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Diversity of stx_2 converting bacteriophages induced from Shiga-toxin-producing *Escherichia coli* strains isolated from cattle

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The presence of bacteriophages encoding Shiga toxin 2 (stx_2 phages) was analysed in 168 strains of Shiga-toxin-producing *Escherichia coli* (STEC) isolated from cattle. Following mitomycin C induction, strains carrying stx_2 phages were screened by plaque blot and hybridization with an stx_2A-probe. In the stx_2-phage-carrying strains, the amounts of phage production, phage DNA extracted and Stx_2 produced after induction were assessed. The induced stx_2 phages were characterized morphologically and genetically. Assays to obtain lysogens from different strains were also carried out and phages induced from the lysogens were compared with those induced from the STEC isolates. Results indicated that 18 % of the strains carried an inducible stx_2 phage. Most of them showed a direct relationship between phage induction and toxin production. Each strain carried only one inducible stx_2 phage, although a few strains had two copies of the stx_2 in the chromosome. The stx_2 phages showed diverse morphology and a wide variability in their genome. Assays to obtain lysogens showed that not all the phages were transduced with the same frequency and only six lysogens were obtained. Phages in the lysogens were the same as those induced from their respective initial STEC host strains, although the induction and relative toxin production of the lysogens varied. Most phages carried the stx_2 gene, while a few carried stx_2 variants. Infectivity of the phages depended on the different hosts, although O157 : H7 was preferentially infected by phages induced from O157 strains. The results show that inducible stx_2 phages are common among STEC of animal origin and that they may enhance the spread of stx_2.

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INTRODUCTION

Cattle are a major reservoir for Shiga-toxin-producing *Escherichia coli* (STEC). Ruminant animals such as cattle, sheep and goats are naturally colonized with STEC and release these organisms into the environment with their faeces (Blanco *et al.*, 2001, 2003; Licence *et al.*, 2001; Widiasih *et al.*, 2004). STEC can cause haemorrhagic colitis and haemolytic uraemic syndrome (HUS) in humans (Ritchie *et al.*, 2003). The most common virulent serotype is *E. coli* O157 : H7, although others have been described (Karmali, 2004). *E. coli* O157 : H7 and over 100 other STEC serotypes can be found on virtually all farms in countries throughout the world (Beutin *et al.*, 1997; Heuvelink *et al.*, 1998; Blanco *et al.*, 2001; Hancock *et al.*, 2001). Transmission of STEC from cattle to humans occurs via food- and waterborne routes. The Shiga toxins 1 and 2 (Stx_1 and Stx_2) and some variants of Stx_2 are, among others, the main virulence factors in the progression of the disease in humans (Nataro & Kaper, 1998; Paton & Paton, 1998; Karmali, 2004).

Several authors have shown that stx_1, stx_2 and some stx_2 variants are encoded in the genome of temperate lambdoid double-stranded DNA phages (*stx*-phages) (O'Brien *et al.*, 1984; Acheson *et al.*, 1998; Neely & Friedman, 1998; Muniesa *et al.*, 2000; Teel *et al.*, 2002). Various patterns of *stx*-phages have been described and since they present a wide range of DNA structures, restriction patterns, host spectra and morphology, they are considered to form a heterogeneous family (Unkmeir & Schmidt, 2000; Schmidt *et al.*, 2000; Johansen *et al.*, 2001).

Abbreviations: STEC, Shiga-toxin-producing *Escherichia coli*; Stx, Shiga toxin.

The GenBank/EMBL/DDBJ accession numbers of the stx_2 genes of phages detailed in this paper are AY633453–AY633473.

The *stx*-phages are inducible from the host strain by DNA-damaging agents such as UV light or certain antibiotics (Kimmitt *et al.*, 2000; Kohler *et al.*, 2000; Zhang *et al.*, 2000). As a result of the induction process, bacterial host cells lyse and release free phage particles that can infect other bacteria. This is especially significant with regard to animal feed, since some policies permit the use of subinhibitory concentrations of antibacterial substances (Kohler *et al.*, 2000; WHO, 2001).

The extent to which STEC strains carrying inducible *stx* prophages can be isolated from animals needs to be evaluated. Furthermore, the infectivity of the stx_2 phages induced from these strains and their ability to infect other bacterial host strains should be studied in order to evaluate the risk of gene exchange in animal husbandry and the emergence of new STEC strains by infection with *stx*-phages.

Here we evaluate the presence of stx_2 phages induced from STEC strains isolated from cattle. Our aim was to characterize the phages induced, to test their infectivity in different hosts and to study the extent to which they could be induced by antibiotic treatment, thus providing information to explain their presence among the STEC population.

METHODS

Bacterial strains, serotyping, bacteriophages and media. To study the presence of Shiga toxin phages, 168 STEC strains were screened for the presence of stx_2 phages. The criteria for selecting the strains for this study were the origin (animal) and the presence of stx_2 genes. Of these, 165 were isolated from cattle (151 from calves and cows and 14 from beef meat) belonging to separate herds and three were isolated from sheep. Isolation and characterization of the strains as STEC were performed as indicated in previous studies (Blanco *et al.*, 2003). Of the 168 strains, 98 were *E. coli* O157 : H7, obtained from the Public Health Agency in Barcelona (Agència de Salut Pública de Barcelona), where several pathogenic strains from different origins are routinely analysed. The remaining 70 strains were obtained from the *E. coli* reference centre in Lugo, Spain (LREC; htpp://www.lugo.usc.es/ecoli) and include different serotypes: O2 : H27, O2 : H29, O2 : H32, O4 : H4, O6 : H34, O6 : H49, O8 : H2, O8 : H9, O8 : H19, O8 : H21, O15 : H16, O15 : H18, O15 : H27, O20 : H19, O21 : H21, O22 : H8, O22 : H16, O26 : H-, O38 : H21, O39 : H21, O39 : H48, O41 : H2, O42 : H21, O54 : H-, O60 : H19, O64 : H34, O65 : H16, O70 : H-, O77 : H41, O88 : H8, O88 : H25, O91 : H19, O91 : H21, O91 : H-, O110 : H2, O113 : H-, O113 : H4, O113 : H21, O116 : H16, O116 : H21, O116 : H-, O120 : H10, O127 : H21, O136 : H16, O136 : H-, O140 : H21, O141 : H8, O146 : H21, O148 : H8, O156 : H8, O156 : H19, O156 : H-, O162 : H4, O162 : H7, O162 : H21, O163 : H19, O163 : H21, O165 : H21, O165 : H25, O166 : H28, O171 : H2, O171 : H25, O174 : H2, O174 : H21, O174 : H-, O177 : H-, O178 : H11, ONT : H2, ONT : H19 and ONT : H21. O and H antigens were determined according to the method described by Guinée *et al.* (1981), employing all available O (O1–O181) and H (H1–H56) antisera. All antisera were obtained and absorbed with the corresponding cross-reacting antigens to remove nonspecific agglutinins. The O antisera were produced in the LREC and the H antisera were obtained from the Statens Serum Institut (Copenhagen, Denmark).

E. coli laboratory strain DH5α, *E. coli* strain C600, a *recA* mutant of *E. coli* K-12 strain DM1187 (James *et al.*, 2001), *Shigella sonnei* clinical isolate 866 (kindly provided by G. Prats, Hospital Vall d'Hebron Barcelona) and stx_2^- strain of *E. coli* O157 : H7 ATCC 43888 were used as host strains in some of the experiments described below and for propagation of the bacteriophages induced from the strains studied.

E. coli O157 : H7 ATCC 43889, which produces Stx_2, and bacteriophage 933W (O'Brien *et al.*, 1984) were used as positive controls for bacteria and phage, respectively. *E. coli* O157 : H7 ATCC 43888, which possesses neither stx_1 nor stx_2, was used as a negative control in some experiments.

Luria–Bertani (LB) broth or LB agar were used for culture of bacteria.

PCR studies. PCRs were performed with a GeneAmp PCR system 2400 (Perkin-Elmer). The DNA template was prepared directly from two colonies of each strain suspended in 50 µl double-distilled water and heated to 96 °C for 10 min prior to addition of the reaction mixture. Purified bacterial or phage DNA was diluted 1 : 20 in double-distilled water. The oligonucleotides used to amplify the stx_2 gene were S2Aup, 5′-ATGAAGTGTATATTATTTA-3′, and S2Alp, 5′-TTCTTCATGCTTAACTCCT-3′, for the A subunit, GK3 and GK4 (Rüssmann *et al.*, 1994) for the B subunit and UP378 and LP378 (Muniesa & Jofre, 1998) amplifying a 378 bp fragment of the stx_2A subunit. Primers 5′-CAGTTAATGTGGTGGCGAAGG-3′ and 5′-ACTGCTAATAGTTCTGCGCATC-3′ were used to amplify stx_1. Five microlitres of each PCR product was analysed by 1 % agarose gel electrophoresis and bands were visualized by ethidium bromide staining.

Preparation of digoxigenin-labelled stx_2A-specific gene probes. A 960 bp fragment corresponding to the stx_2A gene resulting from amplification with primers S2Aup/S2Alp was labelled with digoxigenin by incorporating digoxigenin-11-deoxyuridine triphosphate (Roche Diagnostics) during PCR as described previously (Muniesa *et al.*, 2000), and used as a probe.

Standard DNA techniques. DNA was digested with *Eco*RI and *Cla*I restriction endonucleases (Promega) and restriction fragments were analysed by separation on 0·7 % agarose gels in Tris/borate/EDTA buffer and stained with ethidium bromide. PCR products were purified using a PCR purification kit (Qiagen).

Isolation and detection of stx_2 in bacterial DNA. Chromosomal DNA was isolated from 40 ml cultures of each strain by lysozyme treatment and phenol/chloroform extraction as described by Stewart *et al.* (1998). Purified DNA was diluted 1 : 3 in double-distilled water and digested with *Eco*RI. Electrophoresis, transfer to nylon membranes and hybridization were carried out under the same conditions as described for phage DNA.

Isolation of temperate bacteriophages and preparation of phage lysates. Bacteria were grown from single colonies in LB broth at 37 °C to the exponential growth phase, measured as described below. Mitomycin C was added to the cultures to a final concentration of 0·5 µg ml^{-1}. These cultures were then further incubated overnight. The induced cultures were centrifuged at 10 000 ***g*** for 10 min and the supernatants were filtered through low-protein-binding 0·22 µm membrane filters (Millex-GP; Millipore) and treated with DNase (10 U ml^{-1}; Sigma-Aldrich).

To estimate the level of phage production after induction of each strain, the cultures were analysed with a spectrophotometer (Spectronic 501; Milton Roy, Belgium) at 600 nm until the exponential growth phase (OD_{600} 0·3). At this point mitomycin C was added and the culture was incubated for 14–16 h at 37 °C with shaking in the dark. After incubation the optical density was measured. Inductions and optical density measurements were performed at least in triplicate for each strain.

Screening for the presence of stx_2 temperate bacteriophages. A plate containing *E. coli* DH5α as a host strain was used to detect the presence of temperate bacteriophages in the strains. For this purpose several host strains (*E. coli* DH5α, C600, DM1187, O157 : H7 ATCC 43888 and *S. sonnei* isolate 866) were used in parallel. Five hundred microlitres of an exponential-phase culture of each strain was added to 100 µl 0·1 M $CaCl_2$ and 3 ml molten LB top agar (LB broth with 0·7 % agarose); the mixture was then poured onto LB agar plates and allowed to solidify. Fifteen microlitres of a suspension of each phage lysate and phage 933W, used as a positive control, was spotted onto the plates.

For phage enumeration, the phage lysates obtained after induction were diluted tenfold. One hundred microlitres of each dilution was then mixed with 100 µl 0·1 M $CaCl_2$ and 500 µl of a exponential-phase culture of each host strain and examined by the plaque assay using a double-layer agar method (Muniesa *et al.*, 2004). Controls of the bacterial supernatants without mitomycin C induction were performed. The plates were examined for the presence of a lysis zone or for the presence of plaques after incubation for 18 h at 37 °C.

To determine the presence of the stx_2 gene in phages in the phage lysates, the plaques were transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech) according to a standard procedure (Sambrook & Russell, 2001) and hybridized at 65 °C with a digoxigenin-labelled stx_2A probe prepared as described above. Stringent hybridization was achieved with the DIG–DNA Labelling and Detection kit (Roche Diagnostics) according to the manufacturer's instructions.

Evaluation of the ability of the stx_2 phages to infect different host strains. To evaluate the ability of the stx_2 phages induced to infect host strains other than *E. coli* DH5α, a strain of *E. coli* O157 : H7 stx_2^- (ATCC 43888), *S. sonnei* strain 866, laboratory *E. coli* strain C600 and a *recA* mutant of *E. coli* K-12, strain DM1187, were used as host strains. A drop of phage suspension from the stx_2 phages was spotted onto a monolayer containing each host strain and, after incubation, the spot test was transferred to a nylon membrane and hybridized with the stx_2A-specific probe. If a clear black signal was obtained after hybridization, the corresponding phage was considered positive for infectivity of this strain. A weak signal was considered indicative of low infectivity, whereas no signal was considered as a negative result (i.e. not infectious) for this strain. As a negative control, an agar monolayer containing no host strain was spotted with the phage suspensions, transferred to a nylon membrane and hybridized in the same conditions as described above.

Evaluation of toxin production. To determine the association between phage induction and toxin production, the production of Stx_2 in cultures after phage induction with mitomycin C was determined. An enzyme immunoassay (Premier EHEC) was performed according to the manufacturer's instructions. Production of toxin was compared for each strain with and without mitomycin C induction and with *E. coli* DH5α as a negative control. For this purpose, bacteria were grown from single colonies in LB broth at 37 °C to the exponential growth phase measured as described above. At this point each culture was divided in two aliquots and mitomycin C was added to one of the aliquots as described above for bacteriophage induction. Bacteria were then incubated at 37 °C for 18 h. After incubation, the supernatant of each culture was analysed for the presence of Stx as described by the manufacturer. Results obtained with both aliquots were analysed spectrophotometrically at a dual wavelength (450/630 nm) and compared.

Construction of lysogens. To confirm that no additional prophages were harboured by the STEC strains that can be induced by them and interfere with the results, we purified stx_2 phages from each STEC strain and used them to lysogenize the host strains described above (DH5α C600, O157 : H7 ATCC 43888 and *S. sonnei* strain 866). For this purpose, 0·5 ml of the phage lysate obtained and purified after induction of the STEC strains was mixed with 0·1 ml of an exponential-phase culture of each strain and incubated at 37 °C for 30 min. The mixture was added to 100 µl 0·1 M $CaCl_2$ and 3 ml molten LB top agar (LB broth with 0·7 % agarose), poured onto LB agar plates and further incubated at 37 °C overnight. The following day, the double agar layer was harvested in 1 ml SM buffer, diluted tenfold and plated. The presence of the stx_2 gene in the suspected lysogens was tested by colony hybridization assay (Muniesa *et al.*, 2000) using the stx_2A probe prepared as described above and confirmed by PCR.

Phages induced from the lysogens as described above were analysed and compared with those isolated from the wild STEC strains using the same procedures. Phage induction in the lysogens after mitomycin C treatment was analysed with the spectrophotometer at OD_{600} for 18 h.

Isolation of phage DNA and detection of stx_2 gene. Phage DNA from all the strains harbouring stx_2 phages was isolated from 200 ml cultures after induction with mitomycin C by the polyethylene glycol (PEG) method and phenol/chloroform extraction as described in Sambrook & Russell (2001). DNA was quantified by measuring the absorbance at 260 nm. Phage DNA concentration values were obtained after three isolations of phage DNA in three independent experiments.

Purified DNA was digested with *Eco*RI or *Cla*I, the fragments were separated by 0·8 % agarose gel electrophoresis and bands were visualized by ethidium bromide staining. After electrophoresis, DNA was transferred to nylon membranes (Hybond-N+) by capillary blotting (Sambrook & Russell, 2001). The membranes were hybridized with the DIG-labelled stx_2A fragment probe as described above.

Sequencing of the stx_2 gene encoded by temperate phages. The total length of the stx_2 gene of each phage was sequenced. Sequencing was achieved by PCR amplification using the oligonucleotides S2Aup/S2Alp for stx_2 subunit A and GK3/GK4 for subunit B. Since neither the 5′ and 3′ limits of the amplimer nor the fragment between the two subunits could be correctly sequenced using the same PCR primers, oligonucleotides UP378 and LP378, located within the subunit A sequence, were used to sequence the full length of the gene. Each sequence was performed in triplicate.

Sequencing was performed with the ABI PRISM Big Dye II Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) in an ABI PRISM 3700 DNA Analyser (Applied Biosystems), according to the manufacturer's instructions.

Nucleotide sequence analysis, assembly of the sequences, protein translation and BLAST analyses were performed with the tools available at http://www.ncbi.nlm.nih.gov. Multiple sequence alignment was performed with the software Multalin version 5.4.1 (Corpet, 1988).

Nucleotide sequence accession numbers. The nucleotide sequence of the 1241 bp fragment of the stx_2 gene of each phage showing a sequence that differed from stx_2 of phage 933W was submitted to the GenBank/EMBL database library. The assigned accession numbers were: phage A75 (AY633453), A534 (AY633454), A557 (AY633455), O157-572 (AY633456), VTB75 (AY633457), VTB91 (AY633458), VTB178 (AY633459), VTB324 (AY633460), A312 (AY633461), A315 (AY633462), A549 (AY633463), A580 (AY633464), O157-292 (AY633465), O157-330 (AY633466), O157-310 (AY633467), O157-379 (AY633468), VTB323 (AY633469), VTB46 (AY633470), VTB60 (AY633471), A397 (AY633472) and O157-469 (AY633473).

Electron microscopy. The stx_2-converting bacteriophages present in 10 ml of culture obtained after induction of each strain were

purified by caesium chloride centrifugation (Sambrook & Russell, 2001). The easily visible grey band where the bacteriophages were expected (Franki *et al.*, 1991; Sambrook & Russell, 2001), corresponding to a density of $1{\cdot}45 \pm 0{\cdot}02$ g ml^{-1}, was collected and dialysed to remove the CsCl. A drop of this phage suspension was deposited on copper grids with carbon-coated Formvar films and stained with 2 % KOH phosphotungstic acid (pH 7·2) for 2·5 min. Samples were examined in a Hitachi E.M.800 electron microscope operating at 80 kV.

RESULTS

Screening for temperate bacteriophages and preparation of phage lysates

Infectious *stx*$_2$-converting bacteriophages were detected in 30 strains of the 168 STEC strains analysed. The sources and characteristics of these 30 strains are described in Table 1. These 30 corresponded to an incidence of 18 % of the total number of strains analysed, representing 21 % (21/98) of the O157 : H7 strains and 13 % (9/70) of the non-O157 STEC strains. With the exception of O157-379, which was isolated from a sheep, all strains were isolated from cattle. Strains carrying mitomycin-C-inducible *stx*$_2$-converting bacteriophages were detected by the presence of a positive signal in the spot test area after hybridization with the *stx*$_2$*A*-DIG probe. Of all the host strains used, DH5α followed by C600 and DM1187 gave the best detection results of the *stx*$_2$ phages, allowing lysis to be most clearly detected and giving easily discernible signals after hybridization. Spot test results using DH5α as the host strain are shown in Table 1.

Some of the 168 strains studied carried non-*stx*$_2$ temperate bacteriophages, as shown by the lysis area formed on the semisolid agar with the different hosts studied (data not shown). However, these strains did not hybridize with the *stx*$_2$-specific probe and were discarded from subsequent analyses.

In contrast, some of the phages did not produce a clear lysis zone in the semisolid agar after the spot test with any of the hosts studied, although a positive signal was observed after hybridization. Negative controls using plates without host strain demonstrated that the signals, even if weak, were due to the lysis of the host strain produced by the phages. Results obtained with DH5α (Table 1) showed that not all phages gave the same signal intensity after hybridization. Indeed some phages presented a weak signal (Table 1) while others showed a very strong positive hybridization.

All the phage lysates revealing the presence of *stx*$_2$ phages by the spot test were enumerated using the double agar layer method. Enumeration was performed to correlate prophage induction with low optical densities and the production of p.f.u. and to evaluate whether other non-*stx*$_2$ phages could be observed in the double agar layer. Enumeration was hampered by the fact that the plaques were small and not clearly visible on the agar plate. The largest plaques were obtained with strain DH5α and they were easier to visualize after plaque blot hybridization. A comparison of the induced cultures and the controls (not induced) revealed that there was an increase between 3 and 6 $\log_{10}$ p.f.u. ml^{-1} observed after induction. This finding correlates with the low optical densities detected in the induced strains. There was no evidence of plaque formation by non *stx*-phages.

To confirm that the induced prophages were *stx*$_2$ phages, study of the STEC strains, phage DNA isolation and *stx*$_2$ detection were necessary. The OD_{600} values presented in Table 1 are the mean of three independent induction experiments.

Isolation of phage DNA

The concentration of the phage DNA isolated differed from one strain to another (Table 1), although all phages were induced under the same conditions. The concentrations of phage DNA obtained after induction of the cultures were inversely proportional to the optical densities of the cultures. Those strains showing higher optical densities (due to growth of the bacterial culture) produced lower concentrations of phage DNA. In contrast, strains showing lower optical densities after induction, due to phage replication and release, allowed higher concentrations of phage DNA to be isolated (Table 1). Bacterial strains were classified into three groups according to the phage DNA concentration obtained after induction: those producing less than 0·5 μg DNA μl^{-1}, those producing DNA in the range 0·5–3 μg DNA μl^{-1} and those consistently producing more than 3 μg DNA μl^{-1}. These results were reproducible for each strain after several replicates of the induction process.

Detection of the *stx*$_2$ gene in phage and chromosomal DNA

The *stx*$_2$ gene was located in restriction fragments of different length, as revealed by Southern blotting with the specific *stx*$_2$*A* probe (Table 1). The band detected was the same shown by the chromosomal DNA Southern blot after restriction analyses (Table 1). Some strains showed two copies of the gene in the chromosomal DNA although only one of the copies was detected in phage DNA for all the strains analysed.

The restriction patterns of all the phages studied differed markedly, indicating their distinct genetic composition.

The experiments were also performed using *Cla*I as the restriction enzyme, and again two bands were observed in those samples that presented two bands with *Eco*RI. The same reproducibility of results presented with *Eco*RI was also observed with *Cla*I.

The argument that the isolated phage DNA corresponded to a single *stx*$_2$ phage became more cogent as the band size on the Southern blot corresponded to one of the ethidium bromide stained bands. Additionally, restriction analysis of phage DNA was used to calculate whether a second

Table 1. Characterization of stx_2 phages induced from bovine STEC and analysis of the stx_2 gene

Isolate	Serotype	stx_1	OD_{600}*	Spot test†	Hybridization‡	Phage DNA concn (μg μl^{-1})	Morphology	Phage genome size (kb)	Phage DNA Southern (kb)	Chromosomal DNA Southern (kb)
A9	O157 : H7	+	0·30	ND	+/−	0·5–3	933W-like	50·7	5·2	5·2
A75	O157 : H7	+	0·40	+	+	0·5–3	933W-like	66·4	7·1	7·1
A267	O157 : H7	+	0·38	ND	+/−	<0·5	Curved tail	62·7	5·0	5·0
A312	O157 : H7	−	0·20	ND	+	>3	933W-like	56·4	6·0	6·0
A315	O157 : H7	+	0·44	+	+	0·5–3	933W-like	62·2	7·2	7·2
A397	O157 : H7	−	0·25	ND	+	>3	Straight tail	59·7	4·5	4·5
A494	O157 : H7	−	0·48	ND	+/−	<0·5	933W-like	53·4	11·5	11·5
A521	O157 : H7	+	0·40	+	+	0·5–3	933W-like	55·0	3·9	3·9
A531	O157 : H7	−	0·23	ND	+	>3	Curved tail	58·0	12·0	12·0
A534	O157 : H7	+	0·54	ND	+	0·5–3	933W-like	49·5	7·3	7·3
A549	O157 : H7	+	0·36	ND	+	0·5–3	933W-like	61·1	7·2	7·2
A557	O157 : H7	+	0·40	ND	+	0·5–3	933W-like	55·7	7·2	7·2
A565	O157 : H7	−	0·30	ND	+/−	0·5–3	933W-like	59·6	5·4	5·4
A580	O157 : H7	−	0·53	+	+	<0·5	933W-like	61·3	7·8	7·8
A946	O157 : H7	−	0·16	ND	+	>3	933W-like	52·9	12·0	12·0
VTB6	O116 : H–	−	0·43	+	+	0·5–3	933W-like	50·3	18·0	15·0 and 18·0
VTB46	O171 : H2	−	0·43	+	+	0·5–3	933W-like	55·2	7·2	7·2
VTB55	O2 : H27	−	0·48	ND	+/−	0·5–3	933W-like	52·4	4·5	4·5
VTB60	O136 : H–	−	0·49	ND	+/−	<0·5	933W-like	50·6	5·9	5·9 and 12·0
VTB75	O113 : H4	−	0·31	ND	+	0·5–3	933W-like	49·9	5·7	5·7
VTB91	O77 : H41	−	0·23	+	+	0·5–3	Straight tail	58·8	3·8	3·8
VTB178	O8 : H19	−	0·18	ND	+	>3	Long tail	51·2	18·0	18·0
VTB323	O177 : H–	−	0·39	+	+	0·5–3	933W-like	52·2	4·5	4·5
VTB324	O177 : H25	−	0·32	+	+	0·5–3	933W-like	52·5	6·9	6·9
O157-292	O157 : H7	−	0·41	+	+	<0·5	933W-like	54·3	3·2	3·2
O157-310	O157 : H7	−	0·22	+	+	>3	933W-like	50·3	3·9	3·9 and 6·6
O157-330	O157 : H7	−	0·25	+	+	>3	933W-like	55·3	6·1	6·1
O157-379	O157 : H7	−	0·31	+	+	0·5–3	933W-like	56·1	6·3	6·3 and 8·0
O157-469	O157 : H7	−	0·22	+	+	>3	933W-like	48·6	11·5	11·5
O157-572	O157 : H7	−	0·18	+	+	0·5–3	933W-like	52·6	5·9	4·2 and 5·9

*OD_{600} after 14 h of induction of a culture initially showing an OD_{600} of 0·3. The values are the mean of three independent induction experiments.

†ND, Non-detectable lysis in the spot area; +, clear lysis in the spot area. *E. coli* DH5α was used as host strain.

‡+, Strong signal; +/−, weak signal and −, no signal after hybridization of the spot test with the specific stx_2A-probe.

inducible non-stx_2 phage might be present in any of the strains. The sum of the restriction fragments of phage DNA obtained after induction revealed DNA lengths of the different phages ranging from 49 to 66 kb, which is consistent with the genome size of a single lambdoid phage (Franki *et al.*, 1991). Finally, reproducibility of the results obtained in independent experiments of phage DNA isolation and Southern blotting indicated that only one phage was detectable after induction of these cultures.

Evaluation of the ability of the induced stx_2 phages to infect different host strains

The ability of the induced stx_2 phages to infect diverse host strains is shown in Table 2. As described above, all stx_2 phages induced from STEC isolates infected strain DH5α. This strain was used as a reference recording 100 % positive signals after hybridization with the stx_2A probe. Among these, 80 % of the phages gave a strong signal and 20 % a low signal (Table 1). *E. coli* C600 showed 53·3 % strong signals, 33·3 % low signals and 13·3 % no signals. *E. coli* DM1187 showed 60·0 % strong hybridization signals, 20·0 % low signals and 20·0 % no signals. *S. sonnei* was the second most infected strain, with 67·0 % strong hybridization signals, 23·0 % low and only 10·0 % no signals. Finally, *E. coli* O157 : H7 ATCC 43888 was the least infected strain, with only 36·7 % strong hybridization signals, all of which corresponded to phages isolated from O157 : H7 strains, 16·7 % low signals and 46·7 % no signals (Table 2).

No signals were observed for the membrane used as negative control, transferred from a spot test plate containing no host strain (see Methods).

Table 2. Infectivity of the stx_2 phages in different host bacteria

++, Strong signal; +, low signal; −, no signal after hybridization of the spot test with the specific stx_2A-probe.

Code	Infectivity in:			
	E. coli C600	*E. coli* DM1187	*S. sonnei* 866	*E. coli* O157:H7
A9	++	+	−	−
A75	+	+	++	−
A267	+	−	++	++
A312	++	++	+	+
A315	+	+	++	+
A397	++	++	+	++
A494	−	−	−	−
A521	−	−	++	+
A531	++	++	+	++
A534	+	++	+	−
A549	++	++	++	++
A557	+	+	++	+
A565	+	−	++	−
A580	+	+	++	+
A946	++	++	++	++
VTB6	+	−	+	−
VTB46	++	++	++	−
VTB55	−	−	+	−
VTB60	−	+	−	−
VTB75	+	++	++	−
VTB91	++	++	++	−
VTB178	+	++	+	−
VTB323	++	++	++	−
VTB324	++	++	++	−
O157-292	++	++	++	++
O157-310	++	++	++	++
O157-330	++	++	++	++
O157-379	++	++	++	++
O157-469	++	++	++	++
O157-572	++	++	++	++

Construction of lysogens

We were successful in producing stable lysogens from six of the 30 phages studied. Four phages generated lysogens of DH5α, while the remaining two lysogens were obtained by infecting *S. sonnei* strain 866. Stability of the lysogens to the presence of stx_2 phage was achieved after three subcultivation steps of selected stx_2-positive colonies.

Induction of the stx_2 phage from the six lysogens showed different kinetics (Fig. 1). Some phages showed a high induction from their lysogens (VTB55 and A75), others showed a more reduced induction (315 or 534), while phages 312 and 549 were poorly induced from the host. Interestingly, phages 312 and 549 lysogenized *S. sonnei* but not *E. coli*. Note that phages induced from lysogens VTB55 and A75 seemed to release spontaneously from the host since the strain did not reach an OD_{600} of 1·0 after 18 h incubation with or without mitomycin C treatment. Confirmation that this lack of growth was due to phage release was obtained by the high amount of phage DNA obtained from these two lysogens (more than 1 μg μl^{-1}). In contrast, the concentration of phage DNA obtained from lysogens 866(312) and 866(549) was significantly lower (less than 0·5 μg μl^{-1}). Kinetics of induction of the lysogens showed marked differences from with those of the phages harboured in the original STEC strains (Fig. 1). STEC strains carrying phages VTB55 and A75 produced low phage amounts after induction and the strain did not show spontaneous phage release (OD_{600} greater than 1 without mitomycin C treatment after 18 h). Kinetics for STEC strains A315 and A534 appeared similar to those presented by its lysogens, although phage release was higher. Finally, STEC strains A312 and A549 were highly induced by mitomycin C treatment, whereas, the *S. sonnei* lysogens obtained with these two phages were not.

Phage DNA isolated from the lysogens DH5α(A75), DH5α(A315), DH5α(534), DH5α(VTB55), S866(312) and

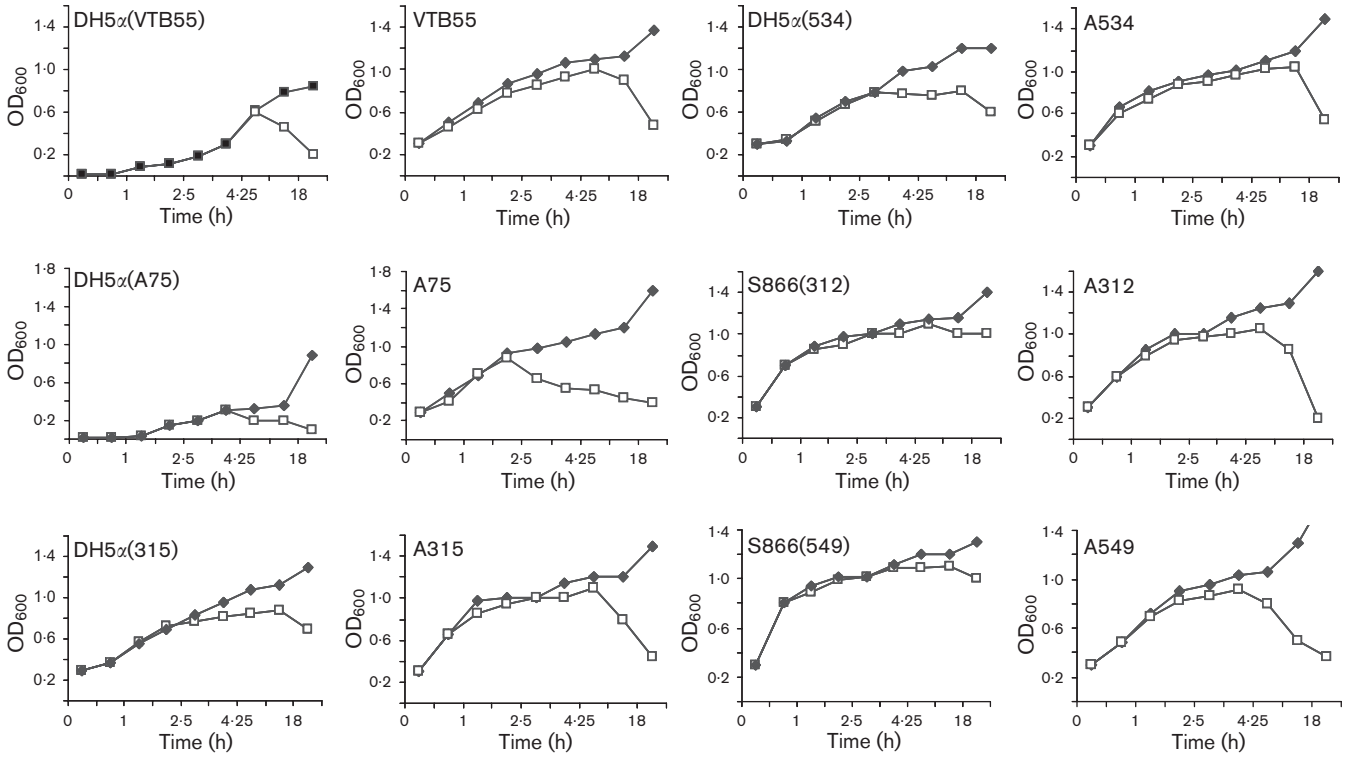

Fig. 1. Kinetics of bacteriophage induction from the lysogens DH5α(VTB55), DH5α(A75), DH5α(A315), DH5α(534), S866(312) and S866(549) and corresponding STEC isolates VTB55, A75, A315, A534, A312 and A549 with (□) and without (■) mitomycin C treatment. Each culture was grown to an OD_{600} of 0·3 and then separated into two aliquots, one of which was induced with mitomycin C; from this point on the kinetics with mitomycin C can be observed in the figure.

S866(549) was restricted with *Eco*RI. No differences were found in their restriction patterns compared with those of the phages induced from the STEC strains. The restricted DNA that was transferred to a nylon membrane and hybridized with the specific *stx*$_2$*A* probe showed a band containing the *stx*$_2$ gene placed in the same location as that in phage and bacterial DNA and it also corresponded to the band observed for the wild phage (Table 1). These results indicate that the same phage was analysed from the wild STEC strains as from the lysogen.

Various attempts to transduce the rest of the phages in any of the host strains proved unsuccessful.

Evaluation of toxin production

Although the method employed here did not allow us to evaluate the toxin concentration in toxin units, the absorbance unit measurements enabled us to compare the toxin produced by the same culture with or without mitomycin C treatment and, furthermore, to identify differences in toxin production between the isolates. The first group of strains (23 isolates from 30) produced an increase in the amount of Stx_2 with mitomycin C, indicating a direct relationship between phage induction and toxin production.

A second group of strains (7 of 30, including strains A9, A267, A549, A557, VTB6, VTB60 and O157-379) showed no variation in toxin production after induction. However, it should be noted that all seven strains possess a second copy of either the *stx*$_2$ or the *stx*$_1$ gene. Since the kit used is also able to detect Stx_1 production, this phage-independent toxin production may have had an influence on the results.

The six lysogens were also analysed for toxin production. All of them showed production of Stx_2 after lysogenization, although the exact proportion varied from one lysogen to another, as it did also with that of the wild STEC strain. The most significant differences were observed with lysogens S866(312) and DH5α(A534), which presented a lower rate of toxin production than that of the STEC hosts. No significant differences were observed among the lysogens when comparing the relative toxin production with or without mitomycin C treatment, although production increased in most cases after treatment, with the exception of lysogens 866(312) and 886(549), where no difference was recorded.

Sequencing of the *stx*$_2$ gene encoded by temperate phages

The nucleotide sequence of the toxin gene indicated that most of the phages carried the *stx*$_2$ gene, and only one *stx*$_2$ variant was detected in this set of phages. Of the 30 phages,

nine carried an stx_2 gene presenting 100 % identity with the sequence of the stx_2 gene carried by phage 933W, GenBank accession number AF125520 (Plunkett *et al.*, 1999). These phages were isolated from strains A9, A267, A494, A521, A531, A565, A946, VTB6 and VTB55. The remainder (21 phages) showed some differences in sequence compared with sequences available in the gene databases for the stx_2 gene of phage 933W. Of these 21 stx_2 gene sequences, some were identical to each other and presented different degrees of identity with the toxin gene of phage 933W (Table 3). The phages showing 100 % stx_2 sequence identity are grouped together in Table 3. Only one of each group was selected for comparison of amino acid sequences (Fig. 2). Sequences from phages induced from STEC strains VTB46, VTB60, A397 and O157-469 were different from other sequences and were considered independently (Table 3). No apparent relationships between these strains in terms of their origin or serotype explained the similarities found between their stx_2 gene sequences.

When compared with the sequence of the stx_2 gene carried by phage 933W (AF125520), the genetic identity of the stx_2 gene among the strains ranged between 97·7 and 99·8 % nucleotide identity (Table 3). The sequences of the stx_2 gene from two phages were more similar to 933W, whereas the gene from five phages showed higher identity with a gene encoding Shiga-like toxin type IIvhc, accession number X61283 (Meyer *et al.*, 1992), currently known as stx_{2c} (WHO, 1991). The remainder showed very high identity with the gene sequence of phage Ni12 (El-Sayed, 2002), induced from an *E. coli* strain isolated from a bovine source.

Comparison with the amino acid sequence for subunits A and B of Stx_2 encoded by phage 933W (Fig. 2) confirmed the results obtained by genetic comparison.

Electron microscopy

Observation of the induced phages by electron microscopy revealed various morphologies (Table 1). Most of the strains had phages with an isometric capsid of approximately 55 nm diameter and a short tail (mostly from 10 nm, phage A75 with a longer tail of 30 nm). These phages are referred to here as 933W-like (Fig. 3a). At least three other phage morphologies were identified. Some strains presented a *Siphoviridae* phage with a long tail of around 120 nm and an isometric capsid of 59 nm (Fig. 3b). Another type was a *Siphoviridae* phage, with a thick straight tail of around 115 nm and an isometric capsid of 65 nm (Fig. 3c). Finally, there was a *Siphoviridae* phage with a curved tail and a capsid of 55 nm diameter (Fig. 3d).

Phages obtained from the seven lysogens – DH5α(A534), DH5α(A9), DH5α(A75), DH5α(A315), C600 (VTB55), S866(A549) and S866(A312) – were also observed by electron microscopy to confirm that the same phage was transduced. The morphology of these phages was similar to that observed for phages induced from the STEC strain, with the exception of lysogen 886(312), which presented low induction and was not isolated in the concentration required for observation by electron microscopy (more than 10^8 p.f.u. ml^{-1}).

DISCUSSION

Eighteen per cent of the strains analysed were identified as carrying a detectable and infectious stx_2 phage, which can be considered as a relatively high prevalence in STEC strains isolated from animals. Moreover, phages carrying one of the stx_2 variants which have also been described in phages induced from strains of animal origin (Muniesa *et al.*, 2000; Schmidt *et al.*, 2000; Koch *et al.*, 2001) were also detected. However, the results may in fact underestimate the real number of inducible *stx*-phages, as we have only been able to consider those phages detectable by the methods used here or those that were able to infect the host strains used. Thus, there may well be others which escaped detection.

The stx_2 phages detected in this study showed quite a high degree of heterogeneity in terms of induction levels, host infectivity range, and the amount of DNA isolated and its characteristics. However, in studying this variability, the possibility that a converting phage other than the stx_2 phage was present in the strains could only be completely ruled out

Table 3. Sequence comparison of stx_2 genes that differed from stx_2 of phage 933W

Strain	Identity to stx_2 gene of 933W (AF125520) (%)	Identity to other stx_2 sequences* (%)
A75, A534, A557, O157-572	97·7	99·1 Phage Ni12 (AJ413274)
VTB75, VTB91, VTB178, VTB324	98·9	99·5 *stx* II*vhc* (X61283)
A312, A315, A549, A580, O157-292, O157-330,	98·3	100·0 Phage Ni12 (AJ413274)
O157-310, O157-379, VTB323	97·7	99·4 Phage Ni12 (AJ413274)
VTB46	99·4	99·4 *stx* II*vhc* (X61283)
VTB60	99·8	–
A397	99·5	–
O157-469	98·3	99·8 Phage Ni12 (AJ413274)

*When the identity to other sequences is higher than identity to stx_2 of phage 933W.

A subunit

```
                 *        20         *        40         *        60         *        80
933W     : MKCILFKWVLCLLLGFSSVSYSREFTIDFSTQQSYVSSLNSIRTEISTPLEHISQGTTSVSVINHTPPGSYFAVDIRGLD :80
VTB60     : ................................................................................ :80
A397      : ..................S............................................................. :80
VTB324    : ................................................................................ :80
VTB46     : ................................................................................ :80
A312      : ................................................................................ :80
O157-310: ...............F.................................................................. :80
O157-469: ..................S............................................................... :80
O157-572: ...............F...S.............................................................. :80

                 *       260         *       280         *       300         *       319
933W     : RVGRISFNNISAILGTVAVILNCHHQGARSVRAVNEESQPECQITGDRPVIKINNTLWESNTAAAFLNRKSQFLYTTGK  :319
VTB60     : .....................................D.......................................... :319 1 (99.7)
A397      : ................................................................................ :319 1 (99.7)
VTB324    : ..............................................................S.....E. :319 2 (99.4)
VTB46     : .....................................D.......................................... :319 1 (99.7)
A312      : .....................................D.......................................... :319 1 (99.7)
O157-310: .....................................D.......................................... :319 2 (99.4)
O157-469: .....................................D.......................................... :319 2 (99.4)
O157-572: .....................................D.......................................... :319 3 (99.0)
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B subunit

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                *        20         *        40         *        60         *        80...
933W     : MKKMFMAVLFALASVNAMAADCAKGKIEFSKYNEDDTFTVKVDGKEYWTSRWNLQPLLQSAQLTGMTVTIKSSTCESGSGFAEVQFNND:89
VTB60     : ................................................................................:89 0(100.0)
A397      : ........................................A.......................................:89 1 (98.9)
VTB324    : ..........V.......................N.......A.....................................:89 3 (96.6)
VTB46     : ..........V.......................N.......A.....................................:89 3 (96.6)
A312      : ..........V.......................N.......A.....................................:89 3 (96.6)
O157-310: ..........V.......................N.......A.....................................:89 3 (96.6)
O157-469: ..........V.......................N.......A.....................................:89 3 (96.6)
O157-572: ..........V.......................N.......A.....................................:89 3 (96.6)
```

Fig. 2. Comparison of the predicted amino acid sequences of Stx_2A and Stx_2B subunits encoded by *stx*$_2$ phages to Stx_2 harboured by phage 933W, subunit A (Entrez accession no.: 9632506) and B (9632507). This figure shows the comparison of residues 1–80 and 240–319 for subunit A and the complete subunit B (89 residues), where differences in the sequence were found. Bold letters in the Stx_2 sequence of phage 933W indicate amino acid residues in which this sequence differs from those of any of the other strains. The differences in the sequence of each strain are specified by letters indicating corresponding amino acid residues; positions containing amino acid residues which are the same in the Stx_2 sequence of the strains with the Stx_2 of 933W are indicated by dots. In the right-hand column, the number of amino acid residues by which the Stx_2A and Stx_2B sequences of each phage differ from that of 933W, and the corresponding percentage of identity (in parentheses) are given.

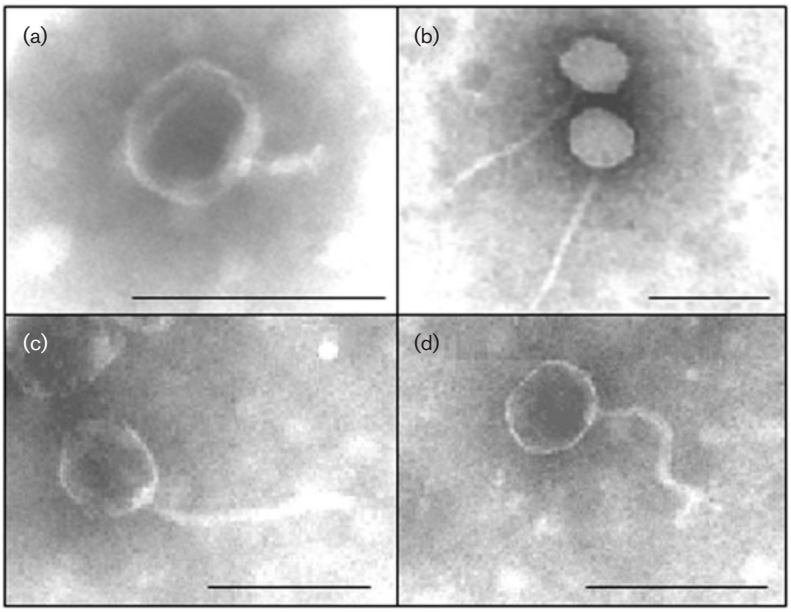

Fig. 3. Electron micrographs of the four main types of bacteriophages detected. (a) Phage 933W-like. (b) *Siphoviridae* phage with a long tail. (c) *Siphoviridae* phage with a thick straight tail. (d) Phage with curved tail. Bars, 100 nm.

in the six lysogens and not in the others. However, for these other strains, several factors indicated the presence of a single inducible stx_2 phage, including the observation of plaques of lysis, the reproducibility of the Southern blot analysis, and the sum of the molecular masses of the phage DNA restriction fragments that points to a DNA molecular mass consistent with a single lambdoid bacteriophage.

Most of the stx_2 phages studied showed a similar morphology to the 933W phage, with an icosahedric capsid and a short tail. The morphology of the phages produced from the lysogens confirmed that they were of the same type as those induced from the original STEC isolates. Only the 933W-like phages were transduced, although this was due to the fact that perhaps more than 83 % of all the phages described here belonged to this morphology. *Siphoviridae* with longer tails were also observed. An stx_2 phage with this morphology has previously been isolated from sewage (Muniesa *et al.*, 2004). With the exception of phage A75, which showed a slightly longer tail than that of the typical 933W morphology, various attempts at transducing the phages with *Siphoviridae* morphology proved unsuccessful. However, from our results it can be stated that the most inducible (or the most abundant) phage present in the supernatant of the induced cultures was observed.

When analysing the phage DNA, the band containing the stx_2 gene was found to belong to the DNA from the most inducible or the most abundant stx_2 phage. This was also the case for those strains presenting more than two copies of the stx_2 gene when their chromosomal DNA was analysed. Since the restriction enzyme employed did not cut the stx_2 gene and the two copies were also observed when using a different restriction enzyme, it can be assumed that each copy, located in a different-sized fragment, was inserted in the genome of a different prophage. However, only one of these fragments corresponded to the fragment detected in the phage DNA, indicating that either the second copy is chromosomal, or it is harboured in the genome of a second prophage that could not be induced, or if induced could not be detected.

The infectivity of different host bacteria must also be considered. The infectivity of stx_2 phages on other host strains has been demonstrated in *E. coli* laboratory strains such as C600 and DH5α (Muniesa & Jofre, 1998; James *et al.*, 2001; Strauch *et al.*, 2001; Tanji *et al.*, 2003) and is of considerable importance given the role played by stx_2 phages in gene transfer between host strains and in the emergence of new Shiga-toxin-producing strains. The strains used in this study were selected because they were sensitive to a wide variety of phages (Muniesa *et al.*, 2003) and our results indicate that these phages could have a diverse host spectrum. The use of DM1187 demonstrates its suitability as a host strain for detecting lytic phages, because this *recA441* mutant produces RecA protease constitutively, thus forcing the phage into the lytic cycle (James *et al.*, 2001). However, not all the phages showed clear lysis plaques even with this strain. It is well known that most lysogenic phages in general, and stx-phages that have been previously described in particular, produce poorly visible or turbid plaques onto the top agar monolayer although infection is produced (Muniesa *et al.*, 2000; Teel *et al.*, 2002). For this reason confirmation of the presence of infective phages is more accurately achieved by hybridization. Phage infection of the different host strains was determined by positive hybridization. Phage infection of *S. sonnei* is noteworthy, as several authors have suggested that *Shigella* might be the origin of the dissemination of *stx* genes (Strauch *et al.*, 2001; Unkmeir & Schmidt, 2000). Strain 866 of *S. sonnei* was also a suitable candidate for phage transduction, since two of the six phage lysogens were preferentially obtained with *S. sonnei* and we obtained a high number of lysogens with these two phages (data not shown). Our results are consistent with previous studies indicating that *Shigella* is a susceptible host for stx_2 phages isolated from sewage (Muniesa *et al.*, 2004). Finally, the low infectivity obtained with *E. coli* O157 ATCC 43888 was expected since previous studies have indicated that ATCC 43888 is infected by fewer phages than other commonly used laboratory strains, and the number of phages producing plaques in this strain was lower than for an *E. coli* C strain (Muniesa & Jofre, 1998, 2000). The results regarding enumeration and transduction of strain O157 : H7 ATCC 43888 with stx_2 phages purified from water (Muniesa & Jofre, 1998; Tanji *et al.*, 2003), together with those reported here, indicate that this strain supports the infection of stx_2 phages.

The degree of difficulty with which lysogens were obtained from the different strains indicates that not all the phages can be transduced in the same host strain and with the same level of effectiveness. Lysogeny is dependent on the frequency of transduction, which is not the same for all the phages and, additionally, we did not use any pressure to select lysogens, as has been done by others (Schmidt *et al.*, 1999). The transduction of these six phages could indicate that they have some differential characteristic that facilitates transduction and that other phages lack this ability.

Indeed, all isolated phages that produced lysogens in our laboratory were induced from STEC strains at a medium to high level, whereas the other phages were induced from the STEC strains at high levels and did not generate lysogens even after several attempts. All stx_2 phages were detectable by hybridization, though not always directly in the spot test. It has been speculated that host- or bacteriophage-encoded factors might influence phage production and the stability of lysogeny (Wagner *et al.*, 1999) and the variability of such factors might explain the variance reported here. This observation also correlates with the fact that two of the lysogens, 866(312) and 866(549), obtained with *S. sonnei*, presented a number of trends in common, namely the low phage release and the low inducibility following treatment with mitomycin C. In contrast, phages VTB55 and A75 showed a high spontaneous phage release and a high inducibility after mitomycin C treatment. Spontaneous induction of *stx*-encoding phages has been correlated with

variations in cellular physiology (RecA protein) (Livny & Friedman, 2004). The differences observed between the lysogens obtained with *S. sonnei* and DH5α could be due to the different host strains and to the fact that DH5α is a *recA* strain, implying a role for *recA* in prophage induction. Although DH5α could be considered as a bad choice as a host to generate lysogens, the best results were still obtained with this strain, and indicate that some other factors are involved. Spontaneous phage induction was not observed in the STEC strains, or in very low amounts which would have had a direct impact on toxin production. STEC strains produced more toxin after induction, but in general, all the lysogens produced toxin in a constitutive manner. Toxin production independent of induction could also be due to spontaneous phage release. Transduction of phage and the increase in toxin production after transduction are again indicators of the potential risk of the stx_2 phages in the emergence of new pathogens. The finding that *S. sonnei* strains transduced with stx_2 phages were able to release low amounts of phage and produced toxin without previous induction merits further study.

Sequencing of the toxin gene also indicated variability in the gene, although these small changes might represent genetic drift. Comparison of the stx_2 sequences showed that the phages can be distributed into three main groups: those which are identical to the stx_2 gene of phage 933W in accordance with previous results; those presenting similarities to stx_{2c}, which has been reported to occur in both human and animal samples (Eklund *et al.*, 2002; Heuvelink *et al.*, 1998); and those with homology to the toxin gene present in phage Ni12 isolated from beef (El-Sayed, 2002). Previous studies (Brett *et al.*, 2003) indicated the prevalence of EDL933 Shiga toxin gene and/or stx2vhb subtypes in bovine isolates. Our results suggest that O157 and some other serotypes from cattle also possess other gene variants, although this study was not focused on specific serotypes and they were not selected for this work, so no specific relationship could be established.

The stx_2 phages showed wide genetic and morphological variation and the extent to which they infect hosts was also variable. Thus they should not be considered as a homogeneous group. This variability may affect the emergence of STEC at two levels. Firstly, it increases the range of host strains that would be susceptible to infection by *stx*-phages, consequently increasing the spread of *stx* genes among bacterial communities. Secondly, it increases the possibility of multiple infections by different phages, as observed in those STEC strains carrying two or more *stx*-phages (Livny & Friedman, 2004; Teel *et al.*, 2002), since the differences between phages do not cause phage immunity. The presence of two or more phages within a bacterial genome facilitates recombination events and could alter the expression of certain genes.

Globalization, sanitation infrastructures, contemporary animal husbandry practices and animal transport increase the possibility of contact between micro-organisms of different origins, thus raising the probability of multiple infections and consequently the spread of the *stx* genes.

The significance of this variability of the *stx*-phages on the origin of STEC should be considered with caution. According to the well-accepted theory of a common bacterial ancestor carrying the stx_2 gene, all stx_2 phages might share a common origin (Strauch *et al.*, 2001; Unkmeir & Schmidt, 2000). However, in the case of the stx_2 gene, the presence of multiple variants argues against a single origin. The general structure of the *stx*-phages is also in conflict with this hypothesis. Theoretically, phage variability could be explained if an original bacterium, susceptible to infection by different phages, was the origin of the gene. These different phages could all have incorporated the gene into their genome. *Shigella* would be a good candidate under this hypothesis, since previous studies have demonstrated that there are many examples of phages that can infect *S. sonnei* and *E. coli* (Muniesa *et al.*, 2003), and cross-infection by phages from *E. coli* to *S. sonnei* has been demonstrated in this study. However there is no conclusive evidence that *Shigella*, other bacteria or even a bacteriophage are the origin of STEC.

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