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Shiga toxin 2 overexpression in *Escherichia coli* O157:H7 strains associated with severe human disease

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Abstract

Variation in disease severity among *E. coli* O157:H7 infections may result from differential expression of Shiga toxin 2 (Stx2). Eleven strains belonging to four prominent phylogenetic clades, including clade 8 strains representative of the 2006 U.S. spinach outbreak, were examined for *stx2* expression by real-time PCR and western blot analysis. Clade 8 strains were shown to overexpress *stx2* basally, and following induction with ciprofloxacin when compared to strains from clades 1-3. Differences in *stx2* expression generally correlated with Stx2 protein levels. Single-nucleotide polymorphisms identified in regions upstream of *stx2AB* in clade 8 strains were largely absent in non-clade 8 strains. This study concludes that *stx2* overexpression is common to strains from clade 8 associated with hemolytic uremic syndrome, and describes SNPs which may affect *stx2* expression and which could be useful in the genetic differentiation of highly-virulent strains.

Keywords

Shiga toxin; *E. coli* O157:H7; Clade 8; Stx2; hemolytic uremic syndrome

1. Introduction

Escherichia coli O157:H7 is an enteric human pathogen which produces a cytotoxin (Shiga toxin) responsible for the life-threatening illness hemolytic uremic syndrome (HUS). Three species of Shiga toxin (Stx1, Stx2 and Stx2c) have been described in association with human disease, each of which is encoded on a distinct lambdoid bacteriophage that forms stable

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Conflict of interest
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lysogens in O157:H7 [1-4]. Production of Stx is linked to induction of the lytic cycle of Stx phage replication, which occurs spontaneously and in response to stressors [5-10].

Variation in disease severity has been reported among O157:H7 phylogenetic lineages (clades) distinguished by single nucleotide polymorphism (SNP) genotyping [11], however, the factors which contribute to this variation are unknown. There is evidence that differential s*tx2* expression may account for differences in virulence. For example, studies have shown the presence of *stx2* to be correlated with a higher frequency of HUS [12-14], and that lysogeny with multiple Stx-phage results in less Stx2 production and reduced rates of HUS relative to strains lysogenized with Stx2-phage alone [15], [16, 17],[18]. In addition, polymorphisms and rearrangements in Stx2-phage DNA have been observed in O157:H7 strains with reduced levels of Stx2 [19-22].

Recently, strains belonging to the clade 8 lineage of O157:H7 have been reported to be associated with HUS [11], cause severe disease in mice [23], and express *stx2* at increased levels following exposure to epithelial cells [24, 25] relative to strains from other clades. This study quantified transcript and protein levels for *stx2* among eleven O157:H7 strains representing four prominent clades, and lysogenized with various Stx-phages.

2. Results and discussion

Among the eleven O157:H7 strains, five demonstrated a 2.9- to 14.6-fold increase in basal *stx2* transcript levels relative to Sakai by quantitative real-time PCR (qRT-PCR), four of which belonged to clade 8 (Fig. 1). Three of four strains from clades 1-3 expressed only a 0.88- to 1.7-fold increase in *stx2* relative to Sakai. Levels of *stx2* were significantly higher in clade 8 strains TW14359, TW14313 and TW02883, as well as in clade 1 strain TW08612, than all other strains ($P<0.05$). Clade 8 strain EC508 was observed, however, to significantly underexpress *stx2* relative to all other strains ($P<0.05$). These results reveal that although increased *stx2* expression is characteristic of clade 8 strains, it is not exclusive to this clade, and not all strains within clade 8 express *stx2*.

Lysogeny with multiple Stx-phages has been observed to both increase and decrease Stx2 production [18, 26]. In the present study however, there was no apparent correlation between the complement of Stx-phage and actual *stx2* expression. Although the reason for this difference in results is unknown, the former studies measured the influence of multiple lysogeny on Stx2 production among EHEC O157:NM strains [26], or in Stx2 lysogens of *E. coli* K-12 [18], which may produce substantially different results when compared to clinical O157:H7 strains. Furthermore, these studies did not measure Stx2 production in strains bearing Stx2-phage in combination with Stx1- or Stx2c-phage.

Differences in basal *stx2* expression were modestly correlated ($r^2=0.70$) to actual Stx2 levels by western blots for proteins extracted from $OD_{600}=0.65$ cultures (Fig. 2). For example, in strains TW08612, TW14359, and TW14313, which were determined to express *stx2* at 14.1-, 13.7-, and 14.6-fold higher than Sakai, corresponding Stx2 protein levels were 2.1-, 3.9-, and 3.4-fold higher, respectively (Figs. 1 and 2). The reason for this difference in fold-change is unknown, but is consistent with studies reporting increased *stx2* expression without an equivalent increase in Stx2 product [9], and disparities between mRNA and protein levels in *E. coli* [27]. Importantly, western blot analysis statistically confirmed *stx2* expression levels by qRT-PCR for 7/8 strains analyzed relative to Sakai, including the overexpression of *stx2* in clade 8 strains TW14359, TW02883 and TW14313 ($P<0.05$). This also included the underexpression of *stx2* in clade 8 strain EC508. This strain originates from a patient with HUS in 1984 (N. Carolina, U.S.), and has been shown to be cytotoxic for HeLa cells [28]. Perhaps significantly, EC508 is a *stx2+ stx2c+* strain (Table 1), and the

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stx2c product is also cytotoxic for HeLa cells [29]. Finally, strains bearing *stx2c* only have also been determined to cause HUS [12]. Only limited cross-reactivity was observed for strain TW09178 (*stx2-stx2c+*) using anti-Stx2 mAbs.

Differences in *stx2* induction following 60- and 120-min exposure to ciprofloxacin (Cip) were observed for a subset of strains shown in this study to differentially express *stx2* at the basal level. Following 60-min, Cip was observed to induce *stx2* in clade 8 strains TW02883 and TW14359, but not in Sakai (clade 1) or EDL933 (clade 3) (Fig. 3) ($P<0.05$). This is an interesting observation in light of evidence that the EDL933 Stx2-phage (933W) is sensitive to lytic induction [30]. After 120-min, *stx2* expression increased substantially for all strains, however the fold increase in *stx2* expression remained significantly higher in clade 8 strains relative to both Sakai and EDL933 (Fig. 3) ($P<0.05$). Moreover, the addition of Cip to cultures at $OD_{600}=0.65$ reduced growth earlier, and in a more dramatic manner for TW02883 and TW14359 when compared to Sakai and EDL933 (Fig. 4). Following 18 h of growth, cell pellets were obtained from Sakai and EDL933 by centrifugation (10,000 × *g*, 2-min), but could not be obtained for clade 8 strains (data not shown). Although strictly correlative, this observation suggests an increased lytic activity in clade 8 strains relative to Sakai and EDL933 when exposed to Cip. Collectively, these results reveal that an inherent difference exists among clade 8 strains in a common regulatory pathway of basal (spontaneous) and inducible *stx2* expression.

Support for this hypothesis comes from the observation that *stx2* expression can be influenced by alterations in upstream regulatory regions [20, 22, 31]. To explore this possibility using the strain set in this study, a DNA region containing $stx2_P$, the phage late promoter $p_{R'}$, the Q utilization site (*qut*), and *q* were examined for each strain by PCR and sequencing, and through analysis of DNA sequences available through GenBank (NCBI). PCR products were obtained for all test strains except TW11029, a *stx2+* strain that expressed low *stx2* levels (Table 1, Fig. 1), despite repeated attempts using different primers, suggesting that sequence variation in the priming sites is preventing amplification. Sequencing revealed a number of SNPs in clade 8 strains which were largely absent in non-clade 8 strains (Supplemental Fig. 1). Ten SNPs were identified within the *q* gene of clade 8 strains TW14313 and TW08609, which were also present in strains EC508 (clade 8) and TW14588 (clade 2). Two of the 10 SNPs in *q* result in non-synonymous mutations: R10→C and Y19→H. In addition, 23 SNPs were identified between *q* and the start codon of *stx2A* in TW14313 and TW08609, which were also present in EC508. Eight of these SNPs were within gene ECs1204, encoding a hypothetical protein, and five of these conferred non-synonymous mutations: A20→S, S32→T, G33→E, N44→D, and V45→L. These 33 SNPs were therefore present in strains TW08609 and TW14313 which overexpressed *stx2*, but also in EC508, in which *stx2* expression was barely detectable (Figs. 1 and 2). Furthermore, the *q-stx2* region of TW08612 (clade 1), which overexpressed *stx2* transcript but not Stx2 product, was identical to 93-111 and EC4501 (clade 2), Sakai (clade 1), and EDL933 (clade 3), together suggesting that these SNPs may partly contribute to differential expression, but are not solely responsible. Two SNPs (one in *argN2*) and a 2-bp deletion were also identified exclusively in clade 8 strains TW02883, TW14359, EC4042, EC4045, EC4076, EC4113, EC4115, EC4196, and EC4206. In concordance with previous studies [24, 32], no difference was identified in the $p_{R'}$, *qut*, or $stx2_P$ sequences. As toxin production is intimately linked to Stx2-phage lytic development [33], it is likely that other determinants of phage replication, not investigated in this study, also contribute to differential *stx2* expression.

To conclude, this study reveals elevated basal and inducible *stx2* transcript and protein levels among O157:H7 clade 8 strains relative to strains of clades 1-3, and describes genetic polymorphisms upstream of *stx2* in regions which may be important for *stx2* expression. The

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results are concordant with studies that show *stx2* upregulation in clade 8 strains exposed to MAC-T bovine epithelial cells in DMEM [24, 25], and suggests that Stx2 production is increased in these strains under a variety of *in vitro* growth conditions. Furthermore, the findings of this research support the hypothesis that differences in disease severity observed between O157:H7 clades can be partly explained by differential Stx2 production. Clade 8 strains have also been observed to overexpress genes of the locus of enterocyte effacement (LEE), a 35.6-kb pathogenicity island encoding structural and regulatory proteins of a type III secretion apparatus [24, 25]. Thus the virulence of clade 8 strains likely reflects the upregulation of several discrete virulence systems. Further research is required to understand the genetic basis and biological significance of differential *stx2* expression.

3. Materials and methods

3.1 Quantitative real-time PCR (qRT-PCR) analysis of stx2 expression

Strains (Table 1) were grown in MOPS media [34] with 4 g/l glucose as described [35]. For basal *stx2* expression, cultures were grown to OD_{600}=0.65 before RNA extraction (RNeasy™ kit, Qiagen, Valencia, CA) and cDNA synthesis (iScript, Bio-Rad, Carlsbad, CA). For inducible *stx2* expression, cultures were grown to OD_{600}=0.4 before induction with ½X MIC ciprofloxacin (Cip, 0.025 μg/ml) for 60-min and 120-min before RNA extraction. Quantitative real-time PCR (qRT-PCR) was performed using primers for *rrsH* (16S rRNA gene) to normalize cDNA levels, and primers Stx2b-6/Stx2b-120 for *stx2B* as described [36]. Reaction conditions included 40 cycles of denaturing at 94°C for 15-sec, followed by annealing at 55°C for 20-sec.

3.2 Growth experiments

MOPS cultures (N=3) were grown to OD_{600}=0.65 before the addition of ½X MIC ciprofloxacin (0.025 μg/ml) or an equal volume of water (controls), and OD was used to measure growth for 4 h at 1 h intervals.

3.3 Western blots and immunodetection of Shiga toxin 2

Strains were grown in MOPS as described for qRT-PCR (above) to OD_{600}=0.65, and following the method of Uzzau *et al*. [37], protein was extracted and resolved by 12% SDS-PAGE, and then transferred to PVDF membranes (Sigma). Mouse anti-Stx2 mAb (Santa Cruz Inc., Santa Cruz, CA), diluted to 1:3000 (v/v) were added to probe these membranes for Stx2, followed by a 1:2000 (v/v) dilution of HRP-conjugated goat anti-mouse IgG (Santa Cruz). Detection was performed using ECL Plus (Amersham Pharmacia, Piscataway, NJ). Relative quantitation of blots representative of three independent experiments was performed using Image J [38].

3.3 DNA sequencing and sequence analysis

For sequencing, purified DNA (Puregene® kits, Gentra, Minneapolis, MN) was used for PCR with primers Q-54 (ACGCTATCGTCAACGGTGTT)/Q+741 (CATTGCTGCTTTGACGCTAC) to amplify a 754-bp product (positions 1265693-1266486 in Sakai, GenBank BA000007) using LA *Taq* polymerase (TaKaRa Inc, Shiga, Japan). Primers Stx2-569 (CGAAGTTTGCGTAACAGCAT)/Stx2+68 (CGGGAATAGGATACCGAAGAA) were used to amplify a 636-bp product (positions 1266396-1267032). PCR products were purified (QIAquick, Qiagen) and sequencing was performed in duplicate using Q-54 and Stx2+68 primers (Eurofins MWG Operon, Huntsville, AL). DNA sequence data was concatenated into a 1272-bp read representing the *q-stx2* region and aligned in MEGA [39]. Because this *q-stx2* region shares only 33%

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nucleotide identity with the *q-stx2c* region in Stx2c-phage 2851 (GenBank AJ605767) [4], no PCR products were generated from the *stx2-stx2c+* control strain TW09178.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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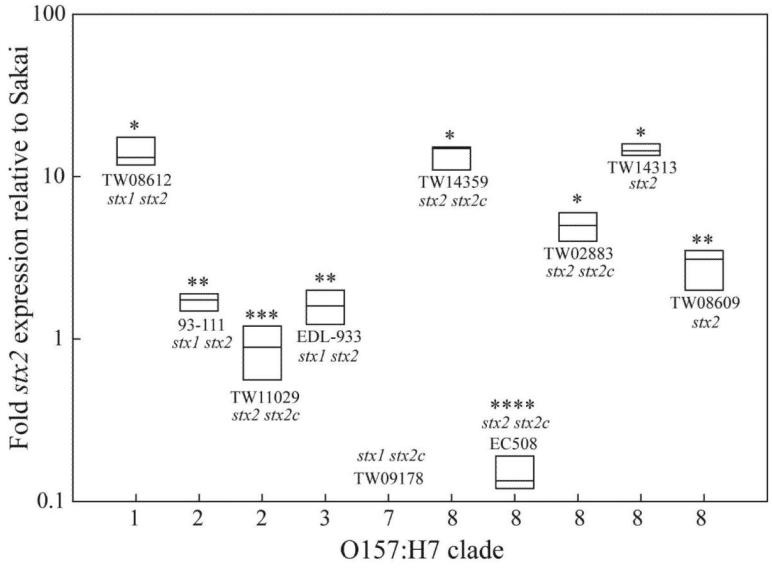

FIG. 1. Basal *stx2* transcript levels

Boxplots of mean fold-change in *stx2* expression relative to strain Sakai for 10 strains representing 5 O157:H7 clades as determined by qRT-PCR. Plot boundaries represent the 25th and 75th percentiles; the median is given by the line. Plots which differ in the number of asterisks, differ significantly by Tukey's HSD following a significant F-test ($P<0.05$). Strain names and *stx* complement are provided in proximity to each respective plot. As expected, no *stx2* transcript was detectable for *stx2-stx2c+* control strain TW09178.

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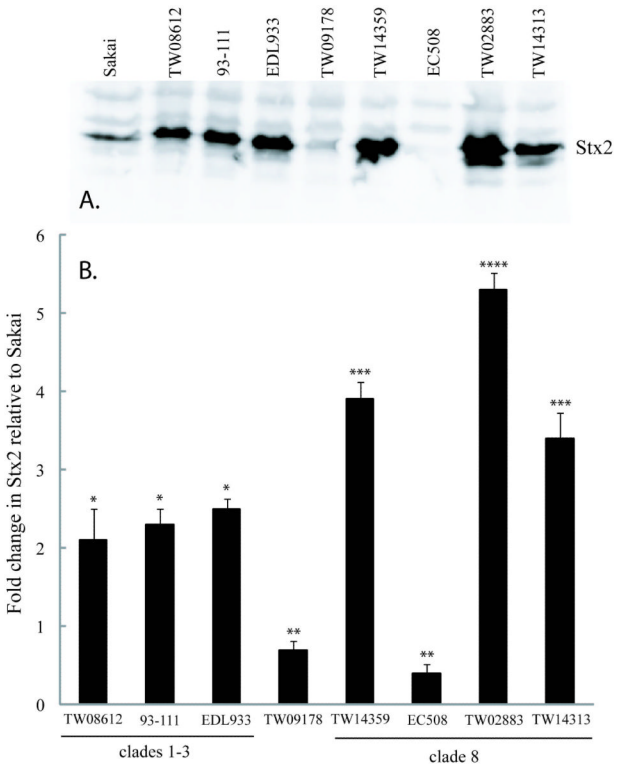

FIG. 2. Basal Stx2 protein levels

(A): Representative western blot measuring Stx2 levels among 9 strains encompassing 5 O157:H7 clades using anti-Stx2 mAbs and visualized with enhanced chemioluminescence. The location of Stx2 is indicated (inset right); (B): Graphical representation of fold-change in Stx2 levels relative to strain Sakai for strains in panel A. Bars represent mean Stx2 levels as determined using Image J. Error bars indicate standard deviation (N=3). Bars which differ in the number of asterisks, differ significantly by Tukey's HSD following a significant F-test ($P<0.05$). Strain TW09178 belongs to clade 7, and is included as a *stx2-stx2c*+ control for cross reactivity of anti-Stx2 mAb with Stx2c.

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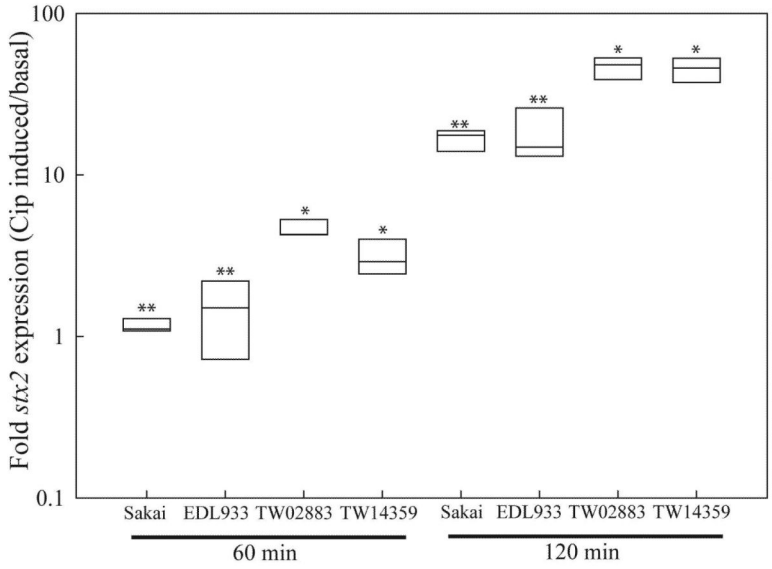

FIG. 3. Ciprofloxacin-induced *stx2* transcript levels

Boxplots of mean fold-change in *stx2* expression following 60- and 120-min exposure to ciprofloxacin (Cip) relative to basal expression levels as determined by qRT-PCR. Plot boundaries represent the 25th and 75th percentiles; the median is given by the line. Plots which differ in the number of asterisks at each time of exposure, differ significantly by Tukey's HSD following a significant F-test ($P<0.05$). Clade designations are: Sakai (clade 1), EDL933 (clade 3), TW02883 (clade 8), and TW14359 (clade 8).

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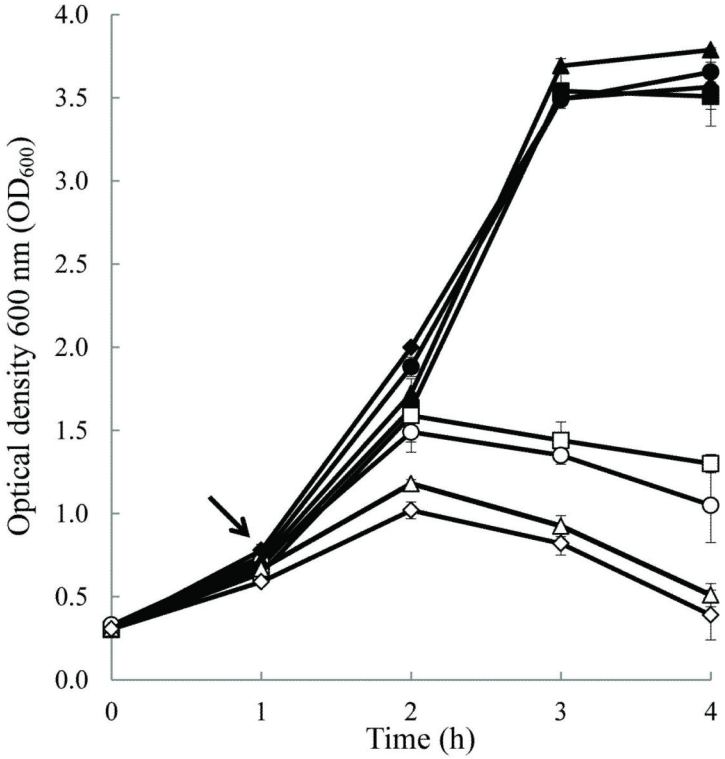

FIG. 4. Bacteriophage induction during growth with ciprofloxacin

Plot of optical density at 600 nm (OD_{600}) as a function of time for uninduced control (filled plots) and ½X MIC ciprofloxacin induced (empty plots) cultures. Arrow indicates point of ciprofloxacin addition. Strains included Sakai (squares), EDL933 (circles), TW02883 (diamonds), and TW14359 (triangles). Measurements represent the mean (N=3) OD_{600} and error bars indicate standard deviation.

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Table 1

List of O157:H7 strains used in this study

Strain[a]	Alias	O157:H7 Clade[b]	SG[b]	Shiga toxin Complement	Clinical presentation, origin and reference
TW08264	Sakai	1	1	*stx1*, *stx2*	Unknown, 1996 outbreak, Sakai, Japan [40]
TW08612	EK4	1	1	*stx1*, *stx2*	HUSc, 2001, WA, USA [41]
TW04863	93-111	2	9	*stx1*, *stx2*	Diarrhea, 1993 outbreak, northwest USA [42]
TW11029		2	ND[d]	*stx2*	HC[e], 2002, MI, USA
TW02302	EDL933	3	12	*stx1*, *stx2*	HC, 1982 outbreak, MI and OR, USA [43]
TW09178	MI03-9	7	29	*stx1*, *stx2c*	
TW14359	MI06-63	8	30	*stx2*, *stx2c*	HC, 2006 outbreak, western USA [11]
TW00885	EC508	8	31	*stx2*, *stx2c*	HUS, 1984, NC, USA [28]
TW02883	E32511	8	31	*stx2*, *stx2c*	HUS, CDC [29, 44]
TW14313	MI06-31	8	33	*stx2*	HUS, 2006, MI, USA
TW08609	EK1	8	33	*stx2*	Diarrhea, 1999, WA, USA [41]

[a] All strains were acquired from the STEC Center at Michigan State University.

[b] Based on the work of Manning et al. [11]. SG is single nucleotide polymorphism (SNP) genotype.

[c] Hemolytic uremic syndrome (HUS).

[d] Not determined (ND).

[e] Hemorrhagic colitis (HC).