Differential expression of *stx*₂ variants in Shiga toxin-producing *Escherichia coli* belonging to seropathotypes A and C

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Only a subset of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) are human pathogens, but the characteristics that account for differences in pathogenicity are not well understood. In this study, we investigated the distribution of the *stx* variants coding for Stx2 and its variants in highly virulent STEC of seropathotype A and low-pathogenic STEC of seropathotype C. We analysed and compared transcription of the corresponding genes, production of Shiga toxins, and *stx*-phage release in basal as well as in induced conditions. We found that the *stx*₂ variant was mainly associated with strains of seropathotype A, whereas most of the strains of seropathotype C possessed the *stx*_{2-vhb} variant, which was frequently associated with *stx*₂, *stx*_{2-vha} or *stx*_{2c}. Levels of *stx*₂ and *stx*₂-related mRNA were higher in strains belonging to seropathotype A and in those strains of seropathotype C that express the *stx*₂ variant than in the remaining strains of seropathotype C. The *stx*_{2-vhb} genes were the least expressed, in basal as well as in induced conditions, and in many cases did not seem to be carried by an inducible prophage. A clear correlation was observed between *stx* mRNA levels and *stx*-phage DNA in the culture supernatants, suggesting that most *stx*₂-related genes are expressed only when they are carried by a phage. In conclusion, some relationship between *stx*₂-related gene expression *in vitro* and the seropathotype of the STEC strains was observed. A higher expression of the *stx*₂ gene and a higher release of its product, in basal as well as in induced conditions, was observed in pathogenic strains of seropathotype A. A subset of strains of seropathotype C shows the same characteristics and could be a high risk to human health.

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INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are food-borne pathogens that cause human diseases ranging from uncomplicated diarrhoea to haemorrhagic colitis (HC) and life-threatening complications, such as the haemolytic-uraemic syndrome (HUS). Cattle and other ruminants appear to be the main reservoir of STEC strains. While most outbreaks are associated with *E. coli* O157:H7, mainly in North America and Japan, approximately half of the sporadic cases may be due to non-O157:H7 serotypes (Banatvala *et al.*, 2001). Many non-O157 strains responsible for HUS do not possess the locus of enterocyte effacement (LEE) (Banatvala *et al.*, 2001), a pathogenicity

island encoding a type III secretion system recognized as a major virulence factor (Girardeau *et al.*, 2005; Garmendia *et al.*, 2005). STEC strains were classified into five seropathotypes (A to E) by Karmali *et al.* (2003) according to their incidence and association with HUS cases and outbreaks. Seropathotypes A and B include strains belonging to serotypes associated with HUS cases and outbreaks and containing the LEE. Seropathotype C includes LEE-negative isolates associated with sporadic cases of HUS but not with outbreaks. The virulence mechanisms of this group of strains are not well understood, and to date it is not possible to distinguish among these strains those which are a high risk to human health. Seropathotype D includes isolates rarely found in humans and associated with less severe disease (diarrhoea and HC). Seropathotype E includes serotypes that have never been found in humans.

The major characteristic of STEC linked to haemolytic symptoms is the production of Shiga toxins (Stx1, Stx2), which inhibit protein synthesis (Karmali *et al.*, 1985; Paton & Paton, 1998). Members of the Stx family are AB holotoxins comprising one A subunit, which is the active

Abbreviations: HC, haemolytic colitis; HUS, haemolytic-uraemic syndrome; LB, Luria-Bertani broth; LEE, locus of enterocyte effacement; q-PCR, quantitative real-time PCR; STEC, Shiga toxin-producing *Escherichia coli*.

Two supplementary figures are available with the online version of this paper.

component of the toxin, covalently bound to five identical B subunits. The B subunits form a pentameric structure required for toxin binding to its receptor, the glycolipid Gb3. Epidemiological studies, together with *in vivo* and *in vitro* experiments, have revealed that Stx2 is the most important virulence factor associated with severe human disease. Indeed, Stx2 is 1000 times more cytotoxic than Stx1 towards human renal endothelial cells, and STEC producing Stx2 are more commonly associated with serious diseases than isolates producing Stx1 or Stx1 plus Stx2 (Louise & Obrig, 1995; Boerlin *et al.*, 1999; Paton & Paton, 1998). Several Stx2 variants have been identified on the basis of sequence homology and immunological cross-reactivity (Ito *et al.*, 1990; Schmitt *et al.*, 1991; Paton *et al.*, 1995; Friedrich *et al.*, 2002; Pierard *et al.*, 1998). The most frequent variants identified so far in strains of human and bovine origin are Stx2, Stx2-vha, Stx2-vhb and Stx2c. Stx2 is the toxin produced by the prototype O157:H7 strains EDL933 and Sakai. Stx2-vha and Stx2-vhb were first described in an *E. coli* O91:H21 strain isolated from a patient with HUS (Ito *et al.*, 1990), and were also named Stx2d1 and Stx2d2, respectively (Teel *et al.*, 2002). Stx2c was found in the *E. coli* O157:H⁻ strain E32511, a clinical isolate associated with HUS (Schmitt *et al.*, 1991). Stx2-vha (AF479828-1), Stx2-vhb (AF479829-1) and Stx2c (M59432-1) have 99%, 97.4% and 100% sequence identity in their mature A subunit and 97.1% identity in their mature B subunit to the corresponding subunit of Stx2 (Y10775). These percentage values correspond to a maximum of three amino acid changes in each subunit. However, it has been suggested that Stx2 sequence variations may affect the capacity of a given Stx2-producing *E. coli* strain to cause disease (Lindgren *et al.*, 1994; Paton *et al.*, 1995).

The genes encoding Shiga toxins (*stx*) are generally carried by lambdoid bacteriophages and can be induced by DNA-damaging agents such as mitomycin C (Muhldorfer *et al.*, 1996). As a result of the induction process, expression of *stx*₂ genes, which is under the control of a late phage promoter (Wagner *et al.*, 2001), is activated. Bacterial host cells lyse and release Shiga toxins and free phage particles into the environment. Epidemiological observations and animal models suggest that the severity of the disease is correlated with the amount of Stx produced during infection (Zhang *et al.*, 2000; Dean-Nystrom *et al.*, 2003). However, expression of *stx* genes and their ability to be induced depending on the *stx*₂ variant or the relative virulence of the STEC strain is not well documented.

Most previous studies investigating the virulence traits of LEE-negative strains were based on the distribution of virulence-associated genes or of genomic islands rather than on their expression and production of the virulence factors. Here, we examined whether seropathotype A and seropathotype C STEC differ in their basal and inducible *stx* expression in an *in vitro* model. Furthermore, association of *stx*₂ and *stx*₂-related genes with an inducible prophage was investigated.

METHODS

Bacterial strains, cell lines, phage induction, and growth conditions. Bacterial strains used in this study are listed in Table 1. The mutants of *E. coli* O157:H7 strain EDL933 lacking the *stx*₁, the *stx*₂, or the *stx*₁ and *stx*₂ genes were constructed in our laboratory (Gobert *et al.*, 2007). Bacterial strains were routinely grown in Luria-Bertani (LB) medium. For Stx2 production under mitomycin C induction, overnight cultures of each isolate were subcultured in 7 ml fresh LB medium or in DMEM low glucose (Invitrogen no. 11880) with 4 mM L-glutamine and grown at 37 °C with rotary shaking at 180 r.p.m. The OD₆₀₀ was measured using 1 cm cuvettes with a Jenway 6300 spectrophotometer. When the OD₆₀₀ reached 0.2–0.3 (*t*₀), the cultures were divided into two tubes, and mitomycin C (250 ng ml⁻¹) was added to one of the tubes. Then the bacteria were grown at 37 °C with rotary shaking up to 6 h. Bacterial growth was monitored spectrophotometrically after appropriate dilution (two- to fivefold) of the samples in LB or DMEM medium. Three hours after mitomycin C treatment (*t*₃), 6 ml of culture was harvested from each tube and centrifuged for 15 min at 3000 g. The supernatants were filtered through 0.45 µm filters (Sartorius) and used for Stx2 ELISA and detection of phage particles; the pellets were used for RNA extraction. For some strains, levels of *stx* mRNA were also measured at *t*_{1.5} and phage DNA at *t*₆.

stx₂ subtyping. *stx*₂ subtyping was performed by PCR-RFLP on the *stx*_{2B} gene as previously described by Bertin *et al.* (2001) to discriminate between *stx*₂ (Y10775), *stx*_{2-vhb} (AF479829-1) and *stx*_{2-vha/stx}_{2c} variants. For distinction between *stx*_{2-vha} (AF479828-1) and *stx*_{2c} (M59432-1) variants, primers were designed in the *stx*_{2A} gene (forward, 5'-GATGGCGGTCCATTATC-3'; reverse, 5'-CGGT-AGAAAGTATTTGTTG-3') that amplify a 529 bp DNA fragment on the genomic DNA of strains carrying *stx*_{2-vha} or *stx*_{2c}. After *EcoRV* digestion of this fragment, *stx*_{2c} variants show two DNA fragments of 329 bp and 200 bp whereas *stx*_{2-vha} DNA remains uncut. The online version of this paper contains a supplementary figure (Fig. S1) showing the nucleotide sequence alignment of the gene encoding the A subunit of *stx*₂, *stx*_{2-vha}, *stx*_{2-vhb} and *stx*_{2c}, with the location of the primers and the *EcoRV* restriction site, and a photograph of the electrophoresis gel showing the DNA fragments obtained with the PCR-RFLP assay that differentiates *stx*_{2c} from *stx*_{2-vha}.

The presence of genes encoding Stx2 activatable by elastase was investigated using PCR with primers SLT-II-vc and CKS2 and restriction analysis of the resulting 890 bp amplicon with *Pst*I as described by Jelacic *et al.* (2003). Absence of the *Pst*I site can be taken as an indicator of the presence of a mucus-activatable *stx*₂ variant (Bielaszewska *et al.*, 2006; Gobius *et al.*, 2003; Jelacic *et al.*, 2003).

RNA extraction and quantitative real-time PCR (q-PCR).

Following mitomycin C induction for 3 h, total RNA was extracted using the Nucleospin RNA II kit (Macherey-Nagel). RNA concentration was determined by measuring the A₂₆₀ in a 96-well plate reader (Biotek µQuant) after 50-fold dilution in RNase-free water. One microgram of each RNA sample was reverse transcribed with Superscript II Enzyme (Invitrogen) and 1 µl of random primers (Invitrogen) in a final volume of 20 µl for 50 min at 42 °C. Three q-PCRs were carried out for each sample on diluted cDNA by using the LightCycler apparatus (Roche) with 0.5 µM of each primer, 4 mM MgCl₂, 1 µl of LightCycler Faststart DNA Master SYBR green I (Roche) and 2 µl of cDNA (1 ng, 100 pg and 10 pg) in microcapillary tubes in a final volume of 20 µl. Primers 2SF (CACATTTACAG-TGAAGTTGA) and 2R (TTCAGCAAATCCGGAGCCTG) allowed amplification of an *stx*₂ fragment but not of an *stx*_{2-vhb} fragment. Primers 2bSF (TACATTCACAGTAAAGTGCGC) and 2R allowed amplification of an *stx*_{2-vhb} fragment but not of an *stx*₂ fragment. *stx*_{2-vha} and *stx*_{2c} fragments were amplified using primers 2a

Table 1. Characteristics of STEC strains

Strain*	Serotype	<i>stx</i> ₂ genotype†	<i>stx</i> ₁ ‡	Origin§	Seropathotype	Source or reference
86-24*	O157:H7	<i>stx</i> ₂	N	H/outbreak	A	T. Whittam
CH1898	O157:H7	<i>stx</i> ₂ <i>stx</i> _{2c}	N	H/HUS	A	V. Livrelli
CHVi-I*	O157:H7	<i>stx</i> _{2c}	N	H/HUS	A	V. Livrelli
Sakai*	O157:H7	<i>stx</i> ₂	P	H/outbreak	A	T. Whittam
EDL 933*	O157:H7	<i>stx</i> ₂	P	Meat/outbreak	A	O'Brien <i>et al.</i> (1983)
CHO75	O157:H7	<i>stx</i> ₂	N	H/HUS	A	V. Livrelli
A8993-CS2	O157:H7	<i>stx</i> ₂	P	H/HC	A	T. Whittam
85-08*	O157:H7	<i>stx</i> ₂	P	H/D	A	T. Whittam
93-111	O157:H7	<i>stx</i> ₂	P	H/outbreak	A	T. Whittam
NV95*	O157:H7	<i>stx</i> _{2c}	P	B	A	Pradel <i>et al.</i> (2000)
NV203	O113:H21	<i>stx</i> ₂ <i>stx</i> _{2-vhb}	N	B	C	Pradel <i>et al.</i> (2000)
NV253	O113:H21	<i>stx</i> ₂ <i>stx</i> _{2-vhb}	N	B	C	Pradel <i>et al.</i> (2000)
NV254*	O113:H21	<i>stx</i> ₂ <i>stx</i> _{2-vhb}	N	B	C	Pradel <i>et al.</i> (2000)
NV166*	O113:H21	<i>stx</i> _{2c}	N	B	C	Pradel <i>et al.</i> (2000)
NV170	O113:H21	<i>stx</i> _{2c}	N	B	C	Pradel <i>et al.</i> (2000)
NV297	O113:H21	<i>stx</i> _{2c} <i>stx</i> _{2-vhb}	P	B	C	Pradel <i>et al.</i> (2000)
NV298	O113:H21	<i>stx</i> _{2-vha} <i>stx</i> _{2-vhb}	N	B	C	Pradel <i>et al.</i> (2000)
NV299	O113:H21	<i>stx</i> _{2-vha} <i>stx</i> _{2-vhb}	N	B	C	Pradel <i>et al.</i> (2000)
NV300	O113:H21	<i>stx</i> _{2-vha} <i>stx</i> _{2-vhb}	P	B	C	Pradel <i>et al.</i> (2000)
NV302	O113:H21	<i>stx</i> _{2-vha} <i>stx</i> _{2-vhb}	N	B	C	Pradel <i>et al.</i> (2000)
NV237*	O113:H21	<i>stx</i> ₂	N	B	C	Pradel <i>et al.</i> (2000)
E-D226*	O113:H21	<i>stx</i> _{2-vhb}	N	H	C	A. Caprioli
CL-15*	O113:H21	<i>stx</i> ₂ <i>stx</i> _{2-vhb}	N	H/HUS	C	T. Whittam
DEC16A*	O113:H21	<i>stx</i> _{2-vhb}	N	H/D	C	T. Whittam
CL-3*	O113:H21	<i>stx</i> ₂ <i>stx</i> _{2-vhb}	N	H/HUS	C	T. Whittam
87-307*	O113:H21	<i>stx</i> ₂ <i>stx</i> _{2-vhb}	N	H/HUS	C	T. Whittam
NV148*	O91:H10	<i>stx</i> _{2-vha}	N	B	C	Pradel <i>et al.</i> (2000)
NV271	O91:H10	<i>stx</i> _{2c}	N	B	C	Pradel <i>et al.</i> (2000)
NV280	O91:H10	<i>stx</i> _{2c}	N	B	C	Pradel <i>et al.</i> (2000)
NV130	O91:H10	<i>stx</i> _{2c} <i>stx</i> _{2-vhb}	N	B	C	Pradel <i>et al.</i> (2000)
NV199*	O91:H10	<i>stx</i> _{2c} <i>stx</i> _{2-vhb}	N	B	C	Pradel <i>et al.</i> (2000)
NV308*	O91:H10	<i>stx</i> _{2-vhb}	N	B	C	Pradel <i>et al.</i> (2000)
CB6775*	O91:H10	<i>stx</i> ₂	N	H	C	L. Beutin
E-D76*	O91:H21	<i>stx</i> ₂ <i>stx</i> _{2-vhb}	P	H	C	A. Caprioli
87-2927*	O91:H21	<i>stx</i> _{2-vha} <i>stx</i> _{2-vhb}	P	H/HUS	C	T. Whittam
VTH-13*	O91:H21	<i>stx</i> ₂	P	H/ HC	C	J. Blanco Alvarez
B2F1*	O91:H21	<i>stx</i> _{2-vha} <i>stx</i> _{2-vhb}	N	H/HUS	C	Ito <i>et al.</i> (1990)
NV200*	O91:H21	<i>stx</i> ₂ <i>stx</i> _{2-vhb}	N	B	C	Pradel <i>et al.</i> (2000)
NV197	O91:H21	<i>stx</i> ₂ <i>stx</i> _{2-vhb}	N	B	C	Pradel <i>et al.</i> (2000)
NV127*	O91:H21	<i>stx</i> _{2c} <i>stx</i> _{2-vhb}	N	B	C	Pradel <i>et al.</i> (2000)
NV32	O91:H21	<i>stx</i> _{2-vhb}	N	B	C	Pradel <i>et al.</i> (2000)
NV74	O91:H21	<i>stx</i> _{2-vhb}	P	B	C	Pradel <i>et al.</i> (2000)

*Strain used for q-PCR and ELISA experiments.

†Variants in bold have the mucus-activatable genotype.

‡N, absence of the *stx*₁ gene; P, presence of the *stx*₁ gene.

§H, human-derived strain; HUS, isolated from a clinical case of HUS; HC, isolated from a clinical case of haemorrhagic colitis; D, isolated from a patient with diarrhoeal symptoms; B, bovine-derived strain.

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(TAAAGTGGCCGAAAAGAG) and 2R. *stx*₂ and *tufA* mRNAs were quantified by noting the fluorescence crossing-point (CP) of the samples on the corresponding standard curve. Results are the mean

ratios between the copy number of *stx*₂ mRNA and the copy number of *tufA* mRNA. Control reactions were performed without reverse transcriptase to confirm that the target detected was RNA. Standard

curves for *stx* quantification were obtained by PCR amplification of genomic DNA from EHEC strains containing only one of the different *stx*₂ variants with the universal *stx*₂ primers 2F-0 (TATATCAGTGCCCGGTGTGA) and 2R-0 (CATTATTAACTGCACTTCAGC). The PCR products (884 bp) were purified with the Strataprep PCR purification kit (Stratagene) and DNA amounts were quantified by measuring the A₂₆₀ in a 96-well plate reader (Biotek µQuant). This amount was converted to molecule number as previously described (Fronhoffs *et al.*, 2002). Then PCR products were 10-fold serially diluted from 5 × 10⁸ to 50 molecules µl⁻¹ and three q-PCRs were carried out in a LightCycler apparatus (Roche) with primers 2SF and 2R, 2bSF and 2R, and 2a and 2R, generating standard curves for *stx*₂, *stx*_{2-vhb} and *stx*_{2-vha/stx}_{2c} respectively. The standard curve for *tufA* quantification was obtained in the same way using the genomic DNA of *E. coli* O157:H7 strain EDL933 and the primers TufAF (CAGGTAGGCGTTCCGTACAT) and TufAR (GTGCAAAAAGGCATCAAAT). After quantification of the molecule number, a q-PCR was done with primers TufAqF (TGGTTGATGACGAAGAGCTG) and TufAqR (GCTCTGGTTCCGGAA-TGTAA) to obtain the standard curve.

Phage particle isolation and *stx*₂ DNA quantification. Phages were purified from non-induced or mitomycin C-induced cultures 3 h or 6 h after mitomycin C treatment as previously described (Fuchs *et al.*, 1999) with slight modifications according to Arthur Donohue-Rolfe, Cummings School of Veterinary Medicine at Tufts University (personal communication). Briefly, 5 ml samples of the filtered culture supernatants were incubated with 10 µg ml⁻¹ RNase A (Amersham) and 40 U ml⁻¹ DNase I (Amersham) for 30 min at 37 °C. Phage particles were pelleted by ultracentrifugation for 16 h at 76 000 g at 4 °C. The pellets were resuspended in 100 µl PBS. Phage suspensions were boiled for 5 min at 95 °C, diluted fivefold in PBS, then *stx*₂ and *stx*₂-related DNA was quantified by q-PCR using the same primers and standards as for mRNA quantification. Absence of bacterial DNA in phage lysates was confirmed by the absence of *tufA* DNA as assayed by PCR. This method of indirect phage quantification detects phage DNA after its release from phage particles upon lysis of the host strain by use of specific primers. The method is very sensitive and circumvents the problem of extreme instability of plaque-forming capabilities of Stx2 phages, which are lost within 2 h of storage (Fuchs *et al.*, 1999; Muniesa *et al.*, 2004b), and the difficulty of identification of *stx*₂-phage plaques, which are very small and not well visible on agar plates (Gamage *et al.*, 2004; Muniesa *et al.*, 2003).

Measurement of Stx2 concentration by ELISA. Sandwich ELISAs were performed in 96-well plates (Maxisorp, Nunc) using a monoclonal antibody against Stx2 (STX2-BB12, Toxin Technology) diluted 1:500 to coat the plates and a rabbit polyclonal antiserum against Stx2 diluted 1:2000 to detect the toxin. A standard curve was obtained with twofold serial dilutions of purified Stx2 (Toxin Technology). Detection was performed with a 1:10 000 dilution of horseradish peroxidase-goat IgG anti-rabbit (Pierce) and *o*-phenylenediamine (OPD, Sigma)-stable peroxidase substrate (Pierce). Absorbance was measured in a 96-well plate reader (Biotek µQuant) at 492 nm.

Statistical analyses. Data are expressed as means ± SEM. To analyse the effect of the mitomycin C treatment on *stx*₂ transcription or Stx2 production, statistical analyses were performed using SAS software (v 8.1). The origin of the strains, the *stx*₂ variant and the serotype were considered to be independent variables. Due to the unequal number of strains in the groups, analysis of variance was performed using the Linear model procedure. Mean multiple comparison tests on log₁₀-transformed datasets were performed. The comparison method used was the Ryan-Einot-Gabriele-Welsh multiple range test. We made pairwise adjustments using the Tukey method. A *P* value ≤ 0.05 was considered significant.

RESULTS

The *stx*₂ variant is mostly associated with the most virulent strains

The prevalence of *stx*₂ and *stx*₂-related genes was investigated among 42 STEC strains. Nineteen strains isolated from humans with diarrhoea, HC or HUS, and 23 strains isolated from healthy cattle were selected (Table 1). Ten O157:H7 strains (nine from humans and one from cattle) belong to seropathotype A and as such possess the LEE. The remaining strains belong to seropathotype C and do not possess the LEE. Among them, 5 strains from humans and 11 strains from cattle belong to the O113:H21 serotype, 4 from humans and 5 from cattle are O91:H21, 1 from humans and 6 from cattle are O91:H10. These ratios between bovine- and human-derived strains for each serotype reflect the actual occurrence quite well, since O157:H7 strains are more frequently isolated from patients than from cattle, whereas O91:H10 strains are rarely found in humans (Caprioli *et al.*, 2005; Pradel *et al.*, 2000; Nataro & Kaper, 1998; Brooks *et al.*, 2005). Among the 42 selected strains, 22 contained only one *stx*₂ variant, and 20 contained two *stx*₂ variants. Only the *stx*₂, *stx*_{2-vha}, *stx*_{2-vhb} and *stx*_{2c} variants were found in these strains. The *stx*₂ variant was mainly associated with human-derived strains, especially the O157:H7 serotype, thus with strains of seropathotype A. This variant was the most frequently found in strains carrying only one *stx*₂ gene. In contrast, 13 strains of seropathotype C among 18 possessed *stx*_{2-vhb}, which was associated in 10 of them with *stx*_{2-vha}, *stx*_{2-vhb} or *stx*_{2c}.

It has been shown that the biological activity of the Stx2-vha and Stx2-vhb toxins is activatable by elastase cleavage of the last two amino acids of the enzymically active A subunit in the human or mouse intestinal mucus (Melton-Celsa *et al.*, 2002). Therefore all the isolates were further characterized for the possession of putative mucus-activatable Stx2 variants by PCR-RFLP analysis as described by Jelacic *et al.* (2003). The genes encoding *stx*_{2-vha} and *stx*_{2-vhb} B subunits were all associated with a putative activatable A subunit. In O157:H7 strains, the B subunits were all associated with a non-activatable A subunit. In non-O157:H7 strains, the association between A and B subunits was heterogeneous. Among 12 strains possessing an *stx*₂ B gene, 6 had a putative activatable A subunit. Among 8 strains possessing an *stx*_{2c} variant (indistinguishable *stx*_{2-vha/stx}_{2c} B gene, A gene with the PCR-RFLP pattern in the 5'-end characteristic of *stx*_{2c}), 7 showed the PCR-RFLP pattern in the A gene 3'-end characteristic of mucus-activatable toxins (Table 1).

Are strains carrying different *stx*₂ variants equally sensitive to mitomycin C?

A subset of 23 strains representative of each serotype and combinations of *stx*₂ variants found in the 42-strain

collection was chosen for a more detailed analysis of *stx*₂ and *stx*_{2c}-related expression (Table 1). Treatment of bacteria with DNA-damaging agents such as mitomycin C results in *stx*-phage induction and cell lysis. It has been shown that the decrease in the culture optical density is a relevant qualitative measurement of prophage induction and *stx*-phage production (Muniesa *et al.*, 2003; Tyler *et al.*, 2004). Thus, to determine whether phages carrying different *stx*₂ variants were equally induced by mitomycin C, we first monitored bacterial growth. Mitomycin C was added to the cultures at *t*₀ when the bacterial concentration reached 10⁷ to 3 × 10⁷ c.f.u. ml⁻¹. On the basis of OD₆₀₀ three growth patterns were observed (Fig. 1). For the first group, which includes the majority of the strains, mitomycin C slightly slowed the growth, and lysis due to lysogenic induction became apparent around 3 h after addition of mitomycin C to the growth medium (Fig. 1a). An early lysis, beginning around 90 min after mitomycin C treatment, was representative of three strains, namely 87-307, 87-2927 and ED-76 (Fig. 1b). The growth of the third group of strains was insensitive or poorly sensitive to mitomycin C (Fig. 1c), indicating that production of phages was limited in these strains. Thus it appeared that all strains were not equally sensitive to mitomycin C. All except one of the strains harbouring only the *stx*₂ variant belonged to the first group, whereas isolates carrying other variants, alone or in combination, were distributed in the three sensitivity groups.

Without lysogenic induction, only the *stx*₂ and *stx*_{2c} variants are expressed

To investigate whether lysogenic induction by mitomycin C was associated with expression of the *stx*₂ variants, mRNA levels were measured using q-PCR. Since 3 h after mitomycin C addition the bacterial cells are just beginning to be lysed for most of the strains, it is possible to isolate *stx*₂ mRNAs at this time from a large population of cells. Therefore, for q-PCR experiments we chose to isolate mRNA from each strain 3 h after mitomycin C treatment and at the same time for non-induced cultures.

In the absence of mitomycin C, only *stx*₂ and *stx*_{2c} mRNAs were detected (Fig. 2). Indeed, basal *stx*₂ mRNA levels were significantly higher than variant-*stx*₂ mRNAs (*P* < 0.001), except for *stx*_{2c} mRNA in two strains, CHVi-I and NV95. It is noteworthy that these strains are the only two O157:H7 strains expressing *stx*_{2c}. No *stx*_{2c} mRNA was detected in the non-O157:H7 strains carrying this variant. However, the *stx*_{2c} variant in these strains was not classical as it showed the genotype associated with mucus-activatable activity. Among the seven strains expressing *stx*₂ only, two did not produce significant mRNA levels. These two strains (VTH-13 and CB6775) belong to non-O157:H7 serotypes, whereas four of the five strains expressing high mRNA levels are O157:H7 strains. In all six strains containing both *stx*₂ and *stx*_{2-vhb}, only *stx*₂ mRNA was detected.

Fig. 1. Growth curves of STEC strains in the absence and presence of mitomycin C. At *t*₀, cultures in LB were divided into two parts, and mitomycin C (250 ng ml⁻¹) was added to one of them. ■, Growth in the absence of mitomycin C; □, growth in the presence of mitomycin C. (a) Growth curve of NV237 (*stx*₂), representative of *E. coli* Sakai (*stx*₂), EDL933 (*stx*₂), 86-24 (*stx*₂), 85-08 (*stx*₂), VTH-13 (*stx*₂), NV237 (*stx*₂), NV308 (*stx*_{2-vhb}), CHVi-I (*stx*_{2c}), NV95 (*stx*_{2c}), CL-3, CL-15 (*stx*_{2-stx2-vhb}), NV254 (*stx*_{2-stx2-vhb}), NV199 (*stx*_{2c-stx2-vhb}) and B2F1 (*stx*_{2-vha-stx2-vhb}). (b) Growth curve of *E. coli* 87-307 (*stx*_{2-stx2-vhb}), representative of E-D76 (*stx*_{2-stx2-vhb}) and 87-2927 (*stx*_{2-vha-stx2-vhb}). (c) Growth curve of *E. coli* NV166 (*stx*_{2c}), representative of CB6775 (*stx*₂), NV148 (*stx*_{2-vha}), DEC16A (*stx*_{2-vhb}), E-D226 (*stx*_{2-vhb}), NV200 (*stx*_{2-stx2-vhb}) and NV127 (*stx*_{2c-stx2-vhb}).

Mitomycin C differentially induces the *stx*₂ variants

Expression of the *stx*₂ variants was differently affected by the mitomycin C treatment depending on the strain (Fig. 2). Globally, induced *stx*₂ mRNA levels were higher than the others (*P* < 0.05), except for the two O157:H7 strains, CHVi-I and NV95, that express *stx*_{2c} and the two strains that express *stx*_{2-vha} and *stx*_{2-vhb}. Transcription of *stx*₂ was stimulated 25- to 100-fold in all strains expressing *stx*₂ only, but when associated with *stx*_{2-vhb}, expression of *stx*₂ was inducible in only two of six strains. In the four

Fig. 2. Relative *stx*₂ and *stx*₂-related mRNA levels and indirect quantification of *stx*₂ and *stx*₂-related phage particles. Left scale: *stx*₂ relative mRNA levels were measured by q-PCR without mitomycin C (solid bars) or with mitomycin C induction (hatched bars). Values are the mean of at least three independent experiments done in triplicate. The standard error of the mean is indicated for each strain. Right scale: copy number of phage-borne *stx*₂ or *stx*₂-related genes in strain supernatants in the absence (●) or presence (○) of mitomycin C for 3 h. The seropathotype of the strains is indicated at the top. *stx*₂ variants indicated under the graph are the variants detected in the q-PCR experiments. The dashed line indicates values of the negative control. *Strains harbouring *stx*₂ + *stx*_{2-vhb} were tested separately for *stx*₂ and *stx*_{2-vhb}.

remaining strains *stx*₂ expression was as high in non-induced as in induced conditions. Three of them (CL-3, CL-15 and NV254) carry a non-classical *stx*₂ variant showing the RFLP pattern associated with mucus-activatable variants. Expression of some *stx*₂ variants was not induced by mitomycin C: *stx*_{2-vhb} mRNA was not produced or was produced at very low levels in all strains expressing *stx*_{2-vhb} either alone or in combination with *stx*₂. However, induction of the lytic cycle was earlier for three of these strains, 87-307, 87-2927 and ED-76 (Fig. 1b). To measure *stx*_{2-vhb} mRNA levels in these strains in a large population of cells, mRNAs were isolated at the beginning of cell lysis, 90 min after mitomycin C treatment. Again, *stx*_{2-vhb} mRNAs were undetectable. *stx*_{2-vha} and *stx*_{2c} mRNA increased 10- to 30-fold after lysogenic induction, reaching high levels in O157:H7 strains only. An increase in *stx*₂-related mRNA levels was also observed in strains expressing *stx*_{2-vhb} in combination with *stx*_{2-vha} or *stx*_{2c}. However, because our attempts to design a primer pair able to differentiate between these mRNAs were unsuccessful, we failed to determine whether expression of both genes or of only one was induced. It is noteworthy that the strains carrying the non-classical *stx*_{2c} variant expressed lower levels of *stx*₂-related mRNA than the strains carrying the classical *stx*_{2c} variant.

Analysis of the growth curves and *stx* mRNA levels showed that mitomycin C-induced cell lysis was associated with high expression of at least one of the *stx*₂ variants carried by the strain. Conversely, when treatment with mitomycin C did not result in cell lysis, the *stx*₂ variants were not or were very poorly expressed. These observations suggest that the *stx*₂ variants are expressed only when they are carried by an inducible prophage.

Only the *stx*₂ variants which are expressed are carried by released phage particles

To investigate whether the *stx*₂ variants are associated with phage DNA, we quantified *stx*-phage DNA in most culture supernatants by q-PCR using the same *stx*₂ and *stx*₂-related specific primers as used for mRNA analysis. Phage particles were harvested from strain supernatants 3 h (Fig. 2) and 6 h (data not shown) after addition of mitomycin C. *stx*-phage DNA was not detected or was detected at low levels in cultures in which no lysis occurred, at 3 h as well as 6 h after addition of mitomycin C. At *t*₃ without treatment, significant amounts of *stx*-phage DNA were only detected in supernatants of strains expressing the corresponding *stx* mRNA in significant amounts. Higher amounts of *stx*-phage DNA were detected under mitomycin C treatment

except for strains in which *stx*₂ transcription was not inducible (CL-15, CL-3 and NV254). Under mitomycin C induction, *stx*_{2-vhb}-phage DNA was detected in low amounts only in the supernatant of the three strains for which *stx*_{2-vhb} mRNA was detected (NV308, DEC16A and NV254) and, surprisingly, in the supernatant of strain 87-307. High *stx* mRNA levels were never observed in the absence of phage particles in the supernatant. The relationships observed between growth curve patterns, *stx* mRNA levels and *stx*-phage release indicate that the *stx* variants carried on prophages able to produce phage particles in the medium were the only ones expressed, and that the promoter of the *stx*_{2-vhb} variant, when it is not carried on such prophages (for example in CL-3, CL-15 and NV200), is inactive in the culture conditions used. In those strains in which expression of *stx*₂ was high but not sensitive to mitomycin C (CL-3, CL-15 and NV254), *stx*₂-phage DNA was detected at similar high levels with or without mitomycin C treatment. Thus it appears that the *stx*₂ prophages in these strains had an unusual level of spontaneous induction. At *t*₆, the amounts of *stx*-phage DNA were two- to fivefold higher than at *t*₃, but the relative amounts of each variant remained similar (data not shown).

To investigate whether *stx*₂ expression and *stx*₂-phage production are affected by nutritional cues, we measured *stx*₂ and *stx*₂-related mRNA and phage DNA when STEC

strains were grown under nutrient-limiting conditions, i.e. in DMEM medium containing a low glucose concentration (1 g l⁻¹). The data obtained in DMEM are presented as a supplementary figure with the online version of this paper (Fig. S2). They were not significantly different from data in LB. As in LB medium, we found that without induction, only the *stx*₂ and *stx*_{2c} variants were expressed. Under mitomycin C treatment, the same levels of mRNA induction as in LB medium were observed for each variant. The *stx*_{2-vhb} genes not expressed in LB remained unexpressed in DMEM. As in LB medium, *stx*-phage production was well correlated with *stx* mRNA.

Shiga toxin release

To confirm that Shiga toxin was synthesized by bacteria expressing the *stx*₂ and *stx*₂-related genes, amounts of Stx2 and Stx2-related variants released in culture supernatants were measured using ELISA (Fig. 3). As Stx2 variants are not immunologically distinct, the assay did not allow differentiation between Stx2, Stx2-vha, Stx2-vhb and Stx2c in the supernatant of strains producing two toxins. Thus differential production of each Stx2 variant could not be assessed by this method.

In non-induced conditions, concentrations of 30–800 ng ml⁻¹ of Stx2 were measured in the culture supernatants,

Fig. 3. Quantification of Stx2 concentration in strain supernatants. Solid bars, concentration reached without mitomycin C treatment; hatched bars, concentration reached under mitomycin C treatment. Values are the mean of at least three independent experiments done in duplicate. The standard error of the mean is indicated for each strain. The *stx*₂ variants carried by each strain are indicated at the bottom. The seropathotype of the strains is indicated above the bars. The dashed line indicates values of the negative control.

depending on the strain. Globally, human-derived strains released significantly higher amounts of Stx2 than bovine-derived strains ($P < 0.05$). Under mitomycin C treatment, toxin release was induced 10- to 140-fold, reaching 3000–100 000 ng ml⁻¹ in most (18 of 24) of the culture supernatants, and the difference in Stx2 release between human and bovine strains was no longer significant. The induction or absence of induction of toxin production correlated quite well with the induction or absence of induction of mRNA expression and phage particle release.

DISCUSSION

It has been suggested that the varied capacity of STEC strains to cause serious disease in humans is associated with the type and/or amount of Stx produced (Paton *et al.*, 1995). However, expression of stx₂ variants in STEC differing in their virulence traits is not well documented. In this study, we determined the distribution of stx₂ variants among 42 Stx2-producing STEC obtained from patients or from healthy cattle. Ten strains belong to seropathotype A, containing O157:H7 LEE-positive strains, which are the most frequently associated with severe outbreaks in the world (Karmali *et al.*, 2003). The remaining strains belong to seropathotype C, which includes mainly non-O157:H7 LEE-negative strains frequently associated with sporadic cases of HUS (Karmali *et al.*, 2003). Then a subset of 24 strains representative of the initial group with regard to the origin, serotype, and stx₂ variants was analysed for the induction by mitomycin C of stx₂ variant expression, Stx2 release and stx-phage particle production.

Heterogeneity of the stx₂ and stx₂-related genotypes

Molecular typing of the stx₂ and stx₂-related variants using previously described or new PCR-RFLP methods revealed mosaic sequences. First, a gene encoding an Stx2 B-subunit can be associated with an A-subunit gene that possesses the *Pst*I site indicative of mucus-activatable toxins. The same observation was made in a recent study on STEC strains isolated in Germany from food samples (Beutin *et al.*, 2007). Second, A-subunit genes primarily typed as stx_{2c} based on PCR-RFLP in their 5' end showed the PCR-RFLP type characteristic of mucus-activatable toxins in their 3' end. However this heterogeneity was not observed in O157:H7 strains, which all showed the non-activatable stx₂ and stx_{2c} genotype, nor for the stx_{2-vha} and stx_{2-vhb} genes, which all showed the activatable genotype.

Heterogeneous expression of stx₂ variants

Several authors have shown that Stx2-encoding prophages are highly variable (Johansen *et al.*, 2001) and present a high degree of heterogeneity in terms of induction levels (Muniesa *et al.*, 2004a; Wagner *et al.*, 1999). In agreement with these reports, we found that expression of stx₂ and

stx₂-related genes is heterogeneous in basal and in induced conditions, depending on the strain and on the stx₂ variant. q-PCR analysis of stx₂-related genes showed that stx₂ mRNA levels were higher than stx_{2-vha} or stx_{2-vhb} mRNA levels. However, a more detailed analysis showed a heterogeneous expression of a given stx₂ variant which correlated with a heterogeneous release of stx-phage particles depending on the strain. This could be due to different regulatory properties of the phages harbouring the same stx variant, or to bacterial host factors such as repair systems, membrane constitution, etc. For example, among nine strains harbouring stx_{2-vhb} alone or in combination with stx₂, stx_{2-vhb} mRNA and phage particles were detected in only three, although at low levels. In Stx2-producing strains, large differences appeared in stx₂-mRNA levels, in basal as well as in induced conditions. stx₂ genes highly expressed without mitomycin C treatment are probably carried by a prophage with a high level of spontaneous induction, leading to high release of phage particles and Stx independently of the SOS system. Previous studies indicate that the 933W prophage, encoding Stx2 in EDL933, induces more readily than lambdoid prophages that do not encode Stx (Livny & Friedman, 2004). Here we broaden this observation to a number of other strains of different serotypes, showing that most of the prophages expressing the stx₂ variant show a high level of spontaneous induction whereas the stx_{2-vha}, stx_{2-vhb} and stx_{2c} variants associated with the mucus-activatable genotype are not or are poorly induced without mitomycin C treatment.

The stx_{2-vhb} variant is probably not associated with an inducible prophage

Upon mitomycin C treatment, stx₂, stx_{2-vha} and stx_{2c} transcription was induced, but stx_{2-vhb} transcription was induced in only two strains out of nine. This differential expression was also seen in strains expressing both stx₂ and stx_{2-vhb}, in which high stx₂ and low stx_{2-vhb} mRNA levels were measured, in induced as well as in non-induced conditions. Thus, our results indicate that in most cases expression of stx_{2-vhb} is not induced upon treatment by mitomycin C, and suggest that in the corresponding strains this gene is not phage-borne, or is carried by a defective prophage. This conclusion is further supported by the absence of stx_{2-vhb}-phages in strain supernatants. Alternatively, in strains harbouring stx_{2-vhb} together with stx₂, stx_{2-vhb}-prophages could be induced later than stx₂-prophages. Thus induction of the lytic cycle of the stx₂-phage could lyse the cells before high stx_{2-vhb} mRNA levels have been produced. This could explain why low amounts of stx_{2-vhb}-phage DNA were detected in the supernatant of strain 87-307 under mitomycin C induction. However, other studies showed that some stx₂-related genes were not associated with an inducible prophage (Teel *et al.*, 2002; Zhang *et al.*, 2005), and that in strains harbouring two stx₂-prophages only one was inducible (Teel *et al.*, 2002; Muniesa *et al.*, 2003). In particular, using two B2F1

isogenic mutants, producing either Stx2-vha only or Stx2-vhb only, Teel *et al.* (2002) have provided evidence that *stx2-vha* expression is bacteriophage associated but *stx2-vhb* is not. In a study including 168 STEC strains, Muniesa *et al.* (2004b) found that 5 strains possessed two *stx2* copies. In each case, only one copy was found to be associated with an inducible prophage. A constitutive, poorly active promoter controlling *stx2* expression in O157 strains has been described (Sung *et al.*, 1990; Plunkett *et al.*, 1999). The very low levels of *stx2-vhb* mRNA detected in induced and non-induced conditions could indicate that this gene was expressed from such a poorly active specific promoter in the conditions tested.

In summary, our results strongly suggest that *stx2* and *stx2*-related genes are expressed only when they are carried by a prophage subject to a high spontaneous induction or/and to SOS-mediated lysogenic induction. In particular, the *stx2-vhb* genes are not or are very poorly expressed, in basal as well as in induced conditions, and in most cases are not found on the genome of phage particles in strain supernatants. Induction levels and amounts of *stx* mRNAs are significantly higher in O157:H7 strains than in strains belonging to the other serotypes ($P < 0.01$). Furthermore, *stx* mRNA levels were higher in strains belonging to seropathotype A and in the strains of seropathotype C that express the *stx2* variant when compared to other strains of seropathotype C.

Stx2 release

We found variable levels of Shiga toxins in supernatants of some STEC strains in LB medium without mitomycin C. This is probably due to spontaneous phage induction, since we detected toxins in the supernatants only when phage particles were also detected. This basal amount of released toxin varied from strain to strain, and was significantly higher in strains isolated from humans than in strains isolated from cattle. There were also marked differences in the effect of mitomycin C on toxin production by different strains, but, in contrast to the findings by Ritchie *et al.* (2003), we found no significant difference between human- and bovine-derived strains, in terms of amounts of released toxin or of induction levels. This discrepancy could be due to the choice of strains included in the study. All but one of the human-derived strains belonged to the highly virulent seropathotype A in the study reported by Ritchie *et al.* (2003), whereas half of our human-derived strains belong to the low-virulent seropathotype C.

For most of the strains, the amount of toxin released in the supernatant correlated well with the measured levels of *stx* mRNA, in basal and in induced conditions. However, this was not the case for some particular strains. Indeed, strains CL-3, CL-15 and NV254 each produced similar high levels of *stx2* mRNA and *stx2*-phage particles independently of the presence of mitomycin C. However, low amounts of toxin were produced without mitomycin C whereas high Stx2 amounts were produced under mitomycin C treatment;

strains NV308, NV127 and NV199 produced moderate levels of *stx* mRNA and *stx*-phage DNA under mitomycin C treatment, but released high amounts of toxin. Post-transcriptional regulation leading to a more efficient translation of *stx* mRNA in the presence of mitomycin C could explain these observations. In contrast, strain NV200 produced high levels of *stx2* mRNA, but did not release Stx2 into the medium, suggesting that *stx2* mRNA was not translated. Although further investigation is required to determine the molecular mechanisms involved, these observations highlight the functional diversity of *stx* phages.

Conclusion

Our results indicate that the *stx2* variant is mainly associated with the most pathogenic human-derived strains belonging to seropathotype A (O157:H7 strains). Furthermore, the data show a relationship between the seropathotype and the expression level of the *stx2* variant, and the amount of *stx*-phages and toxin released, in basal as well as in induced conditions. In seropathotype C, a subset of LEE-negative strains showed the same characteristics as the O157:H7 strains regarding *stx* expression and induction and thus could be a greater risk to human health than the other strains belonging to this seropathotype.

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