Short communication: Characterization of Shiga toxin 2-carrying bacteriophages induced from Shiga-toxigenic *Escherichia coli* isolated from Italian dairy products

G. Volponi,* D. J. Rooks,† D. L. Smith,† C. Picozzi,* H. E. Allison,† I. Vigentini,* R. Foschino,* and A. J. McCarthy†¹

*Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università degli Studi di Milano, via Celoria 2 20133 Milano, Italy

†Microbiology Research Group, Institute of Integrative Biology, Biosciences Building, University of Liverpool, Crown Street, Liverpool, L69 7ZB, United Kingdom

ABSTRACT

Forty samples of raw milk cheese and 25 samples of raw milk itself were subjected to enrichment culture for Shiga-toxigenic *Escherichia coli* (STEC), and a single Shiga toxin 2- (Stx₂) positive strain was obtained from one of the cheese samples. Thus, aged cheeses in which the curd is subsequently heat treated (48°C) cannot be presumed to be STEC free. Infective Stx₂ bacteriophages were induced from 3 STEC strains isolated elsewhere from raw milk and 1 STEC strain from aged cheese sourced in Italy. Data on *E. coli* host range, morphology, genome size, and genetic variation determined by restriction fragment length polymorphism and multi-locus genotyping are presented. Although all 4 bacteriophages were found to be short-tailed *Podoviridae*, they exhibited considerable variation in both genome size and content. This extended to the *Stx*₂ genes themselves, whose sequences contained several point mutations, but these did not translate to amino acid substitutions.

Key words: Shiga-toxigenic *Escherichia coli*, milk and cheese, Shiga toxin bacteriophage, protected designation of origin dairy product

Short Communication

The production of Shiga toxins (Stx) is regarded as the main virulence factor of Shiga-toxigenic *Escherichia coli* (STEC) strains, which are responsible for severe human gastrointestinal diseases, typified by hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) as frequent complications (Cohen and Giannella, 1992). Most pathotypes, among them *Escherichia coli* O157, carry additional virulence markers such as intimin and

enterohemolysin, encoded by *eae* and *hly* genes, respectively (LeBlanc, 2003). Shiga toxin genes are located in the genomes of bacteriophages, which are the natural vector for the spread of Shiga-toxigenic potential among *E. coli* and other *Enterobacteriaceae* through the phenomenon of lysogenic conversion (Herold et al., 2004; Gyles, 2007; Smith et al., 2007a; Strauch et al., 2008). Two Shiga toxin types exist: Stx₁ and Stx₂, which may or may not be present together in STEC strains (Nataro and Kaper, 1998) and although Stx₁ is highly conserved, several variants of Stx₂ exist for which evidence of enhanced pathogenicity exists (Friedrich et al., 2002). The ability of lysogens to give rise to infectious virions and to transduce indigenous *E. coli* strains within the host gastrointestinal tract of mice (Acheson, 1998; Gamage et al., 2003) and sheep (Cornick et al., 2006) has been demonstrated. Therefore, Stx phages are not only responsible for the origin of STEC, but also play a key role in the pathogenesis of HC and HUS with respect to the virulence and diffusion of pathology. In fact, the dissemination of these viral genes by transduction is considered the most likely mechanism for the dynamic emergence of new STEC serotypes (Schmidt et al., 1999; Schmidt, 2001; Bielaszewska et al., 2007).

Shiga toxin phages described thus far comprise double-stranded DNA with isomeric heads and long tails (*Siphoviridae*) or short tails (*Podoviridae*); they are members of the lambdoid family because they all exhibit the organization of genetic modules first described in bacteriophage lambda, even though their genomes have a mosaic structure (Allison, 2007). Unusually for lambdoid phages, the ability of Stx phages to form multiple lysogens has been demonstrated (Fogg et al., 2007; Serra-Moreno et al., 2008) and, thus, the rapid evolution of STEC strains could have been driven by intracellular recombination events between multiple integrated prophages. Moreover, multiple copies of the *Stx* gene in a single *E. coli* genome may lead to enhancement of toxin production in vivo (Fogg et al., 2012).

Received June 12, 2012.

Accepted August 9, 2012.

¹Corresponding author: aj55m@liv.ac.uk

Reports on the prevalence of STEC in different environments reveal that most of the strains recovered produce only 1 type of toxin; 2 to 54% of isolates possessed the *Stx*₁ gene, 16 to 62% the *Stx*₂ gene, and up to 38% carried both *Stx*₁ and *Stx*₂ genes (Muniesa et al., 2004; Dumke et al., 2006; Beutin et al., 2007; Slanec et al., 2009). Shiga toxin 2 and its variants are more important in foodborne infection outbreaks (Bertin et al., 2001; García-Aljaro et al., 2009) and evidence exists that *Stx*₂ is more often associated with the progression to serious disease (Boerlin et al., 1999). Cattle, sheep, and goats are the principal reservoirs of STEC (Pradel et al., 2000; Fratamico et al., 2004; Hussein and Bollinger, 2005; Kaufmann et al., 2006; Gyles, 2007; La Ragione et al., 2009) and although unpasteurized dairy products would, therefore, be expected to represent an infectious source, only isolated reports exist (Vernozy-Rozand et al., 2005; Zweifel et al., 2010). In Italy, the sale of unpackaged raw milk for direct human consumption is authorized because it represents a means to support small farms and maintain economic activities in marginal territories; currently, 1,455 retail distributors of raw milk are on tap, located in 91 districts. Furthermore, across Europe, extensive production exists of protected designation of origin cheeses prepared from raw milk, and at least an implication of semi-hard and hard cheeses as a source of STEC in Switzerland (Stephan et al., 2008; Zweifel et al., 2010). In Italy, a national surveillance system based on voluntary notification was established in 1988 to monitor the incidence of HC and HUS in pediatric patients and to identify the

STEC strains associated with these syndromes (Tozzi et al., 2003). A recent episode of STEC foodborne infection involved 9 children in the Lombardy region, and in recognition of the risk associated with raw milk consumption, a ministerial ordinance requiring milk to be heated before consumption was issued (Ministero del Lavoro, della Salute e delle Politiche Sociali, 2009). To further establish and characterize the potential of raw milk and raw milk cheese to serve as a source of STEC, we describe, for the first time, *Stx* phages induced from *E. coli* isolated from Italian dairy products.

The bacterial strains used in this work are listed in Table 1. Strains were routinely cultured in lysogeny broth (**LB**; Bertani, 2004; BD Difco, Franklin Lakes, NJ), with or without 1.5% (wt/vol) agar (Merck KGaA, Darmstadt, Germany). Phage buffer (**PB**; LB broth supplemented with 10 mM CaCl₂; Sambrook and Russell, 2001) was used for phage propagation. *Escherichia coli* CNCTC 6896, a recA441 mutant of laboratory strain K12, was the indicator strain used for routine propagation of the bacteriophages.

In this study, 40 samples of raw milk cheese (fresh and aged) and 25 samples of raw milk (from goat and cow), collected from different Alpine regions in Northern Italy, were analyzed for STEC presence. Ten grams of each sample was added to 90 mL of LB, supplemented with 20 mg/L of novobiocin (Sigma, St. Louis, MO; Doyle and Schoeni, 1987) homogenized in a Stomacher 400C (Seward, Worthing, UK) for 3 min and incubated at 37°C for 24 h. Two microliters of this preenrichment culture were then subjected to multiplex PCR using

Table 1. Bacterial strains and bacteriophages

Strain/phage	Relevant characteristics	Source	Reference
1D3.07	<i>Escherichia coli</i> NT, ¹ <i>Stx</i> ₂	Fontina cheese	This work
181181/2	<i>E. coli</i> O157:H7, <i>Stx</i> ₂ , <i>eae</i> , <i>hlyA</i>	Cow raw milk	IZSLER ²
208164/2	<i>E. coli</i> O157:H7, <i>Stx</i> ₂ , <i>eae</i> , <i>hlyA</i>	Cow raw milk	IZSLER ²
EC34	<i>E. coli</i> O157:H ⁻ , <i>Stx</i> ₂ , <i>eae</i> , <i>hlyA</i>	Goat raw milk	Picozzi et al. (2005)
SO14	<i>E. coli</i> NT, ¹ <i>Stx</i> ₂ , <i>hlyA</i>	Cow raw milk	CEPIS ³
CNCTC 6896	<i>E. coli</i> Lambda-K12 indicator strain		CNCTC ⁴
CNCTC 6246	<i>E. coli</i> indicator strain		CNCTC ⁴
DH5α	<i>E. coli</i> indicator strain		Muniesa et al. (2004)
DM1187	<i>E. coli</i> K12 derivative, constitutive RecA expression, indicator strain		James et al. (2001)
MC1061	<i>E. coli</i> K12 derivative, indicator strain		James et al. (2001)
NCTC 12079	<i>E. coli</i> O157:H7, <i>Stx</i> ₁ , <i>Stx</i> ₂ , <i>eae</i>		NCTC ⁵
WG5	<i>E. coli</i> C derivative, indicator strain		Muniesa and Jofre (2004)
ΦGV8	<i>Stx</i> phage induced from 1D3.07		This work
ΦGV2412	<i>Stx</i> phage induced from 181181/2		This work
ΦBR2	<i>Stx</i> phage induced from 208164/2		This work
ΦL34	<i>Stx</i> phage induced from EC34		This work

¹Untypeable.

²Istituto Zooprofilattico Sperimentale per la Lombardia e l'Emilia Romagna (Brescia, Italy).

³Centro Enteropatogeni Italia Settentrionale (Milan, Italy).

⁴Czech National Collection of Type Cultures (Prague, Czech Republic).

⁵National Collection of Type Cultures (Salisbury, UK).

specific primers for amplification of *Stx*₁, *Stx*₂, *eaeA*, and *hlyA* genes (Fagan et al., 1999). Amplifications were performed using 1 U of Taq DNA polymerase (5 Prime GmbH, Hamburg, Germany) in a 25- μ L reaction comprising 200 μ M deoxyribonucleotide triphosphate (dNTP) mix (Fermentas, Vilnius, Lithuania), 3 mmol of MgCl₂/L, and 200 nmol/L of each primer. Cycle conditions, carried out in a T-Gradient Biometra Thermocycler (Biometra, Göttingen, Germany) consisted of an initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 40 s and extension at 72°C for 90s, followed by a final extension at 72°C for 5 min. Polymerase chain reaction products were separated on a 1.5% agarose gel stained with 0.3 μ g/mL of ethidium bromide in Tris-acetate-EDTA (TAE) buffer (40 mmol of Tris-acetate/L and 1 mmol of EDTA/L, pH 8.0). Deoxyribonucleic acid fragments were visualized and photographed under UV illumination. A 100-bp DNA Ladder Plus (Fermentas) was included in each gel as molecular size marker.

For the isolation of presumptive STEC strains, *Stx*-positive samples were tested by colony hybridization with a DNA probe. For colony dot-blot hybridization, aliquots of the enrichment culture samples were plated onto Chromocult TBX agar (BD Difco). After incubation at 42°C for 24 h, the appropriate dilution with the highest number of separated colonies was selected. Colonies were transferred to a nylon membrane (Hybond-N+; Amersham Pharmacia Biotech Inc., Piscataway, NJ) according to a standard procedure (Sambrook and Russell, 2001) and hybridized at 65°C with a digoxigenin-labeled *Stx* probe. The *Stx* probes were prepared by labeling PCR amplicons from *E. coli* O157:H7 NCTC 12079 using a DIG High Prime kit (Roche Diagnostics GmbH, Mannheim, Germany) according to Kaufmann et al. (2006). Stringent hybridization was achieved with the DIG-DNA Labeling and Detection Kit (Roche Diagnostic Ltd., Lewes, UK) according to the manufacturer's instructions.

Shiga-toxigenic *Escherichia coli* strains were cultured in PB (optical density at 600 nm of 0.45 to 0.55) and treated with norfloxacin (1 μ g/mL; Matsushiro et al., 1999) at 37°C for 1 h to induce phages. The cultures were diluted 10-fold in PB and allowed to recover at 37°C for 2 h. One hundred microliters of appropriate dilutions was mixed with 100 μ L of PB and 500 μ L of an exponential-phase culture of *E. coli* strain CNCTC 6896 and examined through plaque assay using a double-layer agar method (Muniesa and Jofre, 2004). To detect the presence of the *Stx* genes in phages, the plaques were transferred to a nylon membrane (Hybond-N+; Amersham Pharmacia Biotech Inc.) according to a standard procedure (Sambrook and Russell, 2001) and

hybridized with digoxigenin-labeled *Stx* probe as described above for colony dot-blot hybridization.

After filtration through a 0.45- μ m membrane, phage suspensions were subjected to purification in a CsCl gradient according to a protocol modified from Sambrook and Russell (2001). Phage stock solutions were plated at low dilutions to produce semiconfluent lysis. After overnight incubation (16 h) at 37°C, the plates were overlaid with 3 mL of SM buffer (100 mmol of NaCl/L, 8 mmol of MgSO₄/L, and 50 mmol of Tris-HCl/L, pH 7.5) and stored overnight (16 h) at 4°C. The SM buffer was removed and the top agar was scraped into a sterile glass centrifuge tube, stirred with 10% (vol/vol) SM buffer, centrifuged at 10,000 $\times g$ for 10 min at room temperature, and the supernatant recovered. Chloroform (30 μ L) was added to each 10-mL aliquot of phage suspension to kill any remaining cells. Deoxyribonuclease (5 μ g/mL) and RNase (1 μ g/mL; Sigma) were added to remove any bacterial nucleic acids, and the solution incubated for 1 h at 37°C. Bacteriophages were pelleted by ultracentrifugation at 100,000 $\times g$ for 30 min at room temperature, resuspended in SM buffer, and subsequently purified by CsCl density preformed gradient ($d = 1.3$ – 1.7 g/mL) ultracentrifugation at 100,000 $\times g$ for 1 h at room temperature. The visible band was collected and dialyzed with a 30-kDa cut-off membrane at 4°C overnight (16 h) in dialysis buffer (10 mmol of Tris-HCl/L, pH 7.4; 10 mmol of MgSO₄/L; and 0.5 mol of NaCl/L) to remove CsCl.

To evaluate the ability of these *Stx* phages to infect other hosts in addition to *E. coli* CNCTC 6896, *E. coli* CNCTC 6246, DH5 α , DM1187, MC1061, and WG5 strains were tested. A drop of phage suspension from the *Stx* phage stocks was spotted onto a monolayer plate containing each indicator strain and, after incubation at 37°C overnight (16 h), plates with a clear zone of lysis were scored as positive. The morphology of viral particles was observed by transmission electron microscopy (Philips 201, 80 kV; Philips International BV, Eindhoven, the Netherlands), after laying the suspensions on 300-mesh copper specimen grids coated with carbon film and negatively stained with 2% (wt/vol) uranyl acetate (pH 4.5).

Phage DNA, extracted with a standard protocol (Sambrook and Russell 2001) from purified stocks, was subjected to RFLP analysis by digestion with the *Eco*RI restriction enzyme (Fermentas). Deoxyribonucleic acid fragments were separated by agarose (1% wt/vol) gel electrophoresis in TAE buffer at 5 V/cm and visualized by UV light after staining with ethidium bromide. Deoxyribonucleic acid standard fragments of MassRuler DNA Ladder Mix ready-to-use (Fermentas) were used to calculate the size of the fragments. Genome length

was estimated by the sum of molecular weights of fragments generated by digestion. Purified bacteriophage DNA was also characterized using the multi-loci typing scheme based on lambdoid phage genome organization, using the methods described by Smith et al. (2007b).

The *Stx₂* gene of each phage was sequenced using the oligonucleotides S2Aup/S2Alp for *Stx₂* subunit A and GK3/GK4 for subunit B, as described by Muniesa et al. (2004) and using oligonucleotides VT1/VT2, as described by Kaufmann et al. (2006). Sequencing was provided by Primm Srl (Milan, Italy). Nucleotide sequence analysis, assembly of the sequences, protein translation, and BLAST analyses were performed with the GenDoc Multiple Sequence Alignment Editor (<http://petang.cgu.edu.tw/Bioinformatics/Softwares/SOFTWARES/Soft-index.htm>), BioEdit Sequence Alignment Editor (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>), and the ClustalW Multiple Sequence Alignment Program (<http://www.ebi.ac.uk/clustalw/>). Nucleotide sequences obtained in this work have been deposited in the GenBank database under accession numbers from JN193428 to JN193431.

Only 1 of the 40 cheese samples tested gave a positive result after the multiplex PCR assay on the preenrichment broth culture, showing a band for the *Stx₂* gene only at 779 bp. That same sample yielded a single STEC strain (named 1D3.07), isolated and confirmed as Stx positive by colony-dot-blot hybridization. Amplification products for the *eae* and *hlyA* genes were not detected and the STEC strain could not be serotyped. The positive sample was an Italian protected designation of origin cheese produced from raw cow milk. None of the 25 samples of raw cow milk tested positive for the presence of the *Stx* gene by PCR amplification of extracted DNA. These results can be added to the limited data set that already exists for STEC occurrence in dairy products: Pradel et al. (2000) found that 60 out of 603 (10%) cheese samples examined were positive for *Stx* genes; Vernozy-Rozand et al. (2005) observed a STEC incidence of 13% in 1039 French raw milk cheeses; Rantsiou and Cocolin (2009) used quantitative PCR to report 18% *Stx*-positive samples in Piedmont tradi-

tional cheeses manufactured from raw milk; Stephan et al. (2008) and recently Zweifel et al. (2010) reported STEC prevalences of 4.9% in 796 samples and 5.7% in 1,502 samples, respectively, of soft, semi-hard, and hard cheeses made from Swiss cows, goats, and sheep raw milk collected at the producer level. The recovery here of an STEC strain (1D3.07), lysogenic for an inducible Stx phage (ϕGV8), from a sample of Fontina cheese that had undergone heating of the curd at 48°C and was ripened for no less than 3 mo at 12°C, supports a previous study (Zweifel et al., 2010) in which it was suggested that aged raw milk cheeses are a potential source of STEC.

Other STEC isolated from raw milk samples, obtained in previous studies or kindly supplied by Italian institutions, were also investigated (Table 1). *Escherichia coli* O157:H7 strains 181181/2, 208164/2, and EC34 yielded amplification products of the expected size in multiplex PCR for the *Stx₂*, *eae*, and *hlyA* genes; SO14, an untypable *E. coli* O strain, was positive for *Stx₂* and *hlyA* genes, whereas our ID3.07 isolate, the other untypable strain, was positive for the *Stx₂* gene only. Induction of prophages by norfloxacin treatment was carried out on these 5 STEC strains, and infective bacteriophages were detected in 4 of the 5 strains (not SO14) by plaque assay. A single Stx₂ phage was then purified from each one of the 4 viral lysates; the purified phages (Table 1) were again shown to be Stx positive by plaque hybridization assay. Detection by plaque assay is dependent on the susceptibility of host indicator strains, as evidenced here by the variation in host range found among only 4 purified Stx phages (Table 2). More meaningful data on the prevalence of Stx phages requires the application of direct viral DNA recovery protocols in combination with quantitative PCR-based quantification (Rooks et al., 2010).

All 4 phages had a similar morphology as determined by transmission electron microscopy (Figure 1). The viral particles were identified as members of the *Podoviridae* family, characterized by a short tail and icosahedral head; base plate-like structures were not observed. The heads had a diameter of 45 to 55

Table 2. Characterization of purified Shiga toxin (Stx) phages

Isolated phage	Capsid diameter (nm)	Tail length (nm)	Genome size (kbp)	Host range ¹						% Identity to closest match sequences of <i>Stx₂</i> subunit A and B genes (accession number)
				CNCTC 6246	CNCTC 6896	DH5α	DM1187	MC1061	WG5	
ΦGV8	52	8	61	—	+++	—	+++	++	+++	99.4, Φ933W (AF125520)
ΦGV2412	45	9	65	—	+++	—	+++	—	++	99.6, O157-379 (AY633468)
ΦBR2	55	9	53	—	++	—	++	—	+	100, VTB60 (AY633471)
ΦL34	52	10	58	—	+++	—	+++	+++	++	99.8, VTB324 (AY633460)

¹+++ = strong lysis; ++ = clear lysis; + = weak lysis; — = no lysis in the spot area.

Figure 1. Transmission electron micrographs of the 4 purified Shiga toxin (Stx) phage isolates (ϕ L34, ϕ GV8, ϕ BR2, and ϕ GV2412). Bar markers = 10 nm.

nm with short tails up to 10 nm in length (Table 2). Mean values were obtained by measuring the dimensions of 10 individual viral particles for each of the 4 phage preparations on at least 2 different microscopic preparations. RFLP analysis of phage DNA extracts generated a unique banding pattern for each (Figure 2) and the genome sizes estimated on the basis of the total sum of the restriction fragments were also distinct and in the range of approximately 53 to 61 kbp (Table 2). The ability of Stx phages to infect different host strains was evaluated by spotting the phage preparations on a lawn of each *E. coli* strain and scoring any lysis observed (Table 2). All phages were able to infect and lyse the phage propagation host *E. coli* CNCTC 6896; *E. coli* DM1187, which is a *recA441* mutant that produces RecA protease constitutively and, therefore, cannot be lysogenized; and *E. coli* WG5, which possesses an attenuated host restriction modification system, supporting increased phage sensitivity. In contrast, *E. coli* CNCT 6246 and DH5 α were not infected by any of the 4 phages, whereas *E. coli* MC1061 was sensitive to ϕ GV8 and ϕ L34 phages only. The ϕ GV2412 and ϕ BR2 phages, induced from 2 strains of *E. coli* O157:H7, had a similar host range to one another (Table 2).

The nucleotide sequences of the toxin genes confirmed that all of the 4 phages carried the *Stx*₂ gene. This is in agreement with the suggested predominance of short-tailed *Podoviridae* carrying the *Stx*₂ form of the *Stx* gene among inducible Stx phages (Smith et al., 2007b; McDonald et al., 2010). The *Stx*₂ subunit A and B gene sequences exhibited close homology to various previously described phage-borne *Stx* genes (Table 2; Supplementary Figure 1, available online at <http://www.journalofdairyscience.org/>): the *Stx*₂ sequence of ϕ BR2 was absolutely identical to that of the VTB60 phage induced from an O136:H⁻ *E. coli* strain (Muniesa et al., 2004); the ϕ L34 *Stx*₂ subunit A and B genes were sufficiently similar to the Shiga-like toxin from phage VTB324 induced from an O177:H25 *E. coli* strain (Muniesa et al., 2004) to enable its identification as an *Stx*_{2c} variant (Meyer et al., 1992).

The genotypic variation detected in the 4 purified Stx phages is illustrated in Figure 3. As predicted by the

data of Smith et al. (2007b), confirming the inherent genetic mosaicism of Stx phages, each purified bacteriophage had a unique profile based on the distribution of variants of specific loci. This was confirmed by the construction of a Jaccard single linkage dendrogram (Figure 3). The sequenced Stx phages ϕ 24b, 933W, and P27, and bacteriophage lambda were also included in the analysis. All of the short-tailed phages (*Podoviridae*) share the tail fiber gene type VTTF (Figure 3); otherwise, the genetic heterogeneity indicated by the RFLP analysis (Figure 2) is reflected by the PCR based multi-loci typing.

In spite of abundant literature related to the characterization of Stx phages isolated from the environment, fecal material, sewage, and water (Gamage et al., 2004; Muniesa et al., 2004; Muniesa and Jofre, 2004; García-Aljaro et al., 2009), no data exist on phages induced from STEC isolated from milk or cheese. Here, 4 Stx

Figure 2. Restriction fragment length polymorphism (RFLP) banding patterns of *Eco*RI restriction enzyme- (Fermentas, Vilnius, Lithuania) digested Shiga toxin (Stx) phage DNA. M = molecular markers; 1 = ϕ GV8; 2 = ϕ GV2412; 3 = ϕ L34; 4 = ϕ BR2.

Figure 3. Diagrammatic representation of the presence and absence of genetic loci in lambdoid phages. For full gene variant names and sources, see Smith et al. (2007b). ¹Sequenced phage genomes: Lambda (J02459); ΦP27 (AJ298298); Φ24B (HM208303); Φ933W (AF125520). ²Purified Shiga toxin (Stx) phage isolates (Table 1). The figure indicates the presence (black) and absence (gray) distribution of PCR products for each gene variant, and a Jaccard dissimilarity dendrogram of the data.

phages were induced from STEC strains isolated from different sources and dairy products, yet all had similar morphologies and carried the *Stx₂* gene. Both the RFLP and multi-locus characterization data sets (Figures 2 and 3) confirm that Stx phages are genetically heterogeneous (Muniesa et al., 2004; Allison, 2007). This genetic variation also extended partly to the *Stx₂* gene itself, even in this small subset of induced Stx phages, further emphasizing the dynamic state of lambdoid phage evolution and its potential contribution to the emergence of new foodborne STEC strains.

ACKNOWLEDGMENTS

The authors are grateful to Paolo Boni (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER), Brescia, Italy) and Mirella Pontello (Dipartimento di Sanità pubblica – Microbiologia – Virologia, Università degli Studi di Milano, Milan, Italy) for providing some strains. Many thanks to Franco Faoro (Dipartimento di Scienze Agrarie e Ambientali, Università degli Studi di Milano, Milan, Italy) for his help and advice in the preparation of micrographs. This work was supported by Ministero

Italiano dell'Università e della Ricerca (Rome, Italy), PRIN 2007 national project (prot 2007PWS2PL), and the Department for Environment, Food and Rural Affairs (Defra, London, UK).

REFERENCES

- Acheson, D. W. 1998. Nomenclature of enterotoxins. *Lancet* 351:1003.
- Allison, H. E. 2007. Stx-phages: Drivers and mediators of the evolution of STEC and STEC-like pathogens. *Future Microbiol.* 2:165–174.
- Bertani, G. 2004. Lysogeny at mid-twentieth century: P1, P2, and other experimental systems. *J. Bacteriol.* 186:595–600.
- Bertin, Y., K. Boukhors, N. Pradel, V. Livrelli, and C. Martin. 2001. Stx2 subtyping of Shiga toxin-producing *Escherichia coli* isolated from cattle in France: Detection of a new Stx2 subtype and correlation with additional virulence factors. *J. Clin. Microbiol.* 39:3060–3065.
- Beutin, L., A. Miko, G. Krause, K. Pries, S. Haby, K. Steege, and N. Albrecht. 2007. Identification of human-pathogenic strains of Shiga toxin-producing *Escherichia coli* from food by a combination of serotyping and molecular typing of Shiga toxin genes. *Appl. Environ. Microbiol.* 73:4769–4775.
- Bielaszewska, M., R. Prager, R. Köck, A. Mellmann, W. Zhang, H. Tschäpe, P. I. Tarr, and H. Karch. 2007. Shiga toxin gene loss and transfer in vitro and in vivo during enterohemorrhagic *Escherichia coli* O26 infection in humans. *Appl. Environ. Microbiol.* 73:3144–3150.
- Boerlin, P., S. A. McEwen, F. Boerlin-Petzold, J. B. Wilson, R. P. Johnson, and C. L. Gyles. 1999. Associations between virulence

- factors of Shiga toxin-producing *Escherichia coli* and disease in humans. *J. Clin. Microbiol.* 37:497–503.
- Cohen, M. B., and R. A. Giannella. 1992. Hemorrhagic colitis associated with *Escherichia coli* O157:H7. *Adv. Intern. Med.* 37:173–195.
- Cornick, N. A., A. F. Helgerson, V. Mai, J. M. Ritchie, and D. W. K. Acheson. 2006. In vivo transduction of an Stx-encoding phage in ruminants. *Appl. Environ. Microbiol.* 72:5086–5088.
- Doyle, M. P., and J. L. Schoeni. 1987. Isolation of *Escherichia coli* O157:H7 from retail fresh meats and poultry. *Appl. Environ. Microbiol.* 53:2394–2396.
- Dumke, R., U. Schröter-Bobsin, E. Jacobs, and I. Röske. 2006. Detection of phages carrying the Shiga toxin 1 and 2 genes in waste water and river water samples. *Lett. Appl. Microbiol.* 42:48–53.
- Fagan, P. K., M. A. Hornitzky, K. A. Bettelheim, and S. P. Djordjevic. 1999. Detection of Shiga-like toxin (stx₁ and stx₂), intimin (eaeA), and enterohemorrhagic *Escherichia coli* (EHEC) hemolysin (EHEC hlyA) genes in animal feces by multiplex PCR. *Appl. Environ. Microbiol.* 65:868–872.
- Fogg, P. C. M., S. M. Gossage, D. L. Smith, J. R. Saunders, A. J. McCarthy, and H. E. Allison. 2007. Identification of multiple integration sites for Stx-phage ϕ 24_B in the *Escherichia coli* genome, description of a novel integrase and evidence for a functional anti-repressor. *Microbiology* 153:4098–4110.
- Fogg, P. C. M., J. R. Saunders, A. J. McCarthy, and H. E. Allison. 2012. Cumulative effect of prophage burden on Shiga toxin production in *Escherichia coli*. *Microbiology* 158:488–497.
- Fratamico, P. M., L. K. Bagi, E. J. Bush, and B. T. Solow. 2004. Prevalence and characterization of Shiga toxin-producing *Escherichia coli* in swine feces recovered in the National Animal Health Monitoring System's Swine 2000 study. *Appl. Environ. Microbiol.* 70:7173–7178.
- Friedrich, A. W., M. Bielaszewska, W.-L. Zhang, M. Pulz, T. Kuczias, A. Ammon, and H. Karch. 2002. *Escherichia coli* harboring Shiga toxin 2 gene variants: Frequency and association with clinical symptoms. *J. Infect. Dis.* 185:74–84.
- Gamage, S. D., A. K. Patton, J. F. Hanson, and A. A. Weiss. 2004. Diversity and host range of Shiga toxin-encoding phage. *Infect. Immun.* 72:7131–7139.
- Gamage, S. D., J. E. Strasser, C. L. Chalk, and A. A. Weiss. 2003. Nonpathogenic *Escherichia coli* can contribute to the production of Shiga toxin. *Infect. Immun.* 71:3107–3115.
- García-Aljaro, C., M. Muniesa, J. Jofre, and A. R. Blanch. 2009. Genotypic and phenotypic diversity among induced, stx2-carrying bacteriophages from environmental *Escherichia coli* strains. *Appl. Environ. Microbiol.* 75:329–336.
- Gyles, C. L. 2007. Shiga toxin-producing *Escherichia coli*: An overview. *J. Anim. Sci.* 85(Suppl.):E45–E62.
- Herold, S., H. Karch, and H. Schmidt. 2004. Shiga toxin-encoding bacteriophages—Genomes in motion. *Int. J. Med. Microbiol.* 294:115–121.
- Hussein, H. S., and L. M. Bollinger. 2005. Prevalence of Shiga toxin-producing *Escherichia coli* in beef cattle. *J. Food Prot.* 68:2224–2241.
- James, C. E., K. N. Stanley, H. E. Allison, H. J. Flint, C. S. Stewart, R. J. Sharp, J. R. Saunders, and A. J. McCarthy. 2001. Lytic and lysogenic infection of diverse *Escherichia coli* and *Shigella* strains with a verocytotoxinogenic bacteriophage. *Appl. Environ. Microbiol.* 67:4335–4337.
- Kaufmann, M., C. Zweifel, M. Blanco, J. E. Blanco, J. Blanco, L. Beutin, and R. Stephan. 2006. *Escherichia coli* O157 and non-O157 Shiga toxin-producing *Escherichia coli* in fecal samples of finished pigs at slaughter in Switzerland. *J. Food Prot.* 69:260–266.
- La Ragione, R. M., A. Best, M. J. Woodward, and A. D. Wales. 2009. *Escherichia coli* O157:H7 colonization in small domestic ruminants. *FEMS Microbiol. Rev.* 33:394–410.
- LeBlanc, J. J. 2003. Implication of virulence factors in *Escherichia coli* O157:H7 pathogenesis. *Crit. Rev. Microbiol.* 29:277–296.
- Matsushiro, A., K. Sato, H. Miyamoto, T. Yamamura, and T. Honda. 1999. Induction of prophages of enterohemorrhagic *Escherichia coli* O157:H7 with norfloxacin. *J. Bacteriol.* 181:2257–2260.
- McDonald, J. E., D. L. Smith, P. C. Fogg, A. J. McCarthy, and H. E. Allison. 2010. High-throughput method for rapid induction of prophages from lysogens and its application in the study of Shiga toxin-encoding *Escherichia coli* strains. *Appl. Environ. Microbiol.* 76:2360–2365.
- Meyer, T., H. Karch, J. Hacker, H. Bocklage, and J. Heesemann. 1992. Cloning and sequencing of a Shiga-like toxin II-related gene from *Escherichia coli* O157:H7 strain 7279. *Zentralbl. Bakteriol.* 276:176–188.
- Ministro del Lavoro, della Salute e delle Politiche Sociali. 2009. Misure urgenti in materia di produzione, commercializzazione e vendita diretta di latte crudo per l'alimentazione umana. Gazzetta Ufficiale della Repubblica Italiana, Rome, Italy.
- Muniesa, M., J. E. Blanco, M. de Simón, R. Serra-Moreno, A. R. Blanch, and J. Jofre. 2004. Diversity of stx₂ converting bacteriophages induced from Shiga-toxin-producing *Escherichia coli* strains isolated from cattle. *Microbiology* 150:2959–2971.
- Muniesa, M., and J. Jofre. 2004. Factors influencing the replication of somatic coliphages in the water environment. *Antonie Van Leeuwenhoek* 86:65–76.
- Nataro, J. P., and J. B. Kaper. 1998. Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* 11:142–201.
- Picozzi, C., R. Foschino, A. Heuvelink, and R. Beumer. 2005. Phenotypic and genotypic characterization of sorbitol-negative or slow-fermenting (suspected O157) *Escherichia coli* isolated from milk samples in Lombardy region. *Lett. Appl. Microbiol.* 40:491–496.
- Pradel, N., V. Livrelli, C. De Champs, J.-B. Palcoux, A. Reynaud, F. Scheutz, J. Sirot, B. Joly, and C. Forestier. 2000. Prevalence and characterization of Shiga toxin-producing *Escherichia coli* isolated from cattle, food, and children during a one-year prospective study in France. *J. Clin. Microbiol.* 38:1023–1031.
- Rantsiou, K., and L. Coccolin. 2009. Survey of Shiga-like toxin producing *Escherichia coli* in foods of animal origin of the Piedmont region in Italy. Page 75 in Book of Abstracts (Annals of Microbiology, 59, Special Issue) of II National Congress of Società Italiana di Microbiologia Agro-alimentare ed Ambientale (SIMTREA), Sassari, Italy.
- Rooks, D. J., Y. Yan, J. E. McDonald, M. J. Woodward, A. J. McCarthy, and H. E. Allison. 2010. Development and validation of a qPCR-based method for quantifying Shiga toxin-encoding and other lambdoid bacteriophages. *Environ. Microbiol.* 12:1194–1204.
- Sambrook, J., and D. W. Russell. 2001. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Schmidt, H. 2001. Shiga-toxin-converting bacteriophages. *Res. Microbiol.* 152:687–695.
- Schmidt, H., M. Bielaszewska, and H. Karch. 1999. Transduction of enteric *Escherichia coli* isolates with a derivative of Shiga toxin 2-encoding bacteriophage ϕ 3538 isolated from *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 65:3855–3861.
- Serra-Moreno, R., J. Jofre, and M. Muniesa. 2008. The CI repressors of Shiga toxin-converting prophages are involved in coinfection of *Escherichia coli* strains, which causes a down regulation in the production of Shiga toxin 2. *J. Bacteriol.* 190:4722–4735.
- Slanec, T., A. Fruth, K. Creuzburg, and H. Schmidt. 2009. Molecular analysis of virulence profiles and Shiga toxin genes in food-borne Shiga toxin-producing *Escherichia coli*. *Appl. Environ. Microbiol.* 75:6187–6197.
- Smith, D. L., C. E. James, M. J. Sergeant, Y. Yaxian, J. R. Saunders, A. J. McCarthy, and H. E. Allison. 2007a. Short-tailed Stx phages exploit the conserved YaeT protein to disseminate Shiga toxin genes among enterobacteria. *J. Bacteriol.* 189:7223–7233.
- Smith, D. L., B. M. Wareing, P. C. M. Fogg, L. M. Riley, M. Spencer, M. J. Cox, J. R. Saunders, A. J. McCarthy, and H. E. Allison. 2007b. Multilocus characterization scheme for Shiga toxin-encoding bacteriophages. *Appl. Environ. Microbiol.* 73:8032–8040.
- Stephan, R., S. Schumacher, S. Corti, G. Krause, J. Danuser, and L. Beutin. 2008. Prevalence and characteristics of Shiga toxin-producing *Escherichia coli* in Swiss raw milk cheeses collected at producer level. *J. Dairy Sci.* 91:2561–2565.

- Strauch, E., J. A. Hammerl, A. Konietzny, S. Schneiker-Bekel, W. Arnold, A. Goesmann, A. Pühler, and L. Beutin. 2008. Bacteriophage 2851 is a prototype phage for dissemination of the Shiga toxin variant gene 2c in *Escherichia coli* O157:H7. *Infect. Immun.* 76:5466–5477.
- Tozzi, A. E., A. Caprioli, F. Minelli, A. Gianviti, L. De Petris, A. Edefonti, G. Montini, A. Ferretti, T. De Palo, M. Gaido, and G. Rizzoni., and the Hemolytic Uremic Syndrome Study Group. 2003. Shiga toxin-producing *Escherichia coli* infections associated with hemolytic uremic syndrome, Italy, 1988–2000. *Emerg. Infect. Dis.* 9:106–108.
- Vernozy-Rozand, C., M. P. Montet, M. Berardin, C. Bavai, and L. Beutin. 2005. Isolation and characterization of Shiga toxin-producing *Escherichia coli* strains from raw milk cheeses in France. *Lett. Appl. Microbiol.* 41:235–241.
- Zweifel, C., N. Giezendanner, S. Corti, G. Krause, L. Beutin, J. Danuser, and R. Stephan. 2010. Characteristics of Shiga toxin-producing *Escherichia coli* isolated from Swiss raw milk cheese within a 3-year monitoring program. *J. Food Prot.* 73:88–91.