Microbiology (2015), **161**, 451–462 DOI 10.1099/mic.0.000003

Review

Shiga toxins and *stx* phages: highly diverse entities

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Received 24 September 2014
Accepted 5 November 2014

Shiga toxins are the main virulence factors of a group of *Escherichia coli* strains [Shiga toxin-producing *E. coli* (STEC)] that cause severe human diseases, such as haemorrhagic colitis and haemolytic–uraemic syndrome. The Shiga toxin family comprises several toxin subtypes, which have been differentially related to clinical manifestations. In addition, the phages that carry the Shiga toxin genes (*stx* phages) are also diverse. These phages play an important role not only in the dissemination of Shiga toxin genes and the emergence of new STEC strains, but also in the regulation of Shiga toxin production. Consequently, differences in *stx* phages may affect the dissemination of *stx* genes as well as the virulence of STEC strains. In addition to presenting an overview of Shiga toxins and *stx* phages, in this review we highlight current knowledge about the diversity of *stx* phages, with emphasis on its impact on STEC virulence. We consider that this diversity should be taken into account when developing STEC infection treatments and diagnostic approaches, and when conducting STEC control in reservoirs.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) strains are a diverse group of *E. coli* that cause severe human diseases, such as haemorrhagic colitis and haemolytic–uraemic syndrome (HUS) (Riley *et al.*, 1983; Karmali *et al.*, 1985). The latter was originally defined as a combination of renal failure, thrombocytopenia and haemolytic anaemia affecting mainly infants and children (Gianantonio *et al.*, 1964). Recently, the definition of HUS has come to include documented haemolysis rather than anaemia, platelet consumption rather than thrombocytopenia and signs of renal damage rather than renal failure (Ardissino *et al.*, 2014). HUS lacks specific treatment; HUS patients are generally given supportive care of electrolytes for water imbalance, anaemia, hypertension and renal failure (Mele *et al.*, 2014). The mortality rate has decreased due to improved diagnosis and treatment, yet 1–2 % of patients die during the acute phase of the disease (Loirat, 2013; Mele *et al.*, 2014) and ~30 % of patients evidence long-term renal damage (Garg *et al.*, 2003; Spinale *et al.*, 2013).

Ruminants, especially bovine animals, are the main reservoir of STEC strains (Naylor *et al.*, 2005). These animals are asymptomatic carriers of STEC and so they generally enter the human food chain. As a result of practices during slaughtering, milking or later when handling and packaging the products, meat and milk often become the main sources of human infection in some countries (Riley *et al.*, 1983; Bell *et al.*, 1994; Guh *et al.*, 2010). Other vehicles of STEC infection include lettuce, spinach, sprouts, watercress, strawberries, apple cider, and drinking and recreational water (Robert Koch Institute, 2011; Launders *et al.*, 2013; Luna-Gierke *et al.*, 2014).

We have gained a great deal of new knowledge about STEC over recent decades. However, many questions remain unanswered and some old statements need to be revised, as demonstrated in the HUS outbreak in Germany in 2011. This outbreak was caused by an unusual *E. coli* strain, occurred in a high proportion of adult patients and had no evidence of zoonotic origin (Frank *et al.*, 2011; Mellmann *et al.*, 2011; Piérard *et al.*, 2012). We learnt that strains lacking in typical virulence factors or belonging to infrequent serotypes may be highly virulent. Moreover, this outbreak highlighted the role of mobile elements, especially phages harbouring *stx* genes, in STEC virulence. In particular, regarding O26 strains from Scotland, Chase-Topping *et al.* (2012) suggested that stx_2 phage acquisition would increase the prevalence of those strains in severe human disease.

In this review, we analyse the published literature on the different Shiga toxin subtypes and *stx* phages, with special emphasis on their diversity, which can affect STEC virulence and the dissemination of Shiga toxin genes.

Shiga toxins

All STEC strains are able to produce Shiga toxins (Stx) – their main virulence factor. A single STEC strain may carry one or more Shiga toxin-encoding genes (*stx*) in their genome. Indeed, strains carrying three or more *stx* subtypes have been described (Bertin *et al.*, 2001; Eklund *et al.*, 2002; Krüger *et al.*, 2011). The *stx* genes are generally carried by

Abbreviations: HUS, haemolytic–uraemic syndrome; STEC, Shiga toxin-producing *Escherichia coli*.

prophages (usually called *stx* phages or Stx phages) and the toxins are released when bacteriophage-mediated bacteriolysis occurs.

The Shiga toxin family includes several toxins related to Shiga toxin from *Shigella dysenteriae* that share a similar structure and biological activity. The toxins produced by STEC strains are also called verotoxins ('toxic to Vero cells'), as initially described by Konowalchuk *et al.* (1977). Shiga toxins are AB5 proteins composed of one active A subunit bound to five B subunits. Their mode of action involves binding to a specific glycolipid on target cells via the B subunits followed by A subunit internalization and A1 fragment release. The RNA *N*-glycosidase activity of Shiga toxins inactivates 60S ribosomal subunits, leading to inhibition of protein synthesis (Endo *et al.*, 1988; Saxena *et al.*, 1989). In addition, evidence shows that Shiga toxins induce apoptosis in many cell types (Tesh, 2010).

Shiga toxin subtypes

All Shiga toxins share structural and enzymic characteristics; however, there are differences regarding sequence, biological activity and serological reactivity. Shiga toxins from *E. coli* are classified in two major types: Stx1 and Stx2. Each group comprises several subtypes, with the Stx2 group being more heterogeneous than the Stx1 group. The different Stx subtypes have been described over time by using different methods and criteria. This has led to a great deal of confusion about Stx nomenclature and has also hindered comparisons amongst studies performed with different subtyping approaches.

To standardize Shiga toxin nomenclature, Scheutz *et al.* (2012) developed a system based on phylogenetic sequence-based relatedness of the proteins. According to this scheme, the Stx nomenclature (without numbers) is reserved for Shiga toxins when they are produced by *Shigella* spp., and Shiga toxin subtypes found in *E. coli* are designated Stx1a, Stx1c, Stx1d, Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f and Stx2g. Scheutz *et al.* (2012) also proposed a new PCR protocol to facilitate *stx* subtyping standardization.

Stx subtyping is not only useful for STEC characterization, but also valuable for diagnosis as some types and subtypes of Shiga toxin have been epidemiologically associated with different clinical outcomes after STEC infection. A correlation has been observed between the stx_2 genotype and severity of disease, as several studies have shown that some stx_2 subtypes are frequently associated with a higher risk of developing HUS, whilst others are present mainly in strains isolated from patients with uncomplicated diarrhoea or in those not isolated from humans (Eklund, *et al.*, 2002; Friedrich *et al.*, 2002; Zhang *et al.*, 2002; Jenkins *et al.*, 2003; Leung *et al.*, 2003; Beutin *et al.*, 2004; Girardeau *et al.*, 2005; Bielaszewska *et al.*, 2006; Persson *et al.*, 2007). By applying the recently proposed nomenclature to previous studies, it is possible to correlate stx_{2a} with high virulence and HUS, and stx_{2e}, stx_{2f} and stx_{2g} with low pathogenicity in humans. However, it is not always possible to apply the current nomenclature to results of studies that used previous subtyping methods because the designations are not equivalent. Therefore, it is necessary to apply the new subtyping method and nomenclature to more studies to confirm the risk associated with each *stx* subtype in STEC infections and its clinical significance.

Several studies have also linked some *stx* subtypes to specific reservoirs. Furthermore, particular *stx* subtypes could affect the level of STEC shedding by cattle and consequently the risk of transmission to humans (Matthews *et al.*, 2013). Although most *stx* subtypes have been detected in STEC strains isolated from cattle and beef products, some predominate amongst bovine STEC strains whereas others are rarely detected in such strains (Bertin *et al.*, 2001; Brett *et al.*, 2003a; Gobius *et al.*, 2003; Beutin *et al.*, 2007; Krüger *et al.*, 2011). Examples of the latter include stx_{1c}, which has frequently been detected in STEC isolated from ovine faeces (Koch *et al.*, 2001; Brett *et al.*, 2003b), stx_{2e}, the common *stx* subtype in STEC strains responsible for oedema disease of swine (Linggood & Thompson, 1987; Weinstein *et al.*, 1988), and stx_{2f}, detected in STEC strains isolated from the faeces of feral pigeons (Schmidt *et al.*, 2000). Once again, it is not always possible to assign the new nomenclature to results obtained using previous subtyping methods. Future studies using the standardized protocol will contribute to confirming associations between *stx* subtypes and reservoirs, and enable us to gain a better understanding of the epidemiology of STEC infections.

The variability amongst Shiga toxins and *stx* gene sequences also has several implications for STEC detection. In some epidemiological or clinical studies, STEC presence may be underestimated when using methods that detect only a limited number of *stx* subtypes. To avoid this, PCR and quantitative PCR assays designed for STEC detection should include all *stx* subtypes and, if possible, additional identification of the *stx* subtypes. For example, a recent study in The Netherlands evaluated the presence of stx_{2f} – a generally underdiagnosed subtype. The results showed that whilst this subtype is still associated with mild STEC infections (Friesema *et al.*, 2014), its frequency is higher than expected.

Diversity of Shiga toxin production and toxicity

Cytotoxic activity on Vero cells is a common characteristic of STEC strains due mainly to the production of Shiga toxins. However, verotoxicity assays of supernatants of STEC cultures have shown that titres vary amongst strains. Differences in cytotoxicity, Stx production or clinical outcome have been related to the number and/or type or subtype of *stx* genes carried by the STEC strain (Bertin *et al.*, 2001; Eklund *et al.*, 2002; Krüger *et al.*, 2011).

Despite epidemiological and experimental observations that link some *stx* subtypes with highly pathogenic strains, their molecular basis is not completely understood. At the same time, other factors seem to be involved. On the one

hand, particular characteristics of Stx toxin, like receptor-binding affinity, have been correlated with cytotoxic specificity on different cell lines (Tyrrell *et al.*, 1992). In a study of potency of purified Stx toxins, Fuller *et al.* (2011) found differences amongst Stx subtypes under both *in vitro* and *in vivo* conditions. In particular, Stx2a and Stx2d proved more potent than Stx2b, Stx2c and Stx1. When comparing Stxs to chimeric toxins, Russo *et al.* (2014) found that the different toxicity between Stx1a and Stx2a on cells and in mice relies on the B subunit. On the other hand, there is diversity in *stx* expression amongst STEC strains, which may account for differences in virulence. Neupane *et al.* (2011) reported an overexpression of Stx2 in *E. coli* O157 : H7 strains associated with severe human disease. As discussed in the following sections, Stx production is related to the level of phage production (Köhler *et al.*, 2000; Muniesa *et al.*, 2003; de Sablet *et al.*, 2008; Łoś *et al.*, 2009), and phages with distinct genotypes were found to produce markedly different amounts of Stx2 (Wagner *et al.*, 1999).

Stx phages

The role of bacteriophages in the transference of *stx* genes was identified in the 1980s, and phages carrying *stx* (here named *stx* phages) were soon isolated and analysed (Scotland *et al.*, 1983; Smith *et al.*, 1983, 1984; O'Brien *et al.*, 1984). The first comparative studies showed a relationship between *stx* phages and λ phages (Huang *et al.*, 1987). Complete sequence studies of *stx* phages reported at the end of the 1990s confirmed that *stx* phages have sequence and gene organization levels similar to those of lambdoid phages, and also showed gene clusters with related functions, including recombination, early regulation, replication, late regulation, lysis and head and tail structural gene regions (Makino *et al.*, 1999; Miyamoto *et al.*, 1999; Plunkett *et al.*, 1999). The location of *stx* genes within the phage lysis region, in addition to data from functional and genetic analysis of regulatory regions, indicated a link between Shiga toxin production and phage release during lytic growth (Mühldorfer *et al.*, 1996; Neely & Friedman, 1998; Fuchs *et al.*, 1999; Karch *et al.*, 1999; Miyamoto *et al.*, 1999; Plunkett *et al.*, 1999). Furthermore, Wagner *et al.* (1999) proposed an active role for *stx* phages in STEC pathogenesis.

Induction of *stx* phages

The *stx* phages have a phage cycle regulation similar to bacteriophage λ. In the lysogenic state, the DNA of the *stx* phage is integrated into the STEC chromosome and the expression of most *stx* phage genes, including *stx*, is inhibited. In the absence of external inducing agents, most of the lysogens are stable; however, a small subpopulation is induced spontaneously. Under certain conditions, repression is removed, phage genes are expressed and *stx* phages are produced and released. This switch from the lysogenic state to the lytic state is called induction. Thus, expression of *stx* in STEC depends primarily on prophage induction (Wagner *et al.*, 2001; Tyler *et al.*, 2013), although stx_1 transcription can be also driven by its own promoter under low iron conditions (Calderwood & Mekalanos, 1987; Aertsen *et al.*, 2005b).

A higher level of spontaneous induction has been reported for *stx* phages in comparison with non-*stx* phages. According to Livny & Friedman (2004), this trait may be valuable for STEC population provided Stx production confers an advantage. The non-induced lysogens may benefit from Stx production as this toxin can cause the death of eukaryotic cells, such as unicellular predators or human leukocytes (Steinberg & Levin, 2007; Łoś *et al.*, 2011; Mauro & Koudelka, 2011). This supports the 'model of STEC altruism' described and analysed by Łoś *et al.* (2013). Furthermore, it has been proposed that Stx has evolved as a mechanism of defence against protozoa that confers a selective advantage for bacteria harbouring *stx* phages (Stolfa & Koudelka, 2012).

As in the case of λ, the induction of *stx* prophages has been shown to be controlled by RecA (Mühldorfer *et al.*, 1996; Fuchs *et al.*, 1999) – a regulator of the SOS bacterial response. Furthermore, it is also assumed that agents or conditions that lead to bacterial DNA damage activate RecA, which cleaves the phage repressor and finally causes prophage induction. The role of RecA has also been evidenced in studies showing a higher level of spontaneous *stx* phage induction in *recA*-positive strains in comparison with *recA*-negative strains (Mühldorfer *et al.*, 1996; Fuchs *et al.*, 1999; Livny & Friedman, 2004; Imamovic & Muniesa, 2012). Several studies have reported enhanced production of *stx* phage particles and Stx under typical SOS inducers, such as UV irradiation and mitomycin C. In addition to activated RecA, other mechanisms could contribute to *stx* prophage induction (Muniesa *et al.*, 2004a; Imamovic & Muniesa, 2012; Nassar *et al.*, 2013). In a recent study, Imamovic & Muniesa (2012) described RecA-independent induction of stx_2 phages by EDTA due to its chelating property.

It is important to note that induction efficiency varies amongst the different *stx* prophages (Muniesa *et al.*, 2004a; de Sablet *et al.*, 2008; Karama & Gyles, 2008; García-Aljaro *et al.*, 2009; Łoś *et al.*, 2009). In addition, the *stx* phages are not equally sensitive to the different inducers.

Several factors have been shown to regulate the lysis/lysogeny switch. Such factors include hydrogen peroxide (Wagner *et al.*, 2001; Łoś *et al.*, 2009, 2010), high temperature in combination with UV irradiation (Yue *et al.*, 2012), EDTA (Imamovic & Muniesa, 2012), sodium citrate (Imamovic & Muniesa, 2012; Nejman-Faleńczyk *et al.*, 2012), amino acid starvation (Nejman-Faleńczyk *et al.*, 2012), phenethyl isothiocyanate (Nowicki *et al.*, 2014), DNase colicins (Toshima *et al.*, 2007), high hydrostatic pressure (Aertsen *et al.*, 2005a), sodium chloride (Łoś *et al.*, 2009; Harris *et al.*, 2012), nitric oxide (Vareille *et al.*, 2007), ^{60}Co irradiation (Yamamoto *et al.*, 2003) and several antibiotics, such as azithromycin, ciprofloxacin, fosfomycin, imipenem, gentamicin, norfloxacin and rifampicin (Matsushiro *et al.*, 1999; Köhler *et al.*, 2000; Zhang *et al.*,

2000; Ohara *et al.*, 2002; Herold *et al.*, 2005; Ochoa *et al.*, 2007; Łoś *et al.*, 2009; Nassar *et al.*, 2013), as well as those antibacterials used as growth promoters in animal production (Köhler *et al.*, 2000).

The expression of *stx* phage genes can be regulated by the presence of other phages in the host genome (Serra-Moreno *et al.*, 2008; Fogg *et al.*, 2012) and interactions amongst O157 prophages can complement the functions of defective prophages (Asadulghani *et al.*, 2009). Some studies show that the presence of more than one *stx* phage in the same strain affects Stx production in comparison with strains harbouring only one *stx* phage. Either an increase or a decrease in toxin production has been reported (Muniesa *et al.*, 2003; Serra-Moreno *et al.*, 2008; Fogg *et al.*, 2012).

In addition to the characteristics of the phages, bacterial factors are also involved in the induction process. Several studies indicate a co-regulation between *stx* phages and the bacterial host. On the one hand, genetic and physiological conditions of the lysogen influence phage-inducing capacity (Muniesa *et al.*, 2004a; Imamovic & Muniesa, 2012) and indeed the host effect on phage development seems to be more pronounced on *stx* phages than in λ (Węgrzyn *et al.*, 2014). On the other hand, *stx* phage lysogeny has a direct effect on the global expression of bacterial genes; moreover, an increase in acid tolerance and motility has been reported when bacteria were lysogenized (Su *et al.*, 2010). Tree *et al.* (2014) found that stx_2 bacteriophages encoded an anti-small RNA that can regulate bacterial mRNA translation. Phages can also regulate different steps of STEC interaction with the intestinal epithelium, providing a selective advantage to STEC strains for colonization and persistence. For example, Stx increases the expression of nucleolin, which is one of the receptors for intimin – an adhesin of STEC (Robinson *et al.*, 2006). In addition, it was described that the presence of stx_2 phages represses the type III secretion system and it was hypothesized that this repression is then overcome when appropriate niche signals are detected (Xu *et al.*, 2012). Tozzoli *et al.* (2014a) identified a regulatory region in *stx* phages that downregulates the expression of type III secretion, mainly present in O157 strains isolated from humans.

Diversity of *stx* phages

All phages carrying a *stx* gene are considered, by definition, *stx* phages. Although they commonly share several characteristics, it is not surprising that this is a heterogeneous group. The *stx* phages present different morphologies, e.g. a regular hexagonal head and a short tail, an elongated head and a long tail, and a regular hexagonal head and a long tail (Rietra *et al.*, 1989; Muniesa *et al.*, 2000; Allison *et al.*, 2003; Karama & Gyles, 2008). Furthermore, there is also heterogeneity in the host infectivity range (Gamage *et al.*, 2004; Muniesa *et al.*, 2004a).

The genome size of sequenced *stx* phages ranges from 29.7 to 68.7 kb (Table 1), with most >60 kb. Genomic differences amongst *stx* phages have been made evident by several approaches, including RFLP patterns (Osawa *et al.*, 2000), polymorphic prophage patterns (Park *et al.*, 2013) and a multilocus characterization scheme (Smith *et al.*, 2007). Developments in sequencing technologies over recent years have allowed for complete nucleotide sequencing of several *stx* phages. Table 1 lists *stx* phages whose complete sequences are available in GenBank. Recent comparisons of whole genomes have confirmed that *stx* phages share a general genomic organization, but with a significant degree of sequence diversity, reinforcing the concept of their mosaic nature (Ahmed *et al.*, 2012; Smith *et al.* 2012; Steyert *et al.*, 2012; Cooper *et al.*, 2014; Tozzoli *et al.*, 2014a). Furthermore, some studies identified different types of *stx* phages harbouring even the same stx_2 subtype (Ahmed *et al.*, 2012; Tozzoli *et al.*, 2014b).

In an analysis of loci representing key modules involved in infection and propagation of *stx* phages (*int*, *N*, *cI*, *cro*, *cII*, *Q*, *O*, *P*, *stx*, capsid and tail structural genes, packaging), Smith *et al.* (2012) found a high level of genetic diversity amongst 11 *stx* phages as they observed that no two phages of that group possessed an identical genetic profile.

The genomes of *stx* phages encode many hypothetical proteins and carry genes with poorly understood roles for phage biology, mainly in the late region (Smith *et al.*, 2012). Amongst others, the gene encoding a putative DNA adenine methyltransferase has been identified in some *stx* phages (Cooper *et al.*, 2014). Recently, Nübling *et al.* (2014) described a functional esterase encoded downstream of the stx_{2a} operon in the bacteriophage 933W, and homologue genes are present in many stx_2-encoding phages (Unkmeir & Schmidt, 2000).

Insertion site diversity

The integration sites of *stx* phages in the bacterial chromosome also show great diversity. Considering the *stx* phages present in *E. coli* O157 : H7 strains, two insertion sites were first described as preferred: *wrbA* and *yehV* (Shaikh & Tarr, 2003; Besser *et al.*, 2007). However, there are other integration sites described for *stx* phages in O157, such as *sbcB*, *argW* and *yecE* (De Greve *et al.*, 2002; Mellor *et al.*, 2012; Shringi *et al.*, 2012).

Several integration sites have been described for *stx* phages in non-O157 STEC strains, i.e. *argW*, *potC*, *prfC*, *serU*, *ssrA*, *wrbA*, *yciD*, *yecD*, *yecE*, *yjbM*, *ynfH* and *Z2577* (Recktenwald & Schmidt, 2002; Koch *et al.*, 2003; Ahmed *et al.*, 2012; Steyert *et al.*, 2012; Cooper *et al.*, 2014).

The factors that mediate the integration of a *stx* phage in a specific locus have not been clearly identified and, in contrast to the immunity to superinfection of λ lysogens, double lysogens have been detected (Allison *et al.*, 2003). In an interesting study evaluating chromosomal site specificity, Serra-Moreno *et al.* (2007) detected that phages preferentially use one insertion site depending on the host strain; however, if the preferred locus is unavailable, the

phage integrates into a secondary insertion site. In addition, Steyert *et al.* (2012) observed heterogeneity in integrase genes amongst *stx* phages in LEE (locus of enterocyte effacement)-negative strains. Such variants could be associated with phage insertion at specific genomic locations.

Dissemination of *stx* phages outside the intestine

Despite the difficulties in detecting and isolating free *stx* phages, some studies have described their occurrence in cattle faeces, river water and sewage (Muniesa *et al.*, 2004b; Dumke *et al.*, 2006; Oot *et al.*, 2007; McDonald *et al.*, 2010), demonstrating the circulation of these phages in the environment. It has been shown that *stx* phages can persist longer than their host bacteria in an aquatic environment (Muniesa *et al.*, 1999; Allué-Guardia *et al.*, 2014). In addition, some *stx* phages have a high ability to tolerate exposure to certain disinfectants and can maintain their infectivity under food-processing conditions (Muniesa *et al.*, 1999; Kajiura *et al.*, 2001; Rode *et al.*, 2011).

Considering the role of these phages as vectors of *stx* genes, conditions that augment the replication and release of *stx* phages could facilitate the spread of *stx* genes. Some studies have shown that transmission of *stx* phages may occur in water (Imamovic *et al.*, 2009), in various food matrices (e.g. milk, orange juice, salad and ground beef) (Imamovic *et al.*, 2009; Picozzi *et al.*, 2012) and also in biofilms (Solheim *et al.*, 2013).

STEC strains may encounter several factors in the environment that could activate the lytic cycle of *stx* phages. The fact that different *stx* phages can be differentially induced should be taken into account in future studies to allow a better understanding of factors that enhance *stx* phage dissemination.

Human STEC infections and *stx* phages

Shiga toxins are considered the main virulence factor of STEC and it is accepted that the pathogenicity of STEC in humans depends on phage-regulated Stx production. Moreover, Tyler *et al.* (2013) showed that Stx2 production and disease in an enterohaemorrhagic *E. coli* mouse model were directly related to induction of the 933W prophage. However, the *stx* phage characteristics that contribute to both high virulence and variation in disease severity are poorly understood.

In a recent study, Tozzoli *et al.* (2014a) performed a microarray analysis of O157 STEC strains from Italy, comparing some strains isolated from human infections and others from animal sources. Interestingly, they found that the stx_2 phage was the major source of variability between the two groups. They identified two polymorphic regions, one between the *gam* and *c*II genes, associated with lytic and lysogenic cycles, and the other between *roi* and *s*.

Another phage region that shows diversity and could be related to the pathogenicity of STEC is the *Q* gene, which codes for a transcriptional antiterminator that controls expression of late phage genes in lambdoid phages. This gene is generally present amongst *stx* phages and located upstream of the *stx* genes (Smith *et al.*, 2007, 2012). Amongst the *stx* phages carried by O157 strains, two *Q* variants have been described: *Q*933 and *Q*21. Moreover, a relationship between the *Q* allele and the level of *stx* expression has been suggested (LeJeune *et al.*, 2004; Ahmad & Zurek, 2006; Zhang *et al.*, 2010; Mellor *et al.*, 2012). Recently, Steyert *et al.* (2012) observed phylogenetic diversity of *stx* phages in an analysis of *Q* sequences, identifying seven clusters amongst 15 selected strains. The *Q* sequences associated with the highest level of *stx* expression were found to be clustered together.

To evaluate the association between *stx* phage induction and disease, it is also important to take into account the possible role of intestinal factors, which may also vary amongst hosts. Currently, little is known about the effect of the specific conditions of mammalian hosts on the induction of *stx* prophages (Livny & Friedman, 2004; Łoś *et al.*, 2009). Oxidative stress has been suggested as one of the conditions that may occur in the intestine of an infected human and could influence the induction of *stx* phages (Łoś *et al.*, 2010). This idea is supported by *in vitro* experiments that show that hydrogen peroxide and neutrophils increase Stx2 production (Wagner *et al.*, 2001), and by the fact that *stx* phages are induced in cultures of STEC strains treated with hydrogen peroxide (Łoś *et al.*, 2009, 2010). However, human microbiota and their secreted products can inhibit Stx production (Gamage *et al.*, 2003, 2006; de Sablet *et al.*, 2009). Gamage *et al.* (2003, 2006) suggested that susceptibility of the intestinal flora to *stx* phages could exert either a protective or an antagonistic role in STEC disease and they proposed that toxin production by intestinal flora may represent another strategy of pathogenesis. Recent studies on the mechanisms that could be involved in *stx* expression *in vivo* (Bentancor *et al.*, 2013a, b) have shown that stx_2 can be transcribed and translated in mammalian cells, producing biologically active Stx. The toxin could therefore be produced after the uptake of *stx* phages into eukaryotic cells, but the mechanisms by which the phages are taken up and the DNA transcribed remain unclear.

The role of *stx* phages in STEC pathogenicity impacts directly on therapeutic approaches to treating STEC infections. First, unlike most other bacterial infections, the treatment of human STEC infection with some antibiotics may have adverse clinical consequences (Wong *et al.*, 2000; Zhang *et al.*, 2000; McGannon *et al.*, 2010) due to the effect of several antibiotics on *stx* phage induction and Shiga toxin production (Yee *et al.*, 1993; Kimmitt *et al.*, 1999, 2000; Matsushiro *et al.*, 1999; Zhang *et al.*, 2000). Although some *in vitro* studies show that certain antibiotics eliminate STEC without triggering the phage lytic cycle, they do not necessarily imply the elimination of Stx production in the intestine (McGannon *et al.*, 2010). Additionally, several types of *stx* phage should be included in the studies to generalize results.

Table 1. Characteristics of *stx* phages whose complete sequences have been submitted to the GenBank

All the information is presented as available in the GenBank accession or its linked reference.

stx phage	Genome size (kbp)	*stx* type or subtype	Strain host				GenBank accession no.
			Integration site	Name	Serotype	Origin	
933W	61.7	stx_2	*wrbA*	EDL933	O157 : H7	NA	AF125520
VT2-Sa	60.9	stx_2	NA	Sakai RIMD 0509894	O157 : H7	Japan outbreak	AP000363
VT1-Sakai	47.9*	stx_1	*yehV*	Sakai RIMD 0509952	O157 : H7	Japan outbreak	AP000400
VT2-Sakai	62.7*	stx_2	*wrbA*	Sakai RIMD 0509952	O157 : H7	Japan outbreak	AP000422
CP-933V	48.9*	stx_1	*yehV*	EDL933	O157 : H7	Hamburger, outbreak of haemorrhagic colitis, HUS	AE005174†
P27	42.6	stx_{2e}	*yecE*	2771/97	ONT : H^-	Patient with diarrhoea	AJ298298
Stx2ϕ-I	61.8	stx_2	*wrbA*	Okayama O-27	O157 : H7	Japan outbreak	AP004402
Stx1ϕ	59.9	stx_1	NA	Morioka V526	O157 : H7	NA	AP005153
Stx2ϕ-II	62.7	stx_2	NA	Morioka V526	O157 : H7	NA	AP005154
CP-1639	NA	stx_1	NA	1639/77	O111 : H-	Patient with bloody diarrhoea	AJ304858
BP-4795	57.9	stx_1	*yehV*	4795/97	O84 : H4	Patient with diarrhoea	AJ556162
86	60.2	stx_2	NA	DIJ1	O86 : H-	Japan	AB255436
Min27	63.4	stx_2	NA	Min27	O157 : H7	Piglet with diarrhoea, China	EU311208
2851	57.2	stx_{2c}	*sbcB*	CB2851	O157 : H7	Human	FM180578
1717	59.9*	stx_{2c}	*sbcB*	EC970520	O157 : H7	NA	FJ188381
YYZ-2008	52.7*	stx_1	NA	EC970520	O157 : H7	NA	FJ184280
NA	62.3	stx_2	*argW*	EC4115	O157 : H7	Human, at the time of spinach outbreak, USA	CP001164†
NA	57.2	stx_{2c}	*sbcB*	EC4115	O157 : H7	Human, at the time of spinach outbreak, USA	CP001164†
EC026_P06	55.5	stx_1	*wrbA*	11368	O26 : H11	Patient with diarrhoea, Japan	AP010953†
ECO103_P15	53.9	stx_1	*prfC*	12009	O103 : H2	Sporadic case of diarrhoea, Japan	AP010958†
ECO103_P12	62.6	stx_2	*argW*	12009	O103 : H2	Sporadic case of diarrhoea, Japan	AP010958†
ECO111_P16	29.7	stx_1	*ssrA*	11128	O111 : H-	Sporadic case of diarrhoea, Japan	AP010960†
ECO111_P11	48.1	stx_2	*yecE*	11128	O111 : H-	Sporadic case of diarrhoea, Japan	AP010960†
NA	NA	stx_2	*argW*	TW14359	O157 : H7	Patient, spinach-associated outbreak, USA	CP001368†
NA	NA	stx_{2c}	*sbcB*	TW14359	O157 : H7	Patient, spinach-associated outbreak, USA	CP001368†
NA	NA	stx_1	*yehV*	Xuzhou21	O157 : H7	HUS patient from 1999 outbreak, China	CP001925†
NA	NA	stx_2	*wrbA*	Xuzhou21	O157 : H7	HUS patient from 1999 outbreak, China	CP001925†
VT2ϕ_272	66.0	stx_2	NA	71074	O157 : H7	NA	HQ424691
TL-2011c	60.5	stx_2	*wrbA*	NVH-734	O103:H25	HUS patient, enterohaemorrhagic *E. coli* outbreak, Norway	JQ011318
P13374	60.9	stx_{2a}	*wrbA*	CB13374	O104:H4	Sprouted seeds, Germany	HE664024
NA	68.7	stx_{2a}	*wrbA*	2011C-3493	O104:H4	Patient with HUS, USA	CP003289†
NA	68.5	stx_{2a}	*wrbA*	2009EL-2050	O104:H4	Bloody diarrhoea, Republic of Georgia	CP003297†
NA	68.5	stx_{2a}	*wrbA*	2009EL-2071	O104:H4	Bloody diarrhoea, Republic of Georgia	CP003301†

Table 1. cont.

stx phage	Genome size (kbp)	*stx* type or subtype	Integration site	Strain host: Name	Strain host: Serotype	Strain host: Origin	GenBank accession no.
NA	62.5	stx_{2a}	*argW*	RM13514	O145:H28	Patient, lettuce-associated outbreak, USA	CP006027†
NA	47.1	stx_{2a}	*yecD–yecE*	RM13516	O145:H28	Ice-cream-associated outbreak, Belgium	CP006262†

NA, not available.
*Size of phage sequence calculated from GenBank data.
†Accession number to complete bacterial genome.

Second, the phage regulation of Stx production allows the use of novel therapeutics, like anti-induction strategies, which would not be directly bactericidal, but might lessen the risk of serious complications, such as HUS (Keen, 2012). Consequently, there are studies aimed at detecting conditions that repress phage induction. Nejman *et al.* (2011) have shown that plasmids derived from *stx* phages are not able to replicate in amino acid-starved bacteria, and Nowicki *et al.* (2013) have studied the mechanism responsible for the inhibition of *stx* phage replication under amino acid starvation, identifying the role of the ppGpp alarmone. Nejman-Faleńczyk *et al.* (2012) have suggested that reducing food consumption during illness, or even fasting, and providing minerals and citrate could be an option to manage STEC infections as they found that these conditions can delay and diminish the efficiency of phage particle formation. However, the authors also pointed out that such results correspond to a study involving only one phage. Bearing in mind the variability that exists amongst *stx* phages, more work is needed to generalize these conclusions.

Concluding remarks

In addition to Shiga toxin diversity, there is heterogeneity amongst the phages that carry *stx* genes and regulate their expression. Studies report differences in structure, genomic organization, response to different inducing agents and insertion site specificity. As a result, phage variability may affect the virulence of STEC strains.

In addition to its role in STEC pathogenicity, the diversity of *stx* phages could influence the dissemination of *stx* genes and the emergence of new STEC strains – events that at the same time can be promoted by other factors, such as certain environmental conditions.

There is still much to learn about the virulence of STEC strains and the characteristics and behaviour of *stx* phages, particularly those from non-O157 strains. We consider that studies on STEC virulence and epidemiology need to take into account the diversity of Shiga toxins and *stx* phages, not only to choose methodological approaches but also to draw conclusions. Moreover, the variability in phage induction and Shiga toxin production should be considered when evaluating treatments for STEC infections.

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Edited by: J. Lindsay