 2005). Evidence supports a role for StcE in EHEC disease in clearing the mucus that forms a protective barrier over the colonic epithelium. Following colonization, intimate adherence, and pedestal formation by EHEC, the clinical syndrome progresses from watery diarrhea to hemorrhagic colitis. At this stage, StcE plays an anti-inflammatory role by localizing the human complement regulator, C1 esterase inhibitor (C1-

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INH), to cell surfaces, decreasing the complement-mediated lysis of both bacteria and host cells (Lathem *et al.*, 2004; Grys *et al.*, 2006).

Shigella, another enteropathogen, is indistinguishable from *E. coli* by DNA-DNA hybridization techniques, with the exception of *Shigella boydii* 13 (*Shigella* B13) (Pupo *et al.*, 2000). *Shigella* B13 is more closely related to *Escherichia albertii* than the *E. coli-Shigella* group, and lacks the large virulence plasmid, (pINV), that confers the invasion phenotype in all other *Shigella*. Hyma et al. demonstrated that *Shigella* B13 and *E. albertii* strains carry *eae*, a marker for LEE (Hyma *et al.*, 2005). A small subset of analyzed *Shigella* B13 strains encoding *eae* were more related to the *E. coli-Shigella* group and labeled atypical *Shigella* B13. Many of these strains also carried markers for the pO157 plasmid, such as *ehxA* and *toxB*, suggesting that atypical *Shigella* B13 may be similar to EHEC and, thus, may encode *stcE*. This study describes the identification of *stcE* in atypical *Shigella* B13 strains and the genetic and phenotypic profile of this unique cluster of *Shigella*.

Materials and Methods

Bacterial culture, DNA extraction and PCR amplification

The *S. boydii* 7 and 13 and *E. albertii* strains used in this study are listed in table 2 and were provided by Thomas Whittam. *E. coli* O157:H7 EDL933 and *E. coli* O127:H6 E2348/69 were provided by Alison O'Brien. *E. coli* K12 MG1655 and *S. flexneri* 5a M90T were provided from Fred Blattner. Internal fragments of *Shigella* (Venkatesan *et al.*, 2001) and *E. coli* (Burland *et al.*, 1998) genes were amplified using the primers shown in table 1. Strains stored at −80°C in Luria-Bertani (LB) medium with 50% glycerol were directly inoculated into PCR reactions with GoTaq polymerase (Promega). The *stcE* gene was sequenced from PCR products amplified with primers IR ApaI 5′ 1 and *etpD* 3′ 1803 (table 1) and TripleMaster polymerase (Eppendorf) from plasmid DNA extracted from the atypical *Shigella* B13 strains using a Maxi Prep Kit (Qiagen). The nucleotide sequence for the *stcE* gene from the atypical *Shigella* B13 strains 3556–77, 3557–77, 3052–94, and 3053–94 have been submitted to GenBank under accession numbers EU159265, EU159266, EU159267, EU159268, respectively. For Southern blot analysis, plasmid DNA isolated from the atypical *Shigella* B13 strains was electrophoresed on a 0.6% agarose gel. Gel and *stcE* probe preparation and hybridization were performed as previously described (Lathem *et al.*, 2003).

StcE activity assay

To examine secretion of StcE, strains were grown in 25 ml Lennox L broth overnight at 37°C with aeration and cells removed by centrifugation. Three mls of culture supernatant were precipitated with 10% trichloroacetic acid on ice for ≥1 hour. To measure StcE activity, 12 ml of culture supernatant were incubated with 0.5 μg C1-INH protein (CompTech) overnight at room temperature prior to TCA precipitation. Precipitated protein was separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with polyclonal anti-rStcE' antisera (Grys *et al.*, 2005) or anti-C1-INH IgG (Cedarlane Laboratories).

Invasion assay

The gentamicin protection assay was used to determine the invasion phenotypes of the atypical *Shigella* B13 strains (Elsinghorst *et al.*, 1994). A colony of each strain grown overnight on LB agar was inoculated into 2 ml of LB broth and incubated statically overnight at 37°C. Overnight culture (40 μl) was diluted into a total volume of 1 ml of HEp-2 media (EMEM, 1 mM sodium pyruvate, 10% FBS) prior to the addition to a monolayer of HEp-2 cells in a 24-well tissue culture plate (MOI of 14–95) and incubated at 37°C in 5% CO_2 for 2 hours. Monolayers were washed with Dulbecco's PBS (D-PBS) and

fresh media containing 100 μg/ml gentamicin added for an additional 2 hours. The monolayers were washed with D-PBS and lysed with 1 ml 0.1% Triton X-100 per well. Suspensions were serially diluted and plated onto LB agar. Results are presented as the average percent of inoculum recovered after gentamicin treatment and are representative of duplicate samples in three independent experiments. Statistical analysis was preformed using a one-way ANOVA with a Tukey's post hoc test.

Pedestal formation assay

To determine the ability of atypical *Shigella* B13 strains to form pedestals, HEp-2 cells were seeded onto 8-well microscope slides (Nalge Nunc International) 48 hours prior to infection so that cells would reach 50–80% confluency. Overnight bacterial cultures (10 μl of 2.5×10^8 to 9.0×10^8 CFUs/ml) grown as for the invasion assay were diluted into a total volume of 250 μl with HEp-2 media and added to each well of washed HEp-2 cells. The mixtures were incubated at 37°C in 5% CO_2 for a total of 6–7 hours with a media exchange after 3 hours. Wells were washed with D-PBS and the cells fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100. Bacterial cells were stained with 1:200 goat anti-lipid A (Abcam), followed by 1:200 anti-goat-Alexa 488 and HEp-2 cells stained with 1:100 phalloidin-Alexa 594 (Invitrogen). Preparations were mounted with Prolong Gold (Invitrogen) and analyzed by epifluorescence microscopy (Carl Zeiss MicroImaging Inc.).

Results

PCR screen for presence of *stcE* in atypical *S. boydii* and *E. albertii* strains

We set out to identify *stcE* in other bacterial species recently found to carry *eae*, the gene that encodes the bacterial adhesin (intimin) required for pedestal formation. A PCR screen of numerous *S. boydii* and *E. albertii* strains showed that an internal fragment of *stcE* can be PCR amplified from only a subset of the *S. boydii* strains known as atypical *S. boydii* 13 (table 2). Atypical *Shigella* B13 strains 3557–77, 3556–77, 3052–94, and 3053–94, which form a distinct phylogenetic cluster, were all positive for the *stcE* gene. Atypical *Shigella* B13 strain 5216–70, which is phylogenetically clustered with enteroinvasive *E. coli* strains, was negative for the *stcE* gene. The presence or absence of the *stcE* gene in all strains was confirmed by Southern blot (data not shown). Analysis of isolated plasmid DNA by Southern blot demonstrated that *stcE* was encoded on the large plasmid of the four atypical *Shigella* B13 strains (data not shown). Sequence analysis of the 2.7 kb *stcE* gene showed only three synonymous substitutions shared among the atypical *Shigella* B13 strains and a Q727L substitution in strain 3556–77 compared to the EHEC EDL933 allele (data not shown). Six substitutions within 220 nucleotides of the intergenic region upstream of the predicted *stcE* promoter are present in the plasmids of all four atypical *Shigella* B13 strains compared to pO157.

Activity of StcE in atypical *S. boydii* B13 strains

To determine if the StcE protein was expressed and secreted by the atypical *Shigella* B13 strains, TCA-precipitated supernatants of overnight cultures were analyzed by immunoblot. StcE protein was identified in supernatants from strains 3556–77, 3052–94, and 3053–94, but not from 3557–77 or 5216–70 (table 2). StcE activity in culture supernatants was assayed for C1-INH proteolysis by immunoblots, and detected with all atypical *Shigella* B13 strains except 3557–77 and 5216–70 (Fig. 1, table 2).

Atypical *Shigella* B13 strains encode other EHEC virulence factors

To determine if the atypical *Shigella* B13 plasmid encoding *stcE* is similar to the large invasion plasmid of *Shigella* (pINV), several pINV-encoded virulence factors were sought

by PCR amplification (table 2). None of the pINV-encoded virulence factors could be amplified from the atypical *Shigella* B13 strains. PCR analysis using primers specific for pO157-encoded genes resulted in amplification of *etpD*, but not *katP*. The gene, *traC*, which is an F plasmid gene that is also encoded on the large virulence plasmid of *E. coli* O157:H-, pSFO157, did not PCR amplify from any of the atypical *Shigella* B13 strains tested.

The presence of additional *E. coli*-specific chromosomally-encoded genes was determined by colony PCR (table 2). The LEE-encoded regulator (Ler) is a global virulence regulator that has been shown to positively regulate the expression of LEE (Mellies *et al.*, 1999), *stcE*, and the *etp* operon in *E. coli* O157:H7 (Lathem *et al.*, 2002). PCR analysis of the atypical *Shigella* B13 strains identified the *ler* gene in the four atypical *Shigella* B13 strains encoding *eae* and *stcE*. An additional LEE-encoded gene, *espA*, encodes a subunit of the type III secretion system unique to EPEC and EHEC and is encoded by the atypical *Shigella* B13 strains encoding *eae* and *stcE*. PCR analysis of *cadA*, which encodes lysine decarboxylase and is universally absent in *Shigella* but present in most *E. coli* strains (Day *et al.*, 2001), revealed that none of the atypical *Shigella* B13 strains encoded *cadA*.

Some atypical *Shigella* B13 strains invade and form pedestals on HEp-2 cells like *E. coli*

The abilities of the atypical *Shigella* B13 strains to invade HEp-2 cells were determined. Strains 3556–77 and 3557–77 showed invasion levels below the level of detection, whereas strains 3052–94 and 3053–94 showed relative levels of invasion more similar to *E. coli* than *S. flexneri* strains (Fig. 2A). The presence of the LEE operon and *stcE* suggested that the atypical *Shigella* B13 strains might form pedestals on host cells. We tested this hypothesis by infecting HEp-2 cells and observing for co-localization of bacteria with actin bundles on the surface of cells. Pedestal formation on HEp-2 cells could be detected for atypical *Shigella* B13 strains 3556–77, 3052–94, and 3053–94, but not 3557–77 (Fig. 2B).

Discussion

In this study, we discovered the *stcE* gene in the atypical *Shigella* B13 cluster. The relatively low incidence of three nucleotide substitutions within the 2.7 kb *stcE* gene compared to the six nucleotide substitutions within 220 nucleotides of the upstream intergenic region suggests selection for the preservation of StcE function. The acquisition of the large plasmid carrying *stcE* and the *etp* operon, in combination with the LEE element encoded on the chromosome, may provide a selective advantage by increasing the level of intimate adherence to host cells. A role of StcE in intimate adherence is further supported by the observation that a lack of extracellular StcE coincides with absence of pedestal formation by strain 3557–77.

The current model of *Shigella* evolution proposes that multiple ancestral *E. coli* clones acquired the pINV *Shigella* invasion plasmid, leading to selection for the loss of traits such as motility and lysine decarboxylation (Pupo *et al.*, 2000). In contrast, the atypical *Shigella* B13 strains show loss of *E. coli* traits in the apparent absence of pINV selective forces. Furthermore, strains 3556–77 and 3557–77 display metabolic phenotypes intermediate between Shigella and *E. coli*, and atypical Shigella B13 DNA is more similar to *E.coli* than other Shigella B13 strains based on DNA-DNA hybridization assays (Brenner *et al.*, 1982). These atypical *Shigella* B13 strains also form a distinct phylogenetic cluster and possess intermediate chromosomal genotypes between *E. coli* and *Shigella* groups (Hyma *et al.*, 2005). As was previously suggested by Hyma *et al.*, these data indicate that the atypical *Shigella* B13 strains were misclassified as *Shigella* and that they actually represent a lineage that evolved from ancestral forms of Shigella and attaching and effacing *E. coli*. The data presented here strengthen this argument by showing the acquisition of LEE and a pO157-like plasmid encoding *stcE* which we suggest recapitulates the model of EHEC evolution,

described as the step-wise acquisition of the LEE element, followed by pO157 and then the Shiga toxin phage (Reid *et al.*, 2000). We therefore propose to reclassify the atypical Shigella B13 strains as an *E. coli* group that, through convergent evolution or horizontal transfer of virulence genes on an ancestral background that shared both *E. coli* and Shigella characteristics, has evolved to closely resemble pathotypes of *E. coli* that form attaching and effacing lesions.

Sequelogs of *stcE*, historically named *tagA*, have been functionally characterized in other pathogens, including *Vibrio cholerae* and *Aeromonas hydrophila* (Szabady *et al.*, 2010 and Pillai *et al.*, 2006). Like StcE, *V. cholerae* TagA is a secreted mucinase and contributes to colonization of the intestinal epithelium (Szabady *et al.*, 2010). The *A. hydrophilia* TagA exhibits an additional StcE function by cleaving and localizing C1-INH the surface of bacterium, increasing the serum resistance of the bacterium *in vitro*. An isogenic deletion mutant of *tagA* decreased the mortality of mice compared to wild-type *A. hydrophila* in a mouse model of peritonitis (Pillai *et al.*, 2006). Thus, StcE-like metalloproteases play a role in the virulence phenotypes of *A. hydrophila*, *V. cholerae* and *E. coli* O157:H7. In this study, we identified *stcE* as a possible virulence factor in atypical *Shigella* B13 strains, and further characterized this unique cluster of attaching and effacing pathogens.

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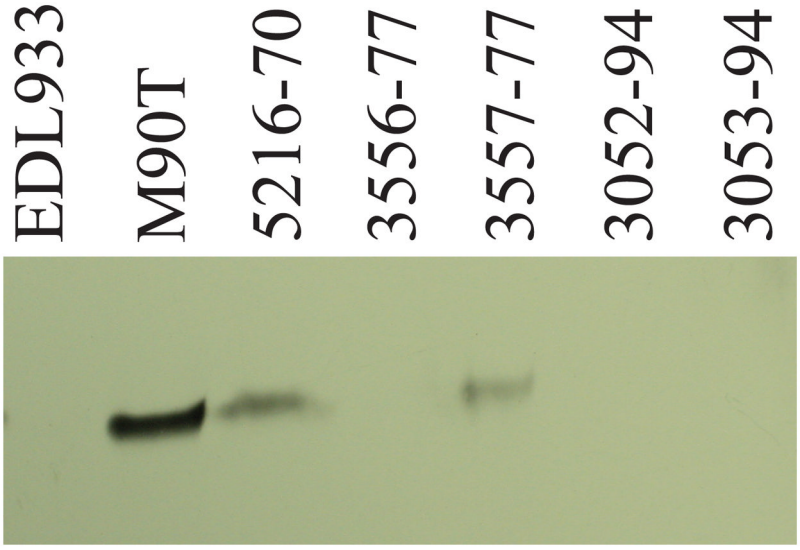

Figure 1. StcE activity of atypical *Shigella* B13 strains

Immunoblot of overnight culture supernatants of atypical *Shigella* B13 strains 5216–70, 3556–77, 3557–77, 3052–94, and 3053–94 incubated overnight at room temperature with purified C1-INH and probed with anti-C1-INH IgG. *E. coli* O157:H7 EDL933 (EHEC) and *S. flexneri* 5a M90T were used as positive and negative controls for C1-INH cleavage, respectively.

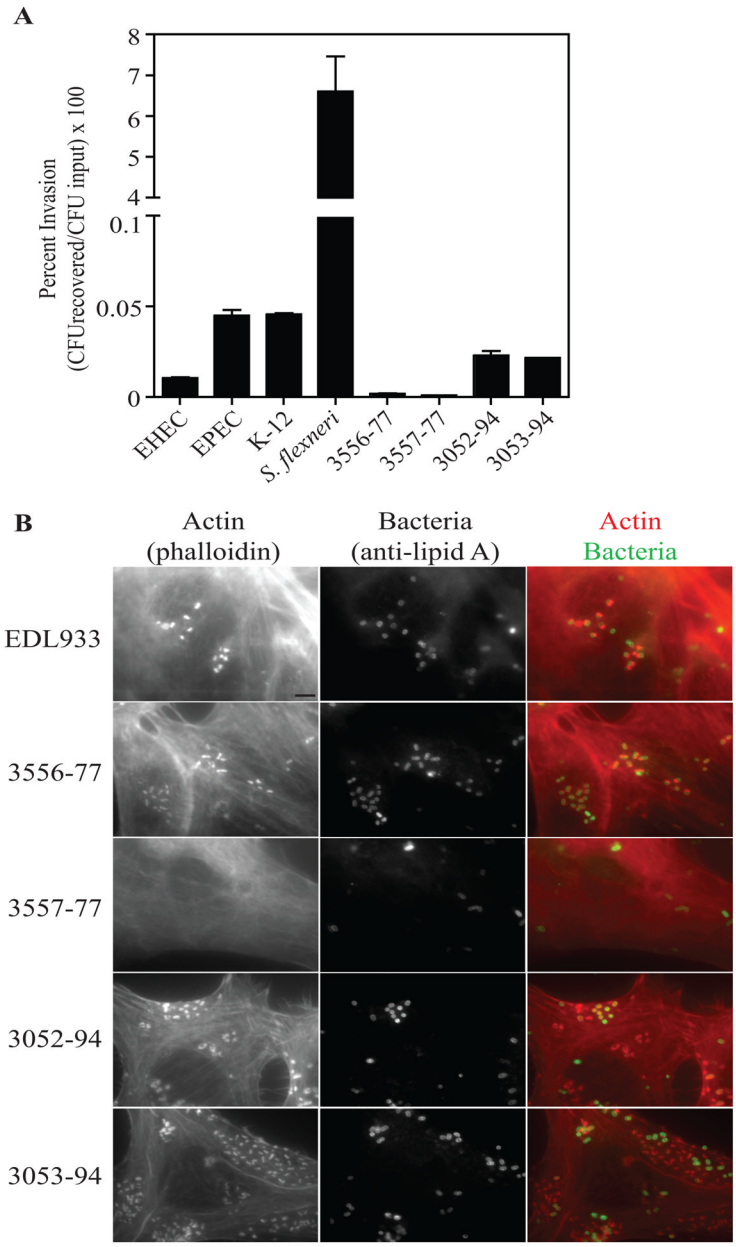

Figure 2. Invasion and pedestal formation of atypical *Shigella* B13 strains

A, Percent HEp-2 invasion by *E. coli* O157:H7 EDL933 (EHEC), *E. coli* O127:H6 E2348/69 (EPEC), *E. coli* K12 MG1655, *S. flexneri* 5a M90T, and the atypical *Shigella* B13 strains 3556–77, 3557–77, 3052–94, and 3053–94 as determined by the gentamicin protection assay with duplicate samples in three independent experiments. Statistical analysis was preformed using a one-way ANOVA with a Tukey's post hoc test. *S. flexneri* invasion is significantly different from all other strains ($P<0.001$). Invasion was not significantly different among all other strains ($P>0.05$). *B*, Pedestal formation of the atypical *Shigella* B13 strains and *E. coli* O157:H7 EDL933. Infected HEp-2 cells were fixed and permeabilized and were stained with anti-lipid A antibody, followed by anti-goat-Alexa 488 and phalloidin-Alexa 594 antibodies. The scale bar located in the lower right corner of the EDL933 actin field represents 5 μm and is applicable to all micrographs.

Table 1

Primers used in this study.

Primer Name	Sequence
stcE 5′ 693	5′-CCGCTCCGGTGAACTGGAGAATA-3′
stcE 3′ 1841	5′-CCTTATCTGCGGAGGCTGTAGGG-3′
etpD SacI 5′ 1	5′-CCGAGCTCCGTGTTCACTACAGTAATTTTG-3′
etpD XbaI 3′ 1929	5′-CCTCTAGATTACATCTCCTGCGCATAAA-3′
katP 5′ 16	5′-CTTCCTGTTCTGATTCTTCTGG-3′
katP 3′ 2141	5′-AACTTATTTCTCGCATCATCC-3′
ler 5′ 38	5′-CACATACAACAAGTCCATACATTCAGC-3′
ler 3′ 378	5′-CAGCGGTATTATTTCTTCTTCAGTGTCC-3′
espA 5′ 81	5′-GTCGAAGGATGAGGTGGTTAAGCTA-3′
espA 3′ 535	5′-ATTGCACATCAGAACGTGCACTCG-3′
cadA 5′ 455	5′-ACATGGGTGGTACTGCATTCCAGA-3′
cadA 3′ 1603	5′-ACAGCAGGTTATACGGACCGGTTT-3′
ipaB 5′ 1	5′-ATGCATAATGTAAGCACCACAACC-3′
ipaB 3′ 1743	5′-TCAAGCAGTAGTTTGTTGCAAAAT-3′
ipaD 5′ 1	5′-ATGAATATAACAACTCTGACTAAT-3′
ipaD 3′ 999	5′-TCAGAAATGGAGAAAAAGTTTATC-3′
virK 5′ 188	5′-TTCTGGCAATACAACCCACGTTGC-3′
virK 3′ 915	5′-TGCATCCAAAGAGCGGATAGCAGT-3′
icsA 5′ 348	5′-AGGTCATGGTGGTGCTGGTGATAA-3′
icsA 3′ 2002	5′-CTGCAATTTCCAGCCGGTCAGTTT-3′
ipaH7.8 5′ 500	5′-ACAGGCTGACAACATTACCCGACT-3′
ipaH7.8 3′ 1624	5′-TCTGCTGTTCAGTCTCACGCATCA-3′
mxiM 5′ 27	5′-TGCTCTGCAGCAAAGATTAAATAGTGAAGA-3′
mxiM 3′ 407	5′-TACCATGTCGAATCATCTGCCTCTCTC-3′
traC 5′ 318	5′-TGGTGACAGGATTGAATACGGGCT-3′
traC 3′ 1732	5′-GCAACAGCAGACCTTCATGCACTT-3′
IR ApaI 5′ 1	5′-AAGGGCCCCTCTGAGGTGTCTGTTAAACCCGTGG-3′
etpD 3′ 1803	5′-CGACTGCACCTGTTCCTGATTA-3′

Table 2

Prevalence of *E. coli* and *Shigella* specific genes detected by PCR and secreted StcE antigen and C1 cleavage activity detected by immunoblot.

		***E. coli* O157:H7**				***S. flexneri* 5a**			
		plasmid		**chromosome**		**plasmid**			
Strain[a]	**Species**	***stcE/etpD***	***katP***	***ler/espA***	***cadA***	***ipaB/ipaD/icsA/mxiM***	***virK/ipaH7.8***	**StcE secretion**	**StcE activity**
EDL933	*E. coli* O157:H7	+	+	+	+	−	−	+	+
M90T	*S. flexneri* 5a	−	−	−	−	+	+	−	−
5216–70	Atypical *S. boydii* 13	−	−	−	−	−	+	−	−
3556–77	Atypical *S. boydii* 13	+	−	+	−	−	−	+	+
3557–77	Atypical *S. boydii* 13	+	−	+	−	−	−	−	−
3052–94	Atypical *S. boydii* 13	+	−	+	−	−	−	+	+
3053–94	Atypical *S. boydii* 13	+	−	+	−	−	−	+	+

[a] *stcE* was not detected in the following additional strains tested: *S. boydii* 13 3103–99, *S. boydii* 13 ATCC 12032, *S. boydii* 13 2045–54, *S. boydii* 13 K-694, *S. boydii* 7 K-1, *E. albertii* 9194, *E. albertii* 10790, *E. albertii* 10457, *E. albertii* 12502, *E. albertii* 19982.