Mosaic structure of Shiga-toxin-2-encoding phages isolated from *Escherichia coli* O157:H7 indicates frequent gene exchange between lambdoid phage genomes

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Shiga-toxin-2 (*stx*₂)-encoding bacteriophages were isolated from Norwegian *Escherichia coli* O157:H7 isolates of cattle and human origin. The phages were characterized by restriction enzyme analysis, hybridization with probes for toxin genes and selected phage DNA such as the *P* gene, integrase gene and IS1203, and by PCR studies and partial sequencing of selected DNA regions in the integrase to *stx*₂ region of the phages. The *stx*₂-phage-containing bacteria from which inducible phages were detected produced similar amounts of toxin, as shown by a Vero cell assay. The results indicate that the population of *stx*₂-carrying phages is heterogeneous, although the phages from epidemiologically linked *E. coli* O157:H7 isolates were similar. There appears to have been frequent recombination of *stx*₂ phages with other lambdoid phages.

Keywords: Stx2-encoding phages, STEC, VTEC

INTRODUCTION

Shiga-toxin-producing *Escherichia coli* (STEC) are potential human pathogens, which are transmitted via food and water from animal reservoirs. STEC O157:H7 is the prototype of the human pathogenic variants, but other STEC belonging to the serogroups O26, O111, O45 and O103 are regarded as emerging in Europe (World Health Organization, 1999). Many STEC serogroups have not yet been associated with disease in humans.

The Shiga toxins (Stxs) are the main virulence factors in the disease progression of haemorrhagic colitis and haemolytic uraemic syndrome caused by STEC O157:H7 and other STEC. The toxin-encoding genes (*stx*) are located on lysogenic lambdoid double-stranded DNA phages, which form a heterogeneous family of *stx* converting phages (O'Brien *et al.*, 1984). Three of the *stx*₂ phages (933W, VT2-Sa and VT2-Sakai) have so far been completely sequenced (Plunkett *et al.*, 1999; Makino *et al.*, 1999; Miyamoto *et al.*, 1999). VT2-Sa and VT2-Sakai are prophages from different *E. coli*

O157:H7 isolates from the Sakai outbreak in Japan, and are very similar. The only major difference is an IS629 insertion in VT2-Sakai. The sequences of these two phages showed considerable variation in the regions encoding replication and early regulation functions as compared to 933W. λ phages are typically mosaic in structure, and it is proposed that they share a common gene pool from which DNA can be frequently exchanged (Muniesa *et al.*, 2000; Hendrix *et al.*, 2000). From the complete genome sequence of the *E. coli* O157:H7 type strain EDL933, 18 multigenic regions related to known bacteriophages were identified (Perna *et al.*, 2001). Only the *stx*₂ phage 933W appeared to be a fully functional phage, while the other λ -like sequences showed a mosaic structure, indicating extensive exchange of genes. Another STEC O157:H7 isolate that is currently being sequenced also has many phage-like sequences in its genome (Yokoyama *et al.*, 2000), and a similar phenomenon has also been observed in *Shigella dysenteriae* (McDonough & Buttermont, 1999). The general position of the *stx* genes in Stx-encoding phages appears to be conserved, as they are located between the genes encoding the transcription antiterminator Q protein and the holin S gene of the lysis cassette (Plunkett *et al.*, 1999; Unkmeir & Schmidt, 2000). Upstream from Q is the gene encoding the cI repressor, which determines immunity, and the genes for the replication proteins O

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

and P. It is assumed that this organization is necessary for controlled expression and release of the toxins together with the infectious phage particles (Neely & Friedman, 1998; Plunkett *et al.*, 1999; Muniesa *et al.*, 2000). The *stx* phages should therefore not only be viewed as passive vectors for the dissemination of *stx*, but as genetic entities where the characteristics of the phage itself influence toxin production and thus virulence of the host bacteria (Wagner *et al.*, 1999).

Pulsed field gels of the Norwegian STEC isolates used in this study have previously been blotted and hybridized with *stx* phage gene probes (Johansen *et al.*, 2000). The results showed that the bacteria tested contained a single copy of *stx*₂, that the *P* gene homologous to that from the phage 933W was present in one to three copies and was associated with *stx*₁ but not with *stx*₂, and that there were 12 or more copies of IS1203-like elements in the bacterial genomes. The findings indicated that the *stx*₂ phages in these isolates were not identical to the published sequences of *stx*₂ phages, and that the phages could play a role in the genome changes observed in the PFGE types. We were interested in examining the diversity and genetic composition of the *stx* phages in these bacteria, and in determining the possible association between the phage type and the toxicity of the host bacteria.

METHODS

Bacterial isolates. The isolates used for phage preparation in this study are presented in Table 1. The five bovine and two human isolates were previously described by Vold *et al.* (1998, 2001) and Heir *et al.* (2000), respectively. The isolates were initially checked by PCR for the presence of *stx*₁ and *stx*₂, IS1203 and the *P* gene from 933W. Only one of the bovine isolates tested positive for *stx*₁ (C21b), although two of the other isolates (H82 and H89) had tested positive when initially isolated. All the isolates were positive for *stx*₂, IS1203-like DNA and the *P* gene. Cultures of *E. coli* O157:H7 EDL933 were obtained from H. Schmidt, University of Würzburg, Germany (designated EDL933HS), and the Culture Collection,

University of Göteborg, Sweden (CCUG 29197B, designated EDL933). EDL933 was used as control strain and to obtain 933W phages, and the EDL933HS strain was used to obtain ϕ 933HS-A phages (see below). *E. coli* DH5 α (Sambrook *et al.*, 1989) was used as an indicator strain for plaque assay.

Isolation of phages and preparation of phage DNA. Phages were induced from the *E. coli* isolates as described by Schmidt *et al.* (1999). Briefly, 2 μ g mitomycin C (Sigma) was added to a 4 ml exponential-phase culture (OD₆₀₀ 0.5) in Tryptic Soy Broth (Difco) containing 5 mM CaCl₂, and the cultivation was continued overnight at 37 °C. Bacterial cells were pelleted at 5000 g for 10 min, and four to five drops of chloroform were added to the supernatants, which subsequently were kept at room temperature for 15–20 min. Ten to one hundred microlitres of supernatant, 100 μ l of an overnight culture of *E. coli* DH5 α and 125 μ l 0.1 M CaCl₂ were incubated for 20 min at 37 °C, mixed with 3 ml LB soft agar (0.5%) and poured onto LB agar plates (2%). Plaques were observed after overnight incubation at 37 °C. One plate for each strain was blotted to Colony/Plaque Screen Hybridization Transfer Membrane (NEN Life Science Products) as described by the manufacturer. The membranes were hybridized with *stx*₁- and *stx*₂-specific probes to confirm the presence of *stx* phages and to estimate the percentage of *stx*-containing phages as compared to the total number of induced phages (data not shown), using the ECL direct nucleic acid labelling and detection system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The following primers were used to generate the probes: *stx*₁-1 and *stx*₁-3 for the *stx*₁ probe, and *stx*₂-1 and *stx*₂-2 for the *stx*₂ probe (Table 2). Three plaques were picked from each positive culture and resuspended in 1 ml 75 mM MgCl₂. The phages were designated by ' ϕ ' in front, and 'A', 'B' or 'C' following, the bacterial isolate number. An aliquot of 0.75–100 μ l of the plaque solutions was plated out a minimum of five times to ensure single plaques. Stocks of bacteriophages were prepared according to the method described by Sambrook *et al.* (1989). Phage DNA was isolated with the Qiagen Lambda System.

RFLP of phage DNA. Phage DNA was digested overnight with the restriction endonucleases *Eco*RI, *Ava*I and *Pst*I according to the manufacturer's recommendations (Boehringer Mannheim). The fragments were separated by gel electrophoresis in a 0.8% agarose gel (SeaKem GTG; FMC BioProducts) overnight, and visualized by ethidium bromide staining.

Table 1. Summary of *E. coli* isolates used in this study

Isolates	Serotype	<i>stx</i> genotype	Source	PFGE type	Reference
H82	O157:H7	<i>stx</i> ₂	Cattle, herd 1	a	Vold <i>et al.</i> (2001)
H89	O157:H7	<i>stx</i> ₂	Cattle, herd 1	a	Vold <i>et al.</i> (2001)
497	O157:H7	<i>stx</i> ₂	Cattle, herd 2	c	Vold <i>et al.</i> (2001)
498	O157:H7	<i>stx</i> ₂	Cattle, herd 2	c	Vold <i>et al.</i> (2001)
C21b	O157:H7	<i>stx</i> ₁ <i>stx</i> ₂	Cattle, herd 3	b	Vold <i>et al.</i> (2001)
1480	O157:H7	<i>stx</i> ₂	Sick child	P24	Heir <i>et al.</i> (2000)
1607	O157:H7	<i>stx</i> ₂	Healthy mother	P25	Heir <i>et al.</i> (2000)
EDL933HS	O157:H7	<i>stx</i> ₁ <i>stx</i> ₂	Human	ND	Unkmeir & Schmidt (2000)
EDL933	O157:H7	<i>stx</i> ₁ <i>stx</i> ₂	Human	ND	CCUG 29197B
DH5 α			Laboratory strain	ND	Sambrook <i>et al.</i> (1989)

* ND, Not determined.

Table 2. Oligonucleotide sequences of primers used in this study

Primer	Oligonucleotide sequence	Gene/DNA*	Reference
<i>stx</i> ₁ -1	5'-CAGTTAATGTGGTGGCGAAG-3'	<i>stx</i> ₁	Olsvik & Strockbine (1993)
<i>stx</i> ₁ -3	5'-CTGCTAATAGTTCTGCGCATC-3'	<i>stx</i> ₁	Olsvik & Strockbine (1993)
<i>stx</i> ₂ -1	5'-CTTCGGTATCCTATTCCCGG-3'	<i>stx</i> ₂	Olsvik & Strockbine (1993)
<i>stx</i> ₂ -2	5'-GGATGCATCTCTGGTCATTG-3'	<i>stx</i> ₂	Olsvik & Strockbine (1993)
IS1	5'-GCCATGAGACGTGCCACTGT-3'	Integrase, IS1203	This study
IS2	5'-TGCGTGTCTGGGTTCGCCAG-3'	Integrase, IS1203	This study
DK1	5'-ACATCGCCGCACAGATGG-3'	<i>P</i>	Datz <i>et al.</i> (1996)
DK2	5'-GCTTCCGGCAATACTCGT-3'	<i>P</i>	Datz <i>et al.</i> (1996)
SsbF	5'-GCTCCGTGTATTCACTCGTT-3'	After <i>ssb</i> , λ -group	This study
Nrev	5'-TTACGCGGCAGGCTACCGGAAAT-3'	<i>N</i> , λ -group	This study
APR	5'-GCTCAGTGATGTAGATGGTC-3'	After <i>P</i> , λ -group	This study
APF	5'-GACCATCTACATCACTGAGC-3'	After <i>P</i> , λ -group	This study
MPQF	5'-CTGCAAATACCTTCCAACGA-3'	Between <i>P</i> and <i>Q</i> , λ -group	This study
MPQR	5'-TCGTTGGAAGGTATTTGCAG-3'	Between <i>P</i> and <i>Q</i> , λ -group	This study
HK97OEF	5'-GCTGAGAGAAGACTCGTTAA-3'	<i>O</i> , HK97	This study
HK97OF	5'-AGCAGCCATGACAGGTCTTC-3'	<i>O</i> , HK97	This study
HK97PR	5'-TGCAAGCTGACTCATGCCGT-3'	<i>P</i> , HK97	This study
PV2F	5'-GCCGATGGATGTTTGAAGAG-3'	<i>P</i> , VT2-Sa	This study
PV2R	5'-GTAAACACCTGACTTCTGTCC-3'	<i>P</i> , VT2-Sa	This study
WbrF	5'-ACAATCACCATGCCGTGATG-3'	<i>wbr</i> , EDL933	This study
WbrR	5'-GCTTTATTATTCCATGTACG-3'	<i>wbr</i> , EDL933	This study
CIIF	5'-TGGCATCAGACACATCAGTC-3'	<i>cII</i> , λ -group	This study
933intF	5'-CCTGGTCACTTTGGGGCACGT-3'	Integrase, 933W	This study
933intR	5'-GCTCCATCGGAGCGACATGG-3'	Integrase, 933W	This study
UinfF	5'-TTATCGCCGAGAAGACGTTGAGCA-3'	Integrase, λ -group	This study
UinfR	5'-AAGAAGGCGAAGTCATGAGCGCCG-3'	Integrase, λ -group	This study
CIWF	5'-GCCACTCCCTTGCTCGATG-3'	<i>cI</i> , 933W/H19	This study
CIWR	5'-GGTTAGTGC GCGTGGTTGGT-3'	<i>cI</i> , 933W/H19	This study
CIVTF	5'-CACAACGGAACAACCTCTCAT-3'	<i>cI</i> , VT2-Sa/HK97/ λ	This study
CIVTR	5'-CTCATGTTTCAGGCTGGGATGT-3'	<i>cI</i> , VT2-Sa/HK97/ λ	This study
CIV2R	5'-ATACCTTGGTATTGATAACA-3'	<i>cI</i> , VT2-Sa	This study
QR	5'-CGCTCAACATACCGATCCATC-3'	<i>Q</i>	This study
QF	5'-CGTGATATCCGGCAGGTTCT-3'	<i>Q</i>	This study
ileZ	5'-GGTGAGACCAGCGACTCATA-3'	tRNA	This study

* λ -Group, conserved DNA from λ , 933W, VT2-Sa, HK97, HK022 and H-19B phages.

Southern blot hybridization of RFLP fragments. DNA fragments from the RFLP agarose gels were transferred to nylon membranes (Hybond N+; Amersham Pharmacia Biotech) according to the manufacturer's instructions. The localization to RFLP fragments of the genes for *stx*₂, IS1203-like DNA and λ integrase (*int*) was determined by hybridization using the ECL direct nucleic acid labelling and detection system (Amersham Pharmacia Biotech) according to the manufacturer's instructions with labelled PCR products. The following primers were used to generate the probes: *stx*₂-1 and *stx*₂-2 for the *stx*₂ probe, IS1 and IS2 for the IS probe and UinfF and UinfR for the λ -*int* probe (Table 2).

PCR. Using a BLAST search (Altschul *et al.*, 1990; <http://www.ncbi.nlm.nih.gov/BLAST/>), regions that are conserved in the genome of as many as possible of the λ -group of phages were determined. Primers were designed so that these conserved regions could be utilized to amplify the regions specific to *stx*₁, *stx*₂, IS1203-like DNA, different *int*, and genome sections of replicative and regulatory genes (*O/P*, *cI* and *Q*) of the individual bacteriophages (Table 2). The presence of these

genes in the host strains and in the isolated phages was determined by PCR carried out in a Minicycler (MJ Research) or Mastercycler gradient (Eppendorf). Bacterial DNA template prepared by suspending two bacterial colonies in 100 μ l sterile water or isolated phage DNA were used as templates. Amplification was carried out in a total volume of 50 μ l containing 10 pmol of each primer, 200 μ M of each dNTP, 2 U DyNAzyme II DNA Polymerase (Finnzymes), 5 μ l 10 $\times$ buffer (Finnzymes) and 5 μ l bacterial or 1–5 ng phage DNA template. After a denaturation step of 3 min at 94 $^{\circ}$ C, the template DNA was denatured at 94 $^{\circ}$ C for 1 min, annealed at the appropriate temperature for 1 min and extended for 1 min at 72 $^{\circ}$ C. This amplification was carried out for 30 cycles. The final step was an extension of 7 min. The amplified products were run on a 1% agarose gel (SeaKem GTG) and visualized by ethidium bromide staining.

Sequencing of phage fragments. Partial sequencing of the PCR products obtained from the isolated phages was used to determine if the sequences showed homology to any known bacteriophage sequences. PCR products were ligated into

pMOSBlue vector using a pMOSBlue blunt-ended cloning kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Plasmids were purified using a QIAprep Spin Miniprep Kit (Qiagen). Nucleotide sequencing was carried out with specific oligonucleotide primers on PCR products, or with universal primers on the vectors using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the standard methods described by the manufacturer. An ABI PRISM 377 automatic sequencer (Perkin Elmer) was used.

Toxin production as evaluated by Vero cell assay. Toxicity was determined using a Vero cell assay (Sandvig & Olsnes, 1982). *E. coli* strains were grown overnight in TSB at 37 °C with moderate agitation, and 50 µl culture supernatant was tested in each Vero cell well. Antibodies (Toxin Technology) against Stx1B and Stx2B were added individually and together to the samples (approx. 0.2 µg) to assess whether the cytotoxicity observed was caused by Stx. All experiments were performed in duplicate and repeated twice.

RESULTS

Induction of phages

All isolates tested produced plaques when induced with mitomycin C. However, no *stx*₁ phages were detected by hybridization with the *stx*₁ probe from the isolates positive for *stx*₁ in PCR, indicating that these phages are noninducible. Analogously, the *stx*₂ probe did not detect any *stx*₂ phages from isolates H82 and H89, although these were *stx*₂-positive in PCR reactions. The induced phages that did not hybridize with the *stx* probes were not included in further PCR studies. Isolates 497 and 498 produced small diffuse plaques, and > 90% of the plaques were from *stx*₂ phages. Isolates C21b, 1480 and EDL933HS gave plaques similar to the EDL933 reference strain, and again, > 90% of the plaques were from *stx*₂ phages. While the plaques from isolate 1607 were visually similar to EDL933, only approximately 60% were from *stx*₂ phages.

RFLP of phage DNA

From each of the five isolates which produced *stx*₂ phages, phages from three plaques were purified and designated by 'φ' in front of, and 'A', 'B' and 'C' following, the bacterial strain number. The RFLP patterns with *EcoRI*, *AvaI* and *PstI* gave equivalent information, except that *PstI* did not digest the *stx*₂ phages from isolates 1480 and 1607. Here, the *EcoRI* RFLP pattern is used to illustrate the results, and only one phage isolate is included in the figure and was used in further studies when all three phages from the bacteria resulted in identical patterns (Fig. 1). Isolates 497 and 498 were from cattle from the same herd, and the phage RFLP patterns were indistinguishable from each other (φ497-A and φ498-A), but different from the other phage RFLPs. From isolates 1607, C21b and 1480, three, two and one phage types were detected, respectively (φ1607-A/B/C; φC21b-A/C; φ1480-A). None of the RFLP patterns from phages isolated from the 'mother isolate' (1607) were identical to the RFLP pattern of the phage from the 'child isolate' (1480) (Table 1). Two phages

Fig. 1. RFLP, using *EcoRI*, of the various isolated phages from *E. coli* O157:H7. Only one phage is presented where the RFLP pattern was indistinguishable from other isolated phages from the same bacterial strain. The symbols ◇ and * show to which band the IS1203-like or *stx*₂ probes, respectively, hybridized.

were isolated from the EDL933 reference strain from CCUG and these were different from the pattern of φ933HS-A phage. The results of blot hybridization of the RFLP gels are shown in Fig. 1. φ497-A and φ498-A had an IS1203-like element located on a band of similar size, while φ1607-A/B/C had the IS1203-like element on different bands and also on different bands to φ1480-A. The *stx*₂ probe hybridized to equivalent bands in the same RFLP pattern groups. φH82-A and φH89-A were negative with both probes.

Determination of the type of replication and regulator genes

The differences in the regions encoding the early gene regulators and replication proteins between VT2-Sa and Sakai-VT2 versus 933W, and the fact that hybridization had shown that the λ-like *P* gene was not associated with *stx*₂ in our isolates, prompted us to determine which O, *P* and *cI* genes were present in the different phages. Phages 933W, VT2-Sakai and VT2-Sa are mosaics of different phages, and are homologous in different regions to various other λ-group phages. We have used

Fig. 2. Schematic diagram of areas of DNA homology in the areas studied between VT2-Sa and 933W and other λ -group phages. The figure is based on the results from BLAST search comparisons. VT2-Sa is used as a basis for comparison in the *ssb*–*N* region. Both 933W and VT2-Sa are used as a basis for comparison in the *cI*–*P* region. Arrows indicate PCR primers that were used to amplify the conserved areas in these regions.

Fig. 3. Diagram of the relative positions and homologies to known sequences of the genes determined in this study. Regions analysed were selected in an attempt to determine homology to important regulatory and replicative genes and areas known to vary in λ phages. The boxes represent regions amplified by PCR using primers based on conserved sequences (grey boxes), and the results of partial sequencing of PCR products to determine homology to known genes (patterned boxes).

933W and VT2-Sa as a basis for comparison (Fig. 2), but it should be kept in mind that the homologies mentioned are not limited only to these two phages, but can include DNA that is homologous to DNA from a number of phages.

O and P genes

PCR products from conserved flanking regions were utilized to obtain PCR products for sequencing and the subsequent design of specific primers. Published sequences from 933W and VT2-Sa O and P regions were utilized to determine homology. As shown in Fig. 3, the sequenced PCR products from the EDL933 phage gave the expected λ -like O and P, while the phage we called ϕ 933HS-A had the VT2-Sa type P gene. ϕ C21b-A, ϕ 1480-A and ϕ 1607-A also had this VT2-Sa-like O and P region. The isolates ϕ 497-A and ϕ 498-A had O and P with homology to HK97 (Juhala *et al.*, 2000), and had in

addition an IS1203-like element inserted immediately after the P gene.

cI genes

Primers were designed to determine if the *cI* gene was 933W (H19B)-like, VT2-Sa (λ)-like or HK97-like (Fig. 2). The phages from the two reference strains and ϕ C21b-A had 933W-like *cI* genes. ϕ 497-A and ϕ 498-A *cI* were homologous to VT2-Sa. We were not able to determine the type of *cI* in ϕ 1480-A and ϕ 1607-A and can only conclude that these phage isolates have a different *cI* type to those mentioned above.

ssb–N region

The primers were selected from regions conserved in 933W, VT2-Sa, H-19B, HK97, HK022 and phi 21 phages in the λ -phage group (Franklin, 1985). The homologies in the region between *ssb* and *N* correlated with the

Table 3. Results of Vero cell assays on bacterial supernatants (controls) and on supernatants after neutralization with Stx1B, Stx2B, and Stx1B and Stx2B antibodies

3+, 2+, + and 0 indicate 75–100, 50–74, 25–49 and < 25% protein synthesis inhibition, respectively.

Strain	Control	After neutralization with:		
		Stx1B	Stx2B	Stx1B and Stx2B
H82	+	+	0	0*
H89	+	+	0	0*
497	2+	2+	+	+
498	2+	2+	+	+
C21b	3+	2+	0	0
1480	2+	2+	0	0*
1607	2+	2+	0	0*
EDL933HS	3+	2+	0	+
EDL933	3+	2+	+	+

* *stx*₁-negative.

results for the *cI* gene, except for ϕ 1480-A and ϕ 1607-A, where an IS1203-like element was inserted in an otherwise 933W homologous DNA region (Fig. 3).

Between *P* and *Q*

Primers based on conserved DNA sequences between *P* and *Q* (MPQR) and directly after the *P* gene (APF) (Table 2) were utilized for PCR. ϕ C21b-A and ϕ 933HS-A gave bands of approx. 1700 bp, while ϕ 497-A, ϕ 498-A and 933W resulted in bands of approximately 2000 bp. No bands were obtained by PCR on ϕ 1480-A and ϕ 1607-A DNA.

Q genes

Primers based on *Q* (QR and QF) (Table 2) from the phages 933W, VT2-Sa and H-19B were used to amplify a 400 bp product inside the *Q* gene. PCR products of the expected size were found in all the phages except ϕ 497-A and ϕ 498-A, which were negative.

*ileZ-stx*₂

PCR using an *ileZ* primer (*ileZ*) and internal *stx*₂ primer (*stx*₂-2) were positive in all the *stx*₂ phages tested (Table 2).

Integrase genes

The phages 933W and VT2-Sa have nearly identical integrase genes, and isolate C21b and the reference strain bacterial cells tested positive in PCR for this integrase. The phages isolated from isolate C21b and the other phages isolated did not have a homologous integrase. 'Universal λ primers' (Uinf + UinfR) (Table 2) based on conserved integrase DNA sequences from λ -group phages HK97, HK022, H19J, λ and 434 (de-

termined using BLAST) were tried in PCR without success on all the isolated phage DNA. These universal integrase primers were used to amplify integrase from λ and this PCR product was used as a probe on RFLP blots, which also gave negative results. These results indicate that the phages studied here show no significant homology with integrases found in the λ -group phages.

Presence of various *O/P* and *cI* in the host bacteria

To determine if there were other λ -group phages present in the host bacteria, which would provide a readily available gene pool for exchange and probable super-immunity of other phages, PCR on host bacterial DNA was performed for the *O/P* and *cI* genes found in this study, which were different to the isolated phage. As mentioned earlier, all bacterial isolates were positive for 933W (λ) *O/P*, while isolates H82 and H89 were also positive for HK97 *O/P*. All isolates except 497 and 498 were positive for *cI* from HK97. Isolates H82 and H89 were positive for 933W *cI*, as were all bacteria from which the *stx*₂ phage was isolated.

Vero cell toxicity assay

The reference strains and isolate C21b gave the highest level of activity in the Vero cell assay, while isolates 497, 498, 1480 and 1607 gave consistently lower values. The Stx1B antibodies reduced the toxicity levels in the *stx*₁-positive strains, which had the highest activity, to the same levels as the *stx*₁-negative strains. Neutralization with Stx2B antibodies reduced the effect of the supernatant on the Vero cells to very low levels (Table 3). From these studies it would appear that the levels of Stx2 production are in the same range for all the bacterial isolates where there is *stx*₂ phage induction, and that Stx1 contributes to the total effect seen on Vero cells. Isolates H82 and H89, from which there was no *stx*₂ phage induction detected, had values that were comparable to the Stx1 contribution in the other strains. This indicates that toxin production in bacteria that carry integrated, but apparently defective, phages is at a lower level than in bacteria from which phages can be induced.

DISCUSSION

Gene exchange seems to be common in *stx* phages

On the basis of conserved DNA sequences in many of the λ -group phages, replication, repressor and integrase genes and other DNA regions of *stx*₂ phages from cattle and human STEC O157:H7 in Norway were examined. Even in this limited material, a range of different gene configurations was observed. The capacity of gene exchange within the λ -group of phages was well illustrated in this study by ϕ 933HS-A, which was originally obtained from an EDL933 strain (thought to carry 933W), but had the *O/P* region homologous to VT2-Sa (HK022-like). The presence of the HK97 *O/P* region found in ϕ 497-A and ϕ 489-A also demonstrates the exchange of comparable genes within this group of phages.

There have been very few clinical STEC cases in Norway, and the 'child-clinical case' (1480) and 'mother-carrier' (1607) isolates were assumed to come from the same, unidentified, source (Heir *et al.*, 2000). While there was at least one band difference in the pattern from ϕ 1607-A/B/C RFLP as compared to ϕ 1480-A, hybridization indicated that there was one copy of *stx*₂ in the same position in the *stx*₂-positive phages. That the RFLP patterns for *stx*₂ phages from the same bacterial isolate, and from the mother and child isolates, were different could explain the differences observed in the PFGE types of the bacterial isolates, and indicate that the gene exchange observed occurs relatively frequently.

Induction of the isolates C21b, 1607 and EDL933 all resulted in more than one phage with different RFLP patterns. The major differences in the RFLP patterns from ϕ 1480 and ϕ 1607 were associated with IS1203-like elements, which could point to the IS element as a source of variation in this case. The IS elements found in phages ϕ 1480, ϕ 1607, ϕ 497 and ϕ 498 were located in non-protein coding DNA and did not seem to have a major effect on the phage functionality. Similar IS elements have been found in *stx* phages which have affected the function of the phage and inactivated the Shiga toxin (Yokoyama *et al.*, 2000; Kusumoto *et al.*, 2000). As many copies of IS1203-like elements have been found in the sequence of *E. coli* O157:H7, these must also be considered as part of the gene pool that are part of the gene exchange of phages (Perna *et al.*, 2001).

As we only examined phage isolated from plaques, only those gene exchanges that did not affect plaque formation were observed. Observations of defective prophages and many copies of viral genes in *E. coli* O157:H7 genomes indicates that 'less successful' gene exchange can also influence the bacterial genome (Perna *et al.*, 2001; Yokoyama *et al.*, 2000; Campbell, 1994). The presence of different immunity genes in the bacteria could influence the immunity of the bacteria to new invasive phages. The availability of numerous phage genes in the bacteria could play an important part in the evolution of λ -group phages and the development of *stx* phages, enabling the ability to produce toxin to spread into new bacteria (Hendrix *et al.*, 2000; Baker *et al.*, 1991).

We had hoped to be able to determine the chromosomal integration site of the various phages, but were hindered by the lack of homology of the integrase in our isolates to the λ -group of integrases. PCR using the WbrF and WbrR primers, spanning the site of 933W integration in the *wrb* gene, indicated that these phages integrate at sites other than those for 933W and VT2-Sa.

PFGE analysis has been established as the gold standard for typing of STEC (*E. coli* O157:H7 in particular) (Barrett *et al.*, 1994). When analysing the PFGE patterns the possibility of rapid recombination among *stx*₂ phage genes, which can possibly affect the clonal turnover of *E. coli* O157:H7 isolates and their genotypic patterns, should be kept in mind. Shifts in PFGE patterns of *E. coli*

O157:H7 isolates during shedding from both humans and experimentally infected cattle have been observed (Karch *et al.*, 1995; Akiba *et al.*, 2000).

A mosaic structure, typical for lambdoid double-stranded DNA phages (Campbell, 1994), has been found in the region between *stx* and the *ssb* genes in the phages we isolated. This supports the hypothesis that such phages share a common genetic pool with frequent exchange of genetic elements (Hendrix *et al.*, 2000; Baker *et al.*, 1991). This type of gene exchange may promote the evolution of mobile *stx* phages which, provided they show a gain in fitness, could contribute to an increased pathogenicity of their host bacterium. Consequently, the phage genotype may influence the transmission of *stx* between bacteria, and thus, the spread and emergence of STEC. Further characterization of the *stx* phage pool is required to determine the role played by these phages in STEC epidemiology and virulence.

Effects of phages on toxicity

The existence of two populations of *E. coli* O157:H7 in cattle, one, a large non-pathogenic population and the other, a smaller population pathogenic to man, has been suggested (Kim *et al.*, 1999). This difference in pathogenicity between populations may, in part, rely upon differences in toxicity of strains. In our study, Vero cell testing showed that the *stx*-positive isolates produced Shiga toxins in appreciable amounts in isolates from which we found induced *stx* phage. The *stx*₁ phages, although not induced with mitomycin C, contributed to total toxin production, as shown by the neutralization experiments. We suggest that the differences shown in pathogenicity in otherwise similar *stx*-positive strains (as discussed above), are associated with the burst size of the *stx* phages. Isolates in which the *stx*₂ phages were inactivated produced less toxin. These results agree with a previous study (Wagner *et al.*, 1999) which showed that in a homogeneous bacterial background the amount of toxin produced was directly correlated with the number of phage induced. The *stx* phages themselves play a major part in toxin production, but the characteristics of the host cell, with its range of other phages and genomic factors, determine if the bacteria themselves survive in environments where they are able to act as pathogens. The pathogenic potential of an STEC strain is therefore partly a result of a complex interplay between the *stx* phages and the host cells, yet to be fully revealed.

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