

Genotypic and Phenotypic Changes in the Emergence of *Escherichia coli* O157:H7

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Escherichia coli O157:H7 is a foodborne pathogen distinguished from typical *E. coli* by the production of Shiga toxins (Stx) and the inability to ferment sorbitol (SOR) and to express β -glucuronidase (GUD) activity. An allele-specific probe for the GUD gene (*uidA*) and multilocus enzyme electrophoresis were used to elucidate stages in the evolutionary emergence of *E. coli* O157:H7. A point mutation at +92 in *uidA* was found only in O157:H7 and its nonmotile relatives, including a SOR⁺ O157:H⁻ clone implicated in outbreaks of hemolytic-uremic syndrome in Germany. The results support a model in which O157:H7 evolved sequentially from an O55:H7 ancestor, first by acquiring the Stx2 gene and then by diverging into two branches; one became GUD⁻ SOR⁻, resulting in the O157:H7 clone that spread worldwide, and the other lost motility, leading to the O157:H⁻ clone that is an increasing public health problem in Europe.

Escherichia coli serotype O157:H7 has emerged as a serious foodborne pathogen that has caused large-scale outbreaks of gastrointestinal illness in developed countries [1, 2]. These bacteria have several factors implicated in pathogenesis, including Shiga toxins (Stx) and a pathogenicity island called LEE that encodes proteins, such as intimin (*eaeA*), involved in attaching effacement [3, 4]. Evolutionarily, the O157:H7 serotype marks a distinct clone that is only distantly related to other Stx-producing enterohemorrhagic *E. coli* (EHEC) and is most closely related to an enteropathogenic *E. coli* (EPEC) clone of serotype O55:H7, a non-Stx-producing strain associated with infantile diarrhea [5].

The clonal nature of *E. coli* O157:H7 has facilitated its identification because these organisms, in contrast to most *E. coli* isolates, do not ferment sorbitol and lack β -glucuronidase (GUD) activity [2]. The gene encoding GUD (*uidA*), however, is intact and is nearly identical to the gene in *E. coli* K-12 with two nucleotide differences: an A→T mutation in the putative -10 promoter region and a T→G mutation at +92 in the structural gene [6]. The *uidA* +92 mutation is conserved among O157:H7 strains and can be detected by an allele-specific oligonucleotide probe [7].

Although O157:H7 is the predominant *E. coli* serotype incriminated in foodborne disease, various nonmotile and cyto-

toxic O157 variants have been isolated [8, 9]. In particular, Karch et al. [10] discovered a novel Stx-producing O157 strain that caused an outbreak of hemolytic-uremic syndrome (HUS) in Germany. In contrast to typical O157:H7 strains, these nonmotile O157 strains ferment sorbitol (SOR⁺) and have DNA patterns distinct from those of typical O157:H7 isolates [10].

In the study reported here, we used multilocus enzyme electrophoresis to assess the clonal relationships among a variety of cytotoxic O157 strains, including nonmotile variants and other Stx-producing serotypes. We characterized strains for the presence of the *uidA* +92 allele, Stx genes, and sorbitol and GUD phenotypes. From a phylogenetic tree based on enzyme allele profiles, we formulated a model for the stepwise changes in virulence factors and phenotypic markers in the recent evolutionary emergence of *E. coli* O157:H7.

Materials and Methods

Bacterial strains. This study is based on analysis of 163 *E. coli* isolates, including 78 O157:H7 strains from contaminated foods and patients with hemorrhagic colitis or HUS, 4 Stx-producing O157:H⁻ strains from HUS patients in Germany, 42 nonmotile O157 strains from the United States and Japan, 10 O157 strains of a variety of H types other than H7, and 33 strains of various O:H serotypes, 14 of which were Stx producers.

Strain characterization. All isolates were examined for the ability to ferment sorbitol and express GUD activity [2]. Shiga toxin genes (*stx1* and *stx2*) were detected by multiplex polymerase chain reaction (PCR) assays [11], and toxin production was confirmed serologically by use of Verotox-F (Denka Seiken, Tokyo).

To identify the +92 allele of the *uidA* gene, strains were examined by colony blots with an allele-specific probe (PF-27) as described previously [7].

Nucleotide sequence analysis. The 5' terminus of the *uidA* gene was amplified by PCR with primers that anneal at -179 and

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+322 from the initiation codon [6]. The amplified 503-bp fragment was sequenced with the Silver Sequence kit (Promega, Madison, WI).

Clonal analysis. The genetic relationships among 46 representative strains were determined from allele profiles at 20 enzyme loci determined by multilocus enzyme electrophoresis [5]. Strains with identical profiles were assigned the same electrophoretic type (ET) and classified as members of a clone. Allele differences were used to estimate genetic distances between strains and to construct dendrograms by the average linkage algorithm [5].

Results

Clonal analysis and phenotypes. The multilocus enzyme genotypes of 46 strains resolved 15 ETs, whose genetic relatedness is shown in figure 1. The top cluster is the O157:H7 clone complex and comprises 5 closely related ETs that differ from one another by one or two enzyme alleles. All O157:H7 and most nonmotile O157 strains were ET1. Two O157 strains differed from this ET by alleles at a single locus; FDA 413 (ET2) had a fast-migrating peptidase and CDC G5101 (ET3) had a slow 6-phosphogluconate dehydrogenase electromorph. CDC G5101 was also unusual in that it was the only O157:H7 strain with a GUD⁺ phenotype [9].

All of the O157:H7 were SOR⁻ and GUD⁻, carried the *uidA* +92 allele, and had genes for Stx1, Stx2, or both. Serologic

tests confirmed that the respective Stx was produced by those strains. Of the total of 42 nonmotile O157 strains examined, 41 carried the *uidA* +92 allele and had Stx genes, and except for 4 strains of ET4 (see below), all were SOR⁻ and GUD⁻. Only 1 nonmotile O157 strain (USDA 7123, ET10) did not have the *uidA* +92 allele, and it was GUD⁻ and Stx⁻ (figure 1).

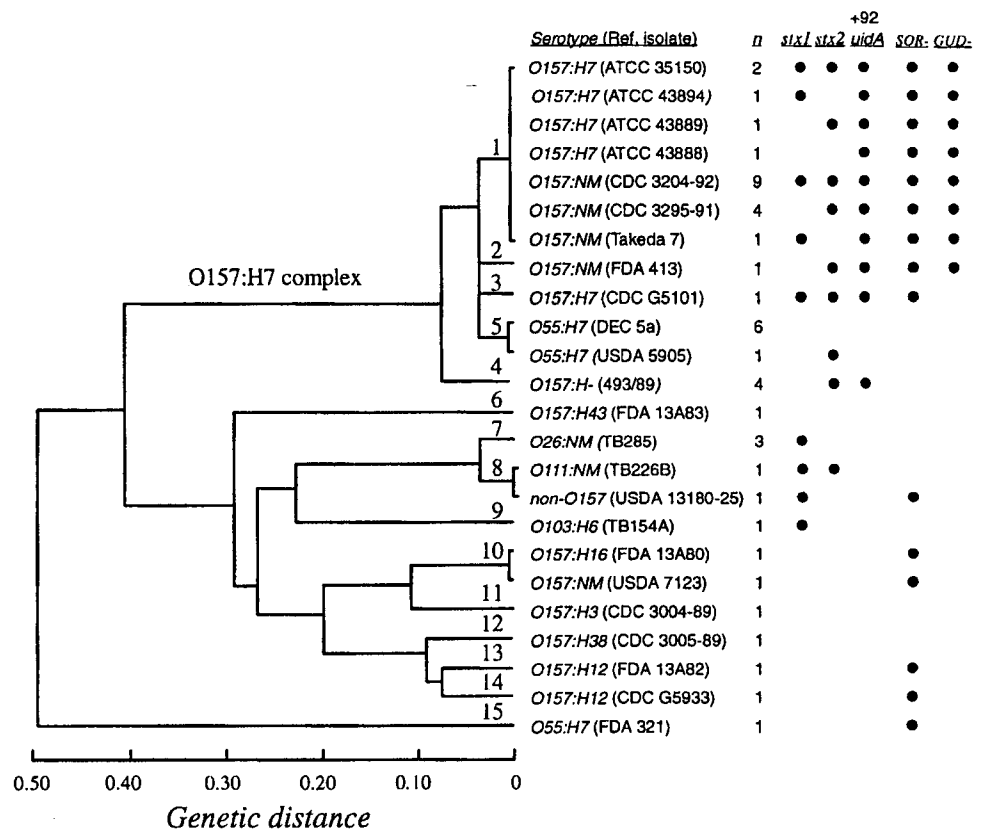
The O157:H7 complex also includes 7 O55:H7 isolates (ET5), which differ by a 6-phosphogluconate dehydrogenase allele from ET1 and are both SOR⁺ and GUD⁺ and, thus, phenotypically distinct from O157:H7 (figure 1). In addition, O55:H7 isolates are typically Stx-negative, although we found that 1 strain (USDA 5905) produced Stx2 (figure 1).

The most divergent member of the O157:H7 complex is ET4, comprising GUD⁺ SOR⁺ O157:H⁻ strains from Germany [12]. These isolates differed from ET1 by two enzyme alleles, a distinct malate dehydrogenase electromorph and a fast mannitol-1-phosphate dehydrogenase electromorph. These strains were positive for Stx2 and also carried the *uidA* +92 allele.

The bottom cluster of the dendrogram (ET6–ET15) is composed of non-H7 flagellar types and nonmotile O157 strains, as well as some representative Stx producers. All of these strains had ETs that differed by multiple alleles from the O157:H7 complex. None of the strains in the bottom half of the dendrogram had the *uidA* +92 allele.

Comparison of *uidA* gene sequences. To test further the genetic relationships in figure 1, we sequenced part of *uidA*

Figure 1. Dendrogram showing genetic distances among 46 representative isolates based on electrophoretic type (ET). ETs are numbered 1–15 and listed by serotype, with reference isolate given in parentheses (*n* = no. of isolates) and presence of specific genes (*stx1*, *stx2*, and *uidA* +92 allele) or phenotypes (SOR⁻ and GUD⁻) indicated by solid circles.



from all 4 ET4 isolates and 4 of the 7 ET5 isolates and compared the sequences with that of O157:H7 [6]. The ET4 strains (O157:H⁻) were identical to O157:H7, whereas the ET5 (O55:H7) strains had the -10 A→T mutation but did not have the T→G +92 mutation in *uidA*.

Discussion

The clonal analysis based on multilocus enzyme electrophoresis shows that O157:H7 and Stx-producing nonmotile variants (O157:H⁻) belong to a genetically distinct clone complex that includes O55:H7 strains. The O157:H7 complex comprises a variety of phenotypic variants that appear to have recently gained and lost motility, Shiga toxin genes, and metabolic traits, such as the ability to ferment sorbitol. The close relationship of these strains is supported by the finding that a point mutation at +92 in *uidA* occurs in O157:H7 and its nonmotile relatives, including an atypical O157:H⁻ clone implicated in outbreaks of HUS in Germany [10, 12] and in central Europe [13]. Comparative sequencing confirmed that these nonmotile

O157 strains carried the same -10 and +92 mutations in the *uidA* as did O157:H7. Interestingly, ET5 (O55:H7) had the -10 but not the +92 mutation, suggesting that the O55:H7 *uidA* allele is an intermediate stage between the wild type allele and the *uidA* allele of O157:H7.

To reconcile the ET profile data, the variety of SOR and GUD phenotypes, and the distribution of the *uidA* mutations, we formulated an evolutionary model that posits a series of steps that have occurred in the emergence of the *E. coli* O157:H7. The model is based on three assumptions: that during divergence, the probability of loss of function greatly exceeds gain of function for metabolic genes; that the gain of function usually occurs via lateral transfer of genes; and that the sequence of events invoking the fewest total is the preferred model.

The evolutionary steps outlined in the model (figure 2) begin at the left with the ancestral or primitive states and progress to the right to the contemporary or derived states. The model begins with an EPEC-like ancestor that we assume, like most present-day *E. coli*, to be able to express β-glucuronidase (GUD⁺) and ferment sorbitol (SOR⁺). From this EPEC-like

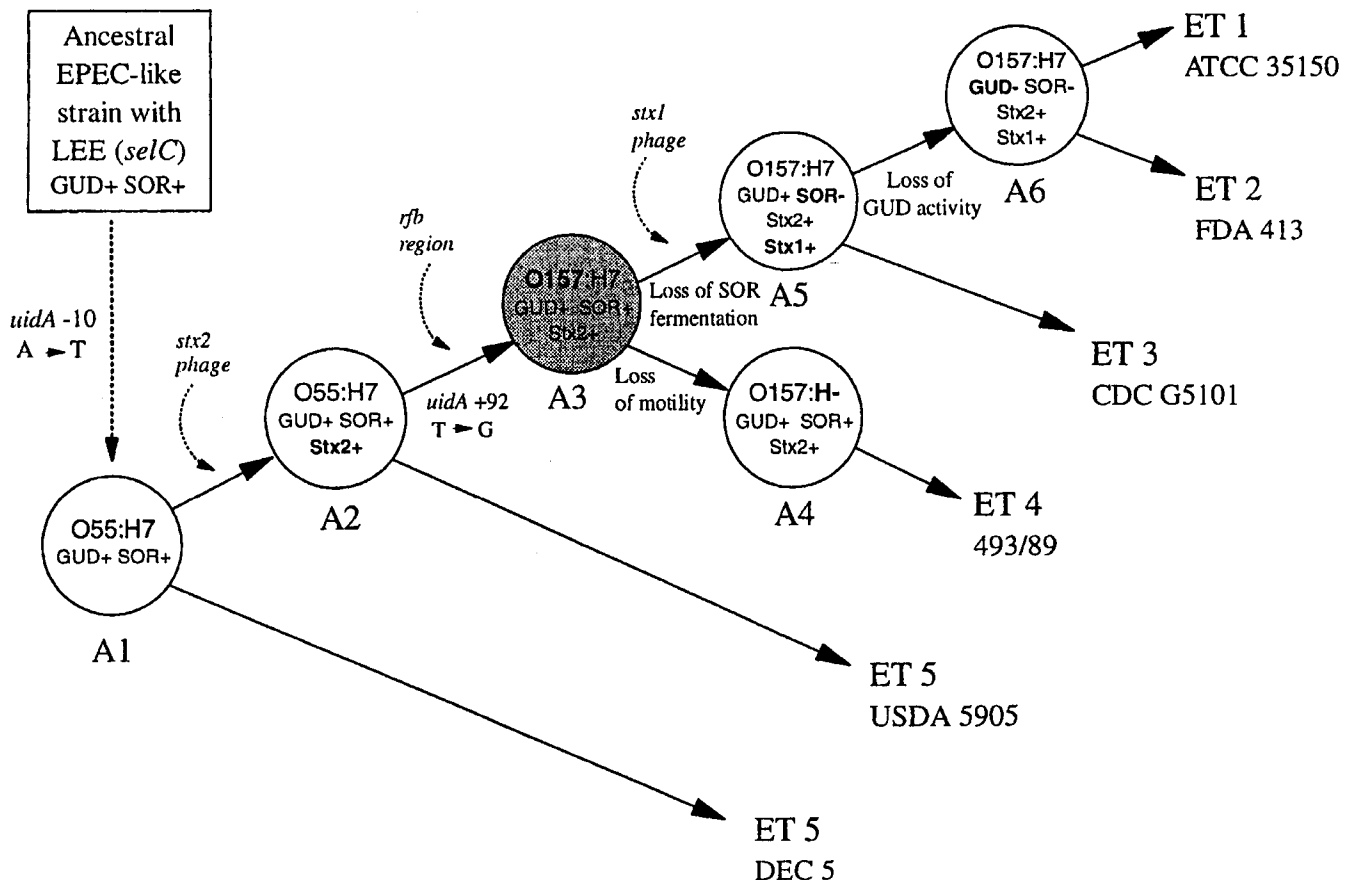


Figure 2. Proposed evolutionary model for emergence of O157:H7 complex based on mutations in *uidA*, Stx production, SOR and GUD phenotypes, and multilocus enzyme electrophoretic profiles of *E. coli* O157:H7 and its relatives. Phenotypes of ancestors A1–A6 are shown; changes predicted to have occurred are in bold. Representative isolates are given below each electrophoretic type (ET). Strain with traits of ancestor A3 (shaded circle) has not been reported.

ancestor, the immediate ancestor with the O55 somatic and the H7 flagellar antigens evolved. This ancestral cell, labeled A1 in figure 2, represents the most recent common ancestor of the ET5 (O55:H7) clone and O157:H7 and its relatives and is assumed to have the *uidA* -10 mutation that occurs in the entire O157:H7 complex. The next step, A1→A2, was the acquisition of *stx2*, presumably by transduction by a toxin-converting bacteriophage, resulting in an Stx2-producing O55:H7 strain. This primitive stage is represented by strain USDA 5905 in ET5 (figure 1). From A2→A3, the *uidA* +92 mutation occurred and the somatic antigen changed from O55 to O157 to give rise to a hypothetical O157:H7 Stx2-producing ancestor (A3) that retained the GUD⁺ SOR⁺ phenotype. Thus far, we are not aware of an actual strain that has been isolated with this combination of traits. Although it is not shown in the model, we also postulate that A3 acquired the EHEC plasmid [14], because the plasmid is present in the descendants of this ancestral cell.

The findings of Bilge et al. [15] suggest that the transition from O55 to O157 antigen occurred as result of a lateral transfer of an *rfbE*-like region with homology to perosamine synthetase of *Vibrio cholerae*. The observation that the *rfbE*-like region is present in O157:H7 and genetically unrelated O157 non-H7 strains, but not in O55:H7, supports the hypothesis that a lateral transfer of this region occurred at this crucial A2→A3 stage in the emergence of O157:H7 from an O55:H7-like ancestor.

From A3, the model proposes that two distinct lines evolved. In the lower path, the lineage lost motility, to yield ancestor A4, which retained the Stx2 and the GUD⁺ SOR⁺ primitive phenotypes. During this divergence from A3, the lineage accumulated two enzyme allele mutations to give rise to the German O157:H⁻ clone represented by ET4 (figure 1). Along the upper path, the lineage lost the ability to ferment sorbitol and acquired the Stx1 gene (presumably by phage conversion) to give rise to an intermediate A5 ancestral stage. The A5 has the primitive traits, GUD⁺ and Stx2⁺, and has the derived states (SOR⁻, Stx1⁺) in the combination of traits seen in strain CDC G5101 (ET3). In the model, A5 then lost GUD activity, producing the immediate ancestor (A6) of the common O157:H7 clone (ET1), which spread globally, and a rare variant (ET2), which differs by a single enzyme allele from ET1. Recent loss of Stx genes and motility in nature, or during isolation and culture, would account for the variants among isolates of the ET1 clone (figure 1).

In summary, the phylogenetic model diagrammed in figure 2 makes specific predictions about the history of descent and the order of acquisition of virulence factors in the emergence of the virulent O157:H7 serotype. The model predicts that both O157:H7 (ET1) and the German O157:H⁻ (ET4) were derived from an EPEC-like O55:H7 ancestor, which carried *eaeA* and acquired the Stx2 gene. This proposition is supported by the similarities between these strains and by the presence of identical mutations in *uidA*. The German O157:H⁻ clone, however,

represents an early diverging member of the O157:H7 clone complex, which retained the ancestral ability to ferment sorbitol and to express β -glucuronidase activity. The model also stipulates that *stx2* was acquired once and at an early stage, before the somatic antigen transition to O157 and prior to the acquisition of the EHEC plasmid and *stx1*. Hence, aside from the *eaeA* and the LEE region, which already existed in the ancestor, the model predicts that Stx2 gene has been evolving in the O157:H7 genome for a longer time than other virulence factors, such as *stx1* and genes on the EHEC plasmid.

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