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Evolution of virulence factors in Shiga-toxin-producing *Escherichia coli*

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Abstract. The major demonstrated or putative virulence factors of Shiga-toxin-producing *Escherichia coli* (STEC) are the Shiga toxins, products of the locus of enterocyte effacement, and products encoded by the EHEC-hemolysin plasmid. Molecular analysis shows that STEC acquired the majority of these virulence factors by horizontal transfer of genetic material. In the case of Shiga toxins, the phages encoding them are probably responsible for this transfer. For the locus of enterocyte effacement, however, it is not clear how often this transfer took place and which parts of the locus were involved in this transfer. The large EHEC-hemolysin plasmid is clearly a mosaic structure, which arose from multiple recombination events with foreign DNA. Two lineages of this plasmid can be distinguished, one of which is associated with chromosomally encoded virulence factors. Despite the wealth of information available, further comparative studies are needed to decipher definitively the evolution of virulence in STEC.

Key words. STEC; SLTEC; VTEC; Shiga toxin; locus of enterocyte effacement; hemolysin; plasmid; evolution; virulence.

Introduction

Shiga-toxin-producing *Escherichia coli* strains (STEC; previously called Shiga-like-toxin-producing *E. coli* or verotoxin-producing *E. coli*) were first associated with severe disease in humans in North America in 1982 [1, 2]. Since then, they have emerged as a major group of foodborne pathogens in industrialized countries [3, 4]. STEC strains often colonize the gastrointestinal tract of cattle and fecal contamination of food represents the major source of infection for humans [4]. Individuals infected by STEC present with a broad range of clinical pictures. These encompass the life-threatening hemolytic uremic syndrome (HUS) and hemorrhagic colitis, but also uncomplicated diarrhea and even transient healthy carriage.

STEC strains have been found in more than 200 *E. coli* serotypes, but only a few (including O157:H7 and O157:H −) are responsible for the majority of recorded outbreaks and sporadic cases of STEC-associated disease in humans [5]. The reasons for the increased propensity of specific serotypes to cause disease are not exactly known. However, STEC isolates are very diverse in terms of virulence factors and epidemiologic studies suggest that the presence of particular virulence factors in some STEC serotypes and strains is associated with increased ability to cause disease in humans [6].

The major virulence factors of STEC are Shiga toxins (previously called Shiga-like toxins or verotoxins). Two major classes of Shiga toxins have been found in STEC, Stx1 and Stx2 [7]. Both types of toxin have similar structures and mode of action. They are formed by five identical B subunits and one A subunit. The B subunits are responsible for the binding of the toxin to the target cells. The A subunit is internalized, activated, and depurinates specific residues of the host cell ribosomes [8, 9]. This modification of the ribosomes finally inhibits protein synthesis in eukaryotic cells. In addition to Shiga toxins, a whole cluster of virulence factors encoded by a chromosomal region called the locus of enterocyte effacement (LEE) is present in many STEC isolates [10]. These factors are responsible for the attaching and effacing lesions typical of many STEC strains and of enteropathogenic *E. coli* (EPEC) [11].

The LEE is composed of at least 41 different genes organized in three major regions [12]. The first region encodes for a type III secretion system [13]. The second region encodes for an adhesin called intimin and for its translocated receptor [14]. The third region of the LEE encodes several secreted proteins (Esp) important in modification of host cell signal transduction during the formation of attaching and effacing lesions [11]. Finally, a large plasmid (EHEC-hemolysin plasmid) encoding several putative virulence factors can also be found in a large proportion of STEC serotypes associated with disease [15]. These factors include the enterohemorrhagic *E. coli* hemolysin [16], a bifunctional catalase/peroxidase (KatP) [17], a protease called EspP [18], and a type II secretion system [19].

The presence of the genes coding for these virulence factors on lysogenic phages (Shiga toxins), on a pathogenicity island (LEE), and on plasmids (EHEC-hemolysin, KatP, EspP, type II secretion system) suggests that they may have been transferred horizontally in bacterial populations. The acquisition of these virulence factors from other bacteria seems therefore a logical explanation for the emergence of STEC as a major pathogen [20].

Shiga toxins of STEC

Since the first description of Shiga toxins in *E. coli* [21, 22], the *stx* genes of many different STEC isolates have been sequenced. Comparison of these sequences shows that Stx1 is highly conserved and is practically identical to Stx from *Shigella dysenteriae* [20, 23, 24]. The *stx2* gene is much less conserved than *stx1* and this has led to the distinction of many Stx2 subtypes, including Stx2, Stx2c, Stx2vha, Stx2vhb, Stx2e, Stx2ev (or Stx2a), and several other variants from STEC isolates of serogroups O111, OX3, and serotype Ount:H12 [24–31]. The reasons for the different diversities of *stx1* and *stx2* remain to be clarified. However, codon usage in *stx* genes differs significantly from that in *E. coli* and suggests that they were transferred only recently to this bacterial species [20]. Stx2 may have entered *E. coli* earlier than Stx1, thus explaining the difference in diversity observed among the two variants. The alignment of the nucleotide sequences for the A and B subunits [20, 24], or for entire *stx* genes [23] from isolates of various STEC serotypes demonstrates four major lineages within *stx2*. The first encompasses the *stx2*, *stx2c*, *stx2vha* and *stx2vhb* variants and is the one most frequently found in STEC isolates from severe disease. The second lineage comprises a subgroup of recently described Stx variants also called VT2d [24]. The third and fourth lineages correspond to the pig-associated *stx2e* and the rare *stx2ev* (or *stx2a*) variants, respectively. The exact significance of these lineages in the evolution of STEC is still unclear and warrants further investigations. However, the diverse *stx* variants seem to be associated with different levels of pathogenicity and may also be found in strains with specific combinations of other virulence factors [24].

Except for *stx2e*, the genes for Shiga toxins of STEC are carried by temperate bacteriophages [32–35]. Conflicting results have been presented on the relatedness and morphology of phages carrying *stx1* and *stx2* [36]. However, they seem to be mainly lambdoid bacteriophages [34, 36–40]. Several *stx*-phages may be present simultaneously on the chromosome of STEC including not only the concomitant presence of *stx1*- and *stx2*-phages [25, 35], but also of different *stx2*-phages [26, 27, 31]. This fact, together with the presence of other non-*stx* lambdoid phages in the chromosome of STEC strains complicate tremendously the characterization of *stx*-phages and the study of their epidemiology and evolution. A mosaic structure of *stx* genes has also been observed and suggests that recombinations between *stx*-phages occurs in nature [23] and further obscure their evolution. Previous studies have shown some association between *stx1* or *stx2* and STEC serotypes [6]. This may indicate a certain stability of *stx*-phages in natural STEC populations. However, the presence of *stx*-phages in the environment [41] suggests that some dynamic in their spread may still exist. *E. coli* strains may be lysogenized by *stx*-phages from the environment, leading to a continuing generation of new STEC strains. This latter hypothesis is supported by the findings of Beutin et al. [42] showing that the *stx* profiles of STEC strains are relatively homogeneous within a population of genetically unrelated but epidemiologically related STEC isolates and differ between epidemiologically separated populations. The stability and pathogenic potential of these new STEC strains remain unknown. Nevertheless, the potential for the emergence of new STEC lineages with a high virulence level seems to be present.

The locus of enterocyte effacement

The LEE was first discovered in EPEC and was later described in STEC, in other categories of pathogenic *E. coli*, in *Hafnia alvei*, and in *Citrobacter freundii* [10]. In STEC, the presence of the LEE is strongly associated with disease [6]. The complete sequences of the LEE were recently published for one EPEC strain of serotype O127:H6 (E2348/69) [43] and for one STEC of serotype O157:H7 (EDL933) [44]. Comparison of these sequences showed that 41 contiguous open reading frames (ORFs) were common to the LEE of the EPEC and STEC strains studied. The GC content of these ORFs

was similar in both strains and significantly lower than in the rest of the *E. coli* chromosome. Codon usage for the two LEE sequences was also atypical for *E. coli* [44]. This clearly shows that the LEE of STEC and EPEC originates from another bacterial species and was transferred horizontally to these pathogens. The LEE of the STEC strain EDL933 contains an additional prophage sequence of approximately 7.5 kb, which is absent from the LEE of the EPEC strain E2348/69 [44]. The role of this prophage sequence in the evolution of the LEE is still not clear. It is also not clear if this represents a consistent difference between STEC and EPEC strains or if the presence or absence of the prophage sequence is associated with only a few particular *E. coli* phylogenetic lineages. The distribution of substitutions between the two sequences for the 41 ORFs common to STEC and EPEC is not homogeneous. Genes encoding factors involved in direct interaction with host cells (i.e., intimin and its receptor, and those factors involved in modification of host cell signal transduction) show more substitutions than other genes of the LEE. These particular virulence factors are under high selective pressure from the immune system of the host and may evolve in a different way than the rest of the LEE [44].

Recently, we studied the diversity of the intimin gene (*eae*) in a broad sample of STEC isolates to obtain some insight into the evolution of the LEE in STEC [45]. For this purpose, the substitution rates between variants of the *eae* in 18 STEC serotypes were estimated for a 1.1-kb PCR product. This PCR product did not encompass the region coding for the binding site of intimin to the host cell surface, which may have introduced some bias in our analysis. Our results revealed two distinct groups of intimin variants. The first contains intimin from the major serotypes O157:H7, O157:H−, O145:H−, O111:H8, and O111:H−, whereas the second group contains intimin from the major serotypes O26:H11, O26:H−, and O103:H2. These results were supported by those obtained by PCR on the *espB* gene, and are in agreement with the suggestion of Kaper et al. [11] that strains with similar *eae* genes may also share similar *esp* genes. Thus, our results on the division of the *eae* gene into two major lineages may be valid for a larger region of the LEE, or even for the whole LEE. Recent reports by others [46–48] have shown that depending on the phylogenetic lineage of *E. coli*, the LEE may be inserted either at the *selC*, at the *pheU* or at another yet unknown locus in the chromo-

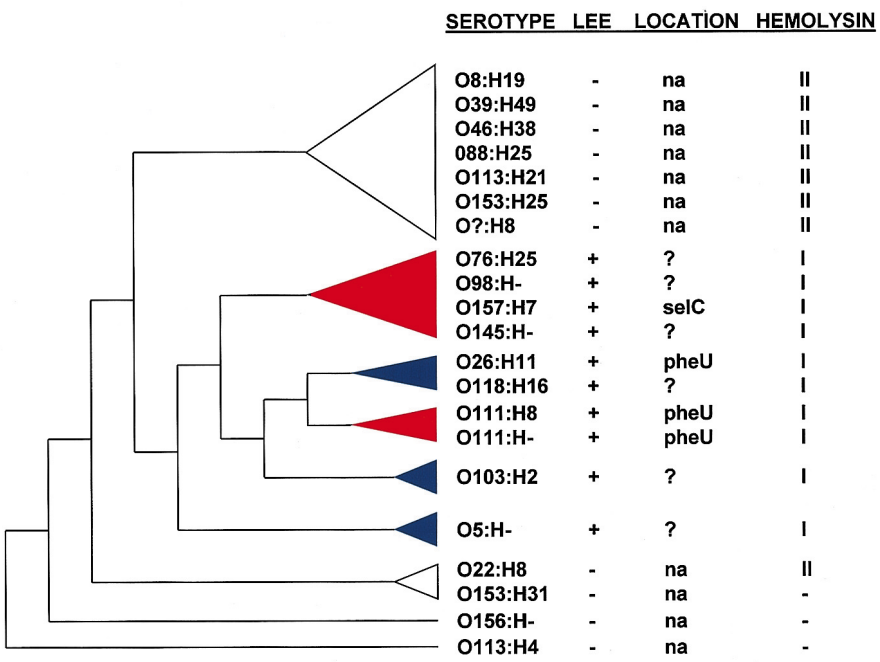

Figure 1. Phylogenetic relationships among 21 different STEC serotypes. The figure represents the most parsimonious tree obtained on the basis of amplified fragment length polymorphism results. Red and blue triangles represent the two major groups of *eae* genes mentioned in the text. LEE, + and − represent presence or absence of the LEE in the respective serotypes; LOCATION, location of the LEE described in the literature for the respective serotypes; na, not applicable; ?, unknown location; HEMOLYSIN, I and II represent the major lineages of EHEC-hemolysin described in the text.

some. The relatively small number of STEC and EPEC serotypes for which the chromosomal location of the LEE is known does not allow extensive comparison with our results on the distinction of two major *eae* lineages. The location of the LEE in the selC locus of the EPEC serotype O127:H6 strain E2348/69 and for STEC O157:H7 serotype strains is in agreement with the similarity of their *eae* genes. However, STEC strains of serotype O111:H8 and O26:H11 are phylogenetically very close [45, 49] and have the LEE inserted at the same locus (*pheU*), but have *eae* genes of different lineages (fig. 1). This lack of congruence in the data suggests that the LEE has been horizontally transferred more frequently than indicated by its insertion sites. The location of the LEE as a function of phylogenetic lineages could be due to a propensity to insert at a lineage-specific site. Alternatively, recombinations and horizontal transfer for only parts of the LEE (including *eae*) may occur. This later hypothesis is in agreement with the observation of Perna et al. [44] showing that the distribution of mutations is not homogeneous within the LEE.

EHEC-hemolysin plasmid

The EHEC-hemolysin plasmid is a non-conjugative F-like plasmid [50], varying in size between STEC serotypes but relatively constant within a single serotype. Isolates of *eae*-negative serotypes generally carry larger plasmids than *eae*-positive serotypes [45]. The smallest EHEC-hemolyin plasmids are approximately 70 kb in size [51] and the largest may be two to three times larger. The EHEC-hemolysin plasmid in STEC isolates of serotype O157:H7 is called pO157. Its complete sequence for two different strains has been recently published [52, 53]. As for many plasmids, pO157 shows a mosaic structure and its base composition is not homogeneously distributed. On the basis of this latter criterion, blocks can be delimited which correspond to functional regions of the plasmid [52]. These regions are often bordered by insertion sequences (IS) or remnants of IS [52] similar to those of the large *Shigella* virulence plasmids [53]. These observations support the hypothesis of the integration of fragments from different evolutionary origins into an F-like plasmid to form the actual pO157 [52]. Thus, virulence factors or putative virulence factors found on the different sectors of pO157 may be of different origins. The EHEC-hemolysin operon (*ehxCABD*) forms a first segment that is probably foreign in origin since it has a different GC% and codon usage than the surrounding regions. The adjacent block of genes encoding a type II secretion system also seems to have a different origin. IS with probably functional transposases can be found around *katP* and *espP* [52], suggesting that these genes represent mobile elements. This hypothesis is supported by the finding that the EHEC-hemolysin plasmids of O157:H7 isolates may sometime carry the *ehxCABD* operon but lack the *espP* gene (P. Boerlin and C. L. Gyles, unpublished observation). Finally, a new putative virulence factor reminiscent of large clostridial toxins seems to form another block of foreign DNA in pO157 [52, 53].

Unfortunately, complete sequences are not available for EHEC-hemolysin plasmids of STEC serotypes other than O157:H7. A direct comparison of the EHEC-hemolysin plasmids of evolutionarily less related STEC strains is therefore impossible. In addition, sequence data for single genes of the EHEC-hemolysin plasmid are sparse or even completely missing for serotypes other than O157:H7. The only gene for which extensive comparative data are available is *ehxA*. Alignment of the amino acid sequences of the EHEC-hemolysin from strains of two serotypes frequently involved in human disease (O157:H7 and O111:H8) and of one serotype rarely isolated from humans (O8:H19) shows that this protein is highly conserved among unrelated STEC [45]. Despite this very low level of diversity, two distinct lineages can be seen for *ehxA* [45]. It is not known if the hemolysins of these two lineages present different biological activities, but the particularly high level of conservation at biologically active sites of the toxin suggests that it may not be the case. Specific sequences of the EHEC-hemolysin plasmid are associated with each of the two EHEC-hemolysin lineages [45]. This shows that not only can two *ehxA* lineages be distinguished, but that two distinct EHEC-hemolysin plasmid lineages are present in STEC populations. One of these specific sequences (ORFs *ecf1–ecf4*) [45] is located between the *espP* gene and the *etp* gene cluster coding for the type II secretion system [19] of pO157. The corresponding region of pO157 presents a codon usage clearly different from the hemolysin operon and slightly different from the surrounding gene clusters [52]. This region may be involved in lipid A biosynthesis [45, 54] and exacerbation of host defenses [55]. Preliminary experiments on cell cultures also indicate that the regulation of expression of factors encoded by this region may be associated with adherence of *E. coli* on eukaryotic cells (P. Boerlin and C. L. Gyles, unpublished results). Altogether, these data strongly suggest that the region between *espP* and the *etp* cluster represents a piece of DNA which was horizontally transferred to the precursor of pO157 at a time when the *ehx* operon was already in place. This incoming segment of DNA may have brought additional virulence-associated factors to only one lineage of the EHEC-hemolysin plasmid.

Relationships between virulence factors and the phylogenetic background of STEC

An interesting relationship can be observed between the EHEC-hemolysin plasmid and the LEE in STEC. The EHEC-hemolysin plasmid lineage mentioned above and carrying the *ecf1–ecf4* ORFs (lineage I) [45] is associated with STEC serotypes carrying the LEE (fig. 1), whereas the other plasmid lineage (II) is found only in LEE-negative serotypes. A clonal and parallel evolution of the EHEC-hemolysin plasmid and of the LEE in STEC populations could explain this correlation. Our data using amplified fragment length polymorphism showed that the EHEC-hemolysin plasmid lineage I is restricted to a single group of phylogenetically related STEC [45] (fig. 1). In addition, the distribution of ehxA subtypes within this plasmid lineage broadly fits to the structure of the global genomic tree of STEC. Thus, pO157 is not only non-conjugative [52, 53] but horizontal transfer probably did not play a major role in the diffusion of the EHEC-hemolysin plasmids in general in STEC populations. EHEC-hemolysin plasmids have probably been present in some particular *E. coli* lineages for a long time and further evolved by integration of horizontally transferred genetic material of foreign origin. These assumptions are in agreement with the hypothesis of Whittam [20], suggesting that pO157 was acquired relatively early in the evolution of O55:H7 to O157:H7. Different approaches suggest that the LEE has been transmitted horizontally on different occasions. Nevertheless, the presence of the LEE in STEC remains apparently restricted to one STEC lineage and associated with strains carrying the same lineage I of EHEC-hemolysin plasmids [45] (fig. 1). Several hypotheses can explain these observations. One suggests that the LEE has been transferred horizontally only once to *E. coli* and subsequently changed its chromosomal location on several occasions without further horizontal transfer. This could be compatible with the lineage-associated location of the LEE [46]. However, it would not explain why only those parts encompassing the factors involved in direct interaction with the host were exchanged by horizontal transfer but not the entire LEE. An alternative explanation is that there is a functional relationship between factors of the LEE and other factors located on the EHEC-hemolysin plasmid or on the chromosome of specific STEC lineages. Such functional relationships between plasmid-encoded factors and chromosomal factors have been described for many pathogens. Relationships of this type have indeed already been described between the LEE and the EAF plasmid in EPEC [56, 57]. It would therefore not be surprising that equivalent but slightly different mechanisms are present in STEC. Finally, factors limiting the horizontal transfer and establishment of foreign DNA in a cell may vary from one bacterial lineage to another, thus explaining the limitation of some virulence factors like the LEE to specific STEC lineages. When preparing plasmids from EHEC-hemolysin-positive STEC, we have, for example, encountered problems with high nuclease activity in LEE-negative but not in LEE-positive isolates [45]. This may indicate that LEE-positive STEC lineages are more prone to uptake and establishment of foreign DNA than others. As suggested by Piérard et al. [24], associations between *stx* variants and other virulence factors (or STEC lineages) may also exist and several other similar associations may be discovered in STEC in the future.

In conclusion, the evolution of virulence factors in STEC represents a very complex and fascinating field. However, the data summarized in the present review show that many points on this subject remain to be clarified. Further work is warranted and comparative studies involving isolates of different STEC serotypes and genetic makeup are particularly needed. The new emerging techniques for DNA sequencing and DNA sequence comparisons will be invaluable to make this kind of work possible in the near future.

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