

## ORIGINAL ARTICLE

Origins of the *E. coli* Strain Causing an Outbreak of Hemolytic–Uremic Syndrome in Germany

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## ABSTRACT

## BACKGROUND

A large outbreak of diarrhea and the hemolytic–uremic syndrome caused by an unusual serotype of Shiga-toxin–producing *Escherichia coli* (O104:H4) began in Germany in May 2011. As of July 22, a large number of cases of diarrhea caused by Shiga-toxin–producing *E. coli* have been reported — 3167 without the hemolytic–uremic syndrome (16 deaths) and 908 with the hemolytic–uremic syndrome (34 deaths) — indicating that this strain is notably more virulent than most of the Shiga-toxin–producing *E. coli* strains. Preliminary genetic characterization of the outbreak strain suggested that, unlike most of these strains, it should be classified within the enteroaggregative pathotype of *E. coli*.

## METHODS

We used third-generation, single-molecule, real-time DNA sequencing to determine the complete genome sequence of the German outbreak strain, as well as the genome sequences of seven diarrhea-associated enteroaggregative *E. coli* serotype O104:H4 strains from Africa and four enteroaggregative *E. coli* reference strains belonging to other serotypes. Genomewide comparisons were performed with the use of these enteroaggregative *E. coli* genomes, as well as those of 40 previously sequenced *E. coli* isolates.

## RESULTS

The enteroaggregative *E. coli* O104:H4 strains are closely related and form a distinct clade among *E. coli* and enteroaggregative *E. coli* strains. However, the genome of the German outbreak strain can be distinguished from those of other O104:H4 strains because it contains a prophage encoding Shiga toxin 2 and a distinct set of additional virulence and antibiotic-resistance factors.

## CONCLUSIONS

Our findings suggest that horizontal genetic exchange allowed for the emergence of the highly virulent Shiga-toxin–producing enteroaggregative *E. coli* O104:H4 strain that caused the German outbreak. More broadly, these findings highlight the way in which the plasticity of bacterial genomes facilitates the emergence of new pathogens.

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**I**N EARLY MAY 2011, AN OUTBREAK OF DIARRHEA with associated hemolytic-uremic syndrome began in northern Germany; cases have subsequently been reported in 15 other countries. As of July 22, a total of 3167 cases of non-hemolytic-uremic syndrome Shiga-toxin-producing *Escherichia coli* (16 deaths) and 908 cases of hemolytic-uremic syndrome (34 deaths) have been reported, according to the German Protection against Infection Act. Several groups reported that the outbreak was caused by a Shiga-toxin-producing *E. coli* strain belonging to serotype O104:H4, with virulence features that are common to the enteroaggregative *E. coli* pathotype.<sup>1-3</sup> This unusual *E. coli* serotype has previously been associated with sporadic cases of human disease<sup>4,5</sup> but not with large-scale outbreaks.

*E. coli* are ordinarily commensal organisms, but six pathotypes of diarrheagenic *E. coli* are recognized, each with distinct phenotypic and genetic traits.<sup>6</sup> Diarrhea associated with the hemolytic-uremic syndrome and neurologic complications is generally caused by *E. coli* that produce Shiga toxins.<sup>6</sup> The majority of such strains, often referred to as enterohemorrhagic *E. coli*, contain the enterocyte effacement pathogenicity island, which facilitates colonization of the large intestine. Unexpectedly, polymerase-chain-reaction assays<sup>1-3</sup> revealed that the German outbreak strain lacked this pathogenicity island, and the rapidly released results of genomic sequencing<sup>7</sup> and cell adherence assays<sup>1</sup> confirmed the initial observations that the German O104:H4 outbreak strain was an enteroaggregative *E. coli* strain rather than a typical enterohemorrhagic *E. coli* strain. The outbreak strain was similar to enteroaggregative *E. coli* O104:H4 strain 55989, isolated from a patient in the Central African Republic who had human immunodeficiency virus infection (HIV) with persistent diarrhea,<sup>8</sup> which did not produce Shiga toxin.

The enteroaggregative *E. coli* pathotype has been implicated as an etiologic agent of diarrhea in travelers,<sup>9</sup> children,<sup>10,11</sup> and HIV-infected patients,<sup>12</sup> as well as of several, probably foodborne outbreaks.<sup>13-16</sup> Enteroaggregative *E. coli* are widespread among human populations, although the basis of the variation in the virulence of enteroaggregative *E. coli* is poorly understood.<sup>17</sup> Enteroaggregative *E. coli* with proven pathogenicity typically contain a large set of virulence-associated genes regulated by the AggR transcription factor. These include plasmid genes encoding the aggregative adherence fimbriae (AAF),<sup>8,18-20</sup> which anchor the bacterium to the intestinal mucosa and induce inflammation,<sup>21</sup> as well as a protein-coat secretion system (Aat), its secreted dispersin protein,<sup>22</sup> and a putative type VI secretion system termed Aai.<sup>23</sup> Enteroaggregative *E. coli* strains often elaborate toxins and a variable number of serine protease autotransporters of Enterobacteriaceae (SPATEs) implicated in mucosal damage and colonization.<sup>24</sup>

Before the current outbreak of enteroaggregative *E. coli* O104:H4, only three enteroaggregative *E. coli* genomes had been sequenced<sup>25-27</sup>; thus, genome-scale knowledge of the phylogeny of enteroaggregative *E. coli* was limited. We used third-generation DNA-sequencing technology to rapidly determine the genome sequences of the *E. coli* O104:H4 outbreak strain, seven other enteroaggregative *E. coli* O104:H4 strains, and four reference strains (Table 1).<sup>28-32</sup>

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## METHODS

### ISOLATES

We isolated an enteroaggregative *E. coli* strain, C227-11, from a 64-year-old woman from Hamburg, Germany, who was hospitalized at Hvidovre University Hospital, Copenhagen. She presented with bloody diarrhea; the hemolytic-uremic syndrome did not develop. We selected five reference isolates (JM221, 17-2, 042, 55989, and C1010-00) to represent the best-studied enteroaggregative *E. coli* strains and to capture the geographic, serotype, and virulence-factor diversity of the enteroaggregative *E. coli* pathotype. We cultured six O104:H4 clinical isolates that we obtained from the Statens Serum Institute, Copenhagen (C35-10, C682-09, C734-09, C754-09, C760-09, and C777-09), the five enteroaggregative *E. coli* reference isolates, and the C227-11 isolate. (Details regarding culture conditions are provided in the Supplementary Appendix, available with the full text of this article at NEJM.org.)

### DNA PREPARATION, SEQUENCING, AND COMPARATIVE ANALYSES

We purified and sequenced the genomic DNA from C227-11, from six African enteroaggregative *E. coli* clinical isolates, and from five prototype enteroaggregative *E. coli* isolates with the use of PacBio RS DNA sequencers (Sequence Read Archive accession number, SRA038239; GenBank accession

**Table 1. Isolates of Enteroaggregative *Escherichia coli* Sequenced or Analyzed in This Study.\***

Isolate†	Serotype	Location of Isolate	Source of Isolate	Reference
O42	O44:H18	Peru	Stool sample from child with diarrhea	Nataro et al. <sup>28</sup>
17-2	O3:H2	Chile	Stool sample from child with diarrhea	Vial et al. <sup>29</sup>
JM221	O92:H33	Mexico	Stool sample from adult with diarrhea	Mathewson et al. <sup>30</sup>
C1010-00	Orough:H-	Denmark	Stool sample from child with diarrhea	Olesen et al. <sup>31</sup>
55989	O104:H4	Central African Republic	Stool sample from adult with diarrhea	Bernier et al. <sup>8</sup> and Touchon et al. <sup>26</sup>
C35-10‡	O104:H4	Africa	Stool sample from child with diarrhea	SSI collections
C682-09‡	O104:H4	Africa	Stool sample from child with diarrhea	SSI collections
C734-09‡	O104:H4	Africa	Stool sample from child without diarrhea	SSI collections
C754-09‡	O104:H4	Africa	Stool sample from child without diarrhea	SSI collections
C760-09‡	O104:H4	Africa	Stool sample from child without diarrhea	SSI collections
C777-09‡	O104:H4	Africa	Stool sample from child with diarrhea	SSI collections
<b>TY2482</b>	O104:H4	Germany	Stool sample from adult with diarrhea	Rohde et al. <sup>32</sup>
<b>LB226692</b>	O104:H4	Germany	Diarrhea in adult	GenBank accession number, AF0B0000000
<b>H112180280</b>	O104:H4	Germany	Diarrhea in adult	www.hpa-bioinformatics.org.uk/lgp/genomes
<b>C227-11</b>	O104:H4	Denmark	Diarrhea in adult	This study

\* All the isolates listed were sequenced (55989 and O42 were resequenced, and the others were newly sequenced) in this study, with the exception of TY2482, LB226692, and H112180280. SSI denotes Statens Serum Institute.

† Isolates shown in bold type are from the current outbreak (May 2011).

‡ These isolates are not from a single clonal outbreak and thus represent population-based diversity in the O104:H4 group.

number, AFST00000000). We also carried out a comparative analysis with the sequence of another outbreak isolate, TY2482. (See the article by Rohde et al. elsewhere in this issue of the *Journal* for an analysis of sequence data obtained from TY2482.<sup>32</sup>) The Supplementary Appendix provides additional details regarding DNA sequencing,<sup>33,34</sup> assembly, resequencing analysis, and detection of DNA structural variations, as well as the construction of phylogenetic trees and the characterization of lambda-like phage elements, virulence factors, plasmids, and regions unique to the outbreak strain.

## RESULTS

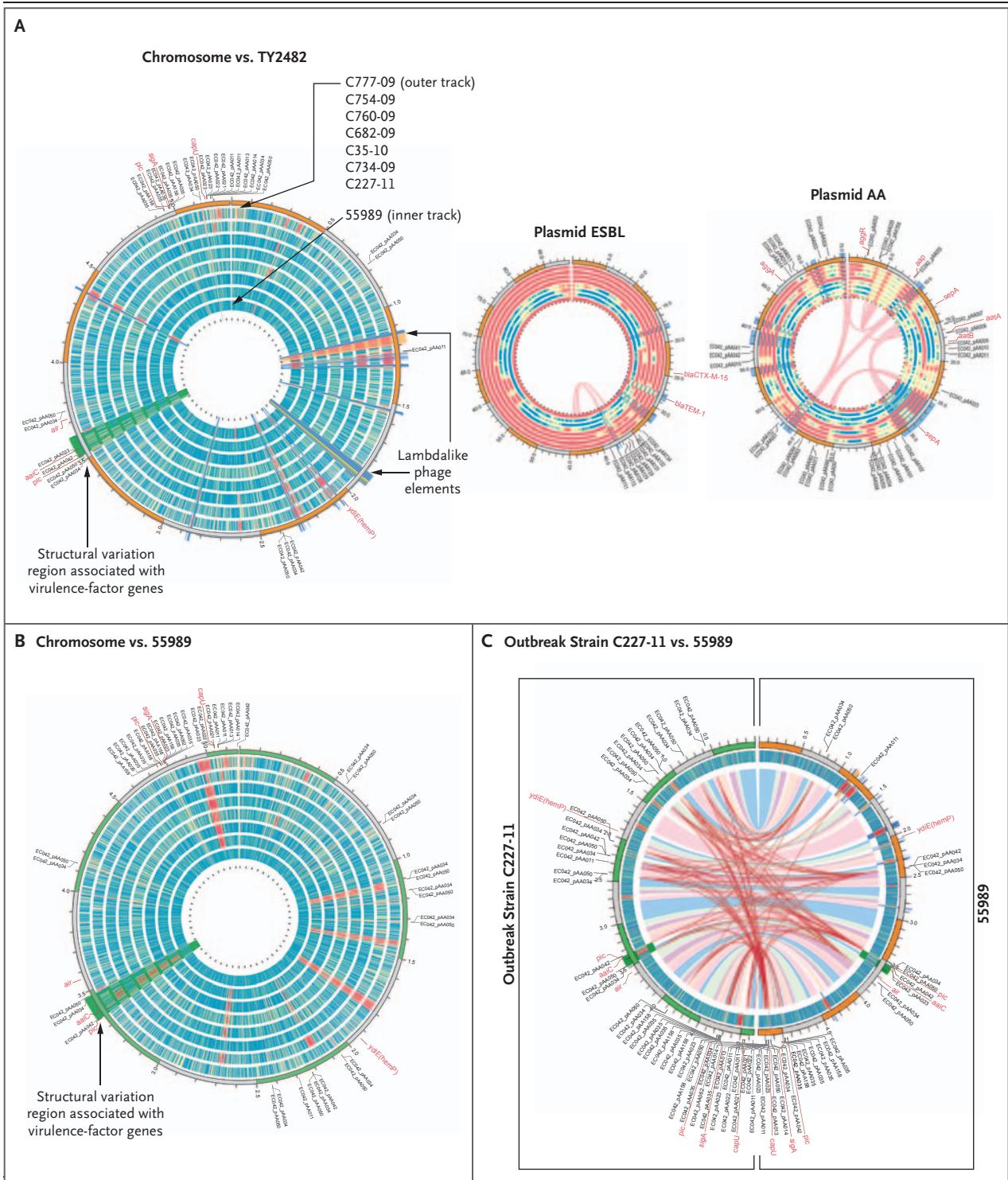
### GENOME SEQUENCING AND ASSEMBLY OF 13 *E. COLI* GENOMES

Using three sequencing instruments in parallel, we obtained coverage by a factor of approximately 75 for each of the isolates sequenced (mean read length, 2067 bases), in approximately 5 hours per isolate (Table 1 in the Supplementary Appendix).

We used an integrative process (Fig. 1 in the Supplementary Appendix) to assemble the C227-11 genome and obtained 33 contigs (N50 of 402 kb

and a maximum contig size of 622 kb), covering 99.97% of the bacterial chromosome at a level of accuracy of 99.97% (Table 3 in the Supplementary Appendix). (A contig is a contiguous sequence of DNA with no gaps; N50 is the contig length so that all contigs of that length or greater cover 50% of the bases in the genome.) Four additional contigs covered two large plasmids (approximately 88 kb and 75 kb) and a third 1.5-kb plasmid. The 1.5-kb plasmid could be fully resolved with two reads (Fig. 1 and Table 2 in the Supplementary Appendix). One of the plasmids is similar to the pAA plasmid found in typical enteroaggregative *E. coli* isolates.<sup>25</sup> The pAA plasmid of C277-11 (referred to here as pAA C277-11) encodes aggregative adherence fimbriae (the AAF/I variant), the Aat complex, the dispersin protein, AggR, and other virulence factors characteristic of enteroaggregative *E. coli* (Fig. 1).

We compared the sequence of C227-11 with the six O104:H4 strains from Africa, the previously released sequence data from outbreak-linked isolates, the available reference-genome sequences for the outbreak isolate TY2482, and the African enteroaggregative *E. coli* 55989 strain,<sup>8,26</sup> in order to



identify copy-number variations and small nucleotide variations among all the isolates (Fig. 1, and Table 3 and Fig. 3 in the Supplementary Appendix). (See animation at NEJM.org.) The genomes of the isolates from the current outbreak (C227-11,

TY2482, LB226692, and H112180280) were very similar, with only 236 differences in single-nucleotide variants detected between TY2482 and C227-11 (Table 3 and Fig. 3 in the Supplementary Appendix), suggesting that the outbreak is clonal. We observed

**Figure 1 (facing page). Comparison of Genomes of Eight Enteroaggregative *Escherichia coli* O104:H4 Isolates.**

In Panel A, the eight circular bands represent the tracks for the different enteroaggregative *E. coli* (EAEC) O104:H4 isolates sequenced in our study (from inner to outer tracks: 55989, C227-11, C734-09, C35-10, C682-09, C760-09, C754-09, and C777-09). These different bands represent coverage plots mapped against the reference genome TY2482, with blue or green indicating a high degree of coverage, yellow moderate coverage, and orange or red little or no coverage. The C227-11 coverage plot indicates near-complete sequence identity with the TY2482 genome. The annotations on the outside of the chromosome plot highlight positions of known virulence-factor genes and genes previously characterized on the AA plasmid in the *E. coli* O42 strain. The band ordering and color coding for the chromosome plot. The thin red lines in the pAA plot show internal repeats between 55989 and C227-11 that highlight shuffling of these elements around the plasmid sequence. In Panel B, the reference genome is 55989. Panel C shows a comparison between the genome of the outbreak strain C227-11 (left semicircle) and the 55989 genome (right semicircle), mapped against the 55989 genome and the genome of the outbreak strain TY2482, respectively. The colored ribbons inside the innermost track (blue, pink, and purple) represent homologous mappings between the two genomes, C227-11 and 55989, indicating a high degree of similarity between these genomes. The red lines show the chromosomal positioning of repeat elements, such as insertion sequences and other mobile elements, which reveal more heterogeneity between the genomes than is evident from mapping regions of homology.

structural variations between the C227-11 genome and the complete genome sequence of TY2482, based in part on the recently described draft assembly,<sup>32</sup> that seem unlikely to be the result of misassemblies in the C227-11 genome, because these regions are identical in the C227-11 and H112180280 genomes (Fig. 4 and Fig. 5 in the Supplementary Appendix). However, we observed larger-scale deletions, insertions, inversions, and other structural variations between the O104:H4 outbreak isolates and the seven other enteroaggregative *E. coli* O104:H4 isolates we sequenced (Fig. 1).

Several of the structurally divergent regions house genes that encode virulence factors. For example, C227-11 contains two lambda-like prophage elements, one of which contains the genes for Shiga toxin. The toxin-encoding phage is observed only in the outbreak isolate (Fig. 1, orange highlight), whereas the other prophage element (Fig. 1, blue highlight) is present in several additional O104:H4 isolates. Figure 1 also reveals regions unique to the outbreak-strain chromosome, including structural variation in regions that include

virulence-factor genes such as *pic* and the *aaI* pathogenicity island (Fig. 1, green highlight). This particular region includes differences between the outbreak isolates and 55989 (Fig. 6 in the Supplementary Appendix).

**WHOLE-GENOME PHYLOGENY**

We used approximately 2.56 Mb of DNA, representing the conserved core genome found in 53 *E. coli* and shigella genomes, including C277-11, other German outbreak strains, and the African O104:H4 strains, to generate a phylogenetic tree depicting the evolution of *E. coli* (Fig. 2, and Fig. 4 and Table 4 in the Supplementary Appendix). We observed enteroaggregative *E. coli* isolates spread through the entire phylogenetic tree (blue boxes), suggesting that, with respect to genome composition, they are the most diverse of the pathotypes. However, the enteroaggregative *E. coli* strains of O104:H4 serotype (orange) form a distinct clade, with highly conserved core genomes (with one O104:H4 isolate, C35-10, being an outlier). The genetic similarity between the Shiga-toxin–encoding strains isolated in the German outbreak and numerous enteroaggregative *E. coli* O104:H4 strains lacking the Shiga-toxin–encoding phage suggests that acquisition of the phage was a relatively recent event (Fig. 4 in the Supplementary Appendix). The presence of the O104:H4 outbreak strain within the enteroaggregative *E. coli* O104:H4 clade confirms that the outbreak strain is not a prototypical enterohemorrhagic *E. coli* strain that has acquired the virulence features of enteroaggregative *E. coli*.

We analyzed the phylogeny of only the enteroaggregative *E. coli* isolates, on the basis of 3.48 Mb of shared genome sequences (Fig. 7 in the Supplementary Appendix). The O104:H4 isolates, including the outbreak isolates, clustered into a single, closely related clade — again, with the exception of the C35-10 isolate. The four isolates from the German outbreak (TY2482, C227-11, LB226692, and H112180280), three of which were sequenced by other groups, are particularly closely related.

**THE LAMBDA-LIKE PHAGE CONTAINING THE STX2 GENES**

A polymerase-chain-reaction assay revealed that the outbreak isolates contained genes encoding Shiga toxin 2,<sup>3</sup> a toxin that inhibits protein synthesis and causes the hemolytic–uremic syndrome.<sup>6</sup> The insertion site of the Shiga-toxin–encoding lambda-like prophage in the outbreak strain is similar to that of the Shiga-toxin–encoding lambda-like pro-



An animation comparing the outbreak strain genome with genomes of other strains is available at [NEJM.org](http://NEJM.org)



outbreak suggests that the enteroaggregative *E. coli* O104:H4 strain that caused the outbreak has additional virulence factors or a particular combination of virulence factors that make it extremely pathogenic (Table 5 in the Supplementary Appendix). The genome of the outbreak strain has many virulence-factor genes commonly found in enteroaggregative *E. coli*, including several for which production is coordinately regulated by the AggR transcription activator (Fig. 11 in the Supplementary Appendix); these include the genes encoding AAF/I (*aggA-D*), dispersin (*aap*), and the dispersin translocator (*aatPABCD*). With the exception of *stxAB2*, genes characteristic of enterohemorrhagic *E. coli* are not present in the C227-11 genome. Together, these observations provide support for classifying the outbreak strain as an enteroaggregative *E. coli* strain.

Among the virulence factors of the German outbreak strain that are common in enteroaggregative *E. coli* and other diarrheagenic *E. coli* strains (and shigella species) are the SPATEs.<sup>37</sup> However, the C227-11 genome encodes a combination of SPATEs (SepA, SigA, and Pic) rarely reported in enteroaggregative *E. coli* strains.<sup>37</sup> This combination was also present in three of the African strains we studied, as well as in 55989. It is also unusual for an enteroaggregative *E. coli* strain to encode more than two SPATEs.<sup>37</sup> Thus, the number and combination of SPATEs in the outbreak strain may contribute to its heightened virulence. Other potential virulence factors identified in the German outbreak strain include long polar fimbriae (*lpf*) and IrgA homologue adhesion (*iha*),<sup>1</sup> which have previously been identified both in enterohemorrhagic *E. coli* and in other *E. coli* pathotypes.<sup>38,39</sup>

#### UNIQUE REGIONS IN THE C227-11 GENOME

The C227-11 genome contains 58 regions longer than 100 bp that are missing in at least 1 of the other 11 prototype enteroaggregative *E. coli* genomes (Table 6 in the Supplementary Appendix). These 58 regions contain 180,088 bp (about 3.4% of the genome), but more than 120,000 bp of this unique DNA comprise the two lambda-like phages mentioned above (61,022 and 59,914 bp, respectively). Most of the remaining sequence (about 60,000 bp) is made up of genes of unknown function that are also found in commensal *E. coli* and thus probably do not contribute to virulence. This paucity of novel DNA further supports the recent emergence of the O104:H4 lineage. As depicted in Figure 1, sequences absent from C227-11 relative to the reference strain 44898 are also absent from

other O104:H4 isolates. Thus, our sequence analyses suggest that acquisition of *stx2*, perhaps enhanced by an unusual complement of SPATEs, could account for the elevated virulence of C227-11.

#### PLASMID SEQUENCES AND OTHER MARKERS OF CLINICAL SIGNIFICANCE

We mapped raw sequences from the 12 isolates to the three plasmid sequences assembled as part of the TY2482 outbreak genome (Fig. 1) (GenBank accession number, AFOG01000000). The largest and smallest plasmids did not contain genes encoding any known virulence factors. However, the largest plasmid, pESBL C227-11, which encodes the extended-spectrum beta-lactamase CTX-M-15, is very similar to the pEC\_Bactec plasmid found in several clinical *E. coli* isolates.<sup>40</sup> The pESBL C227-11 plasmid was not identified in most of the O104:H4 genomes sequenced, suggesting that this plasmid might be a recent acquisition by some strains or, alternatively, might be relatively unstable and consequently lost by numerous strains.

The intermediate-size plasmid pAA C227-11 (Fig. 1) is approximately 75,000 bp and resembles the pAA plasmid identified in enteroaggregative *E. coli* isolates,<sup>25,26</sup> although marked diversity is also evident within this family of plasmids. Comparison of the pAA C227-11 plasmid with the prototype pAA plasmid from enteroaggregative *E. coli* 042 identified only about 34,000 bp of shared sequence, and pAA C227-11 shares only 28,000 bp of the sequence with the 55989 virulence plasmid. The sequence heterogeneity among pAA plasmids in enteroaggregative *E. coli* O104:H4 is much greater than that between chromosome sequences, with conservation found in core virulence features. The pAA C227-11 plasmid encodes a number of enteroaggregative *E. coli*-specific virulence factors (Fig. 1, and Tables 8 and 9 in the Supplementary Appendix), such as *aggR* and genes that it regulates, including AggR, AatPA-D, Aap, AggA-D, and SepA, and thus presumably is critical for C227-11 pathogenicity.

#### DISCUSSION

The outbreak clone is a dramatic example of gene acquisition by means of lateral transfer that resulted in an accretion of synergistic virulence factors. By all molecular definitions, it is an enteroaggregative *E. coli* strain but one that has acquired a Shiga-toxin-encoding phage — a hallmark of Shiga-toxin-producing *E. coli*. Our findings

suggest that critical events in the evolution of the German enteroaggregative *E. coli* outbreak strain included the acquisition of a Stx-encoding prophage and a plasmid bearing an extended-spectrum beta-lactamase gene by an ancestral precursor of this strain (Fig. 11 in the Supplementary Appendix). However, analyses of the genomes of contemporary isolates cannot reveal the true complexity of the evolutionary pathway that yielded the Shiga-toxin-producing enteroaggregative *E. coli* O104:H4 outbreak strain. The outbreak is not the first clinically linked instance of an enteroaggregative *E. coli* acquiring a Shiga-toxin-encoding phage,<sup>41,42</sup> but it is a clear case of such a strain causing a major outbreak of disease. Whether the current outbreak is due to a particularly virulent Shiga-toxin-positive enteroaggregative *E. coli*, a rare epidemiologic opportunity, or both remains unclear.

The similarity between the Shiga-toxin-encoding prophage in the outbreak isolate and such prophages in enterohemorrhagic *E. coli*, particularly in the toxin promoter region, suggests that toxin-inducing conditions might be similar for these pathogens. Our observation that toxin production by the O104:H4 outbreak strain is induced by a quinolone antibiotic, as previously seen with enterohemorrhagic *E. coli* strains,<sup>36</sup> suggests that caution is warranted in the use of certain classes of antibiotics to counteract this newly emerged pathogen.

The current isolate diverges from common enteroaggregative *E. coli* isolates in the number and nature of its SPATE proteases. Most diarrheagenic *E. coli* strains encode a single SPATE, and enteroaggregative *E. coli* strains ordinarily encode two SPATEs<sup>37</sup>; the presence of three SPATE-encoding genes in the outbreak strain is unusual. One of these SPATEs, Pic, is common among enteroaggregative *E. coli* strains and cleaves host intestinal epithelial-cell mucins, thereby promoting intestinal colonization,<sup>43</sup> and mucin-related glycoproteins associated with leukocyte immune functions.<sup>44</sup> The outbreak strain also encodes SigA, a SPATE that cleaves the cytoskeletal protein spectrin, inducing

rounding and exfoliation of enterocytes.<sup>45</sup> The third SPATE, SepA, is associated with increased enteroaggregative *E. coli* virulence, but its function is unknown (unpublished observations). We speculate that the combined activity of these SPATEs, together with other enteroaggregative *E. coli* virulence factors, accounts for the increased uptake of Shiga toxin into the circulation, resulting in the high rates of the hemolytic-uremic syndrome.

Finally, the worldwide efforts to sequence and analyze the genome of the German enteroaggregative *E. coli* outbreak strain illustrate the power of emerging high-throughput DNA-sequencing technologies. The ability to sequence genomes within hours (rather than days to weeks) with unprecedented read lengths is the emerging hallmark of third-generation DNA sequencing; such long reads facilitate new genome-assembly efforts and deeper insights into structural variations. The rapid sequencing of isolates from this outbreak (and from related strains) has yielded critical insight into its causative agent. In addition, the rapid release of DNA sequence data by the Beijing Genomics Institute (an analysis of which is described by Rohde and colleagues<sup>32</sup>) allowed analyses of a bacterial outbreak by communities of researchers to proceed with extraordinary speed, providing a glimpse into a new era in which communities of researchers rapidly share large-scale data sets and analyses that are vital for public health.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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