Genome sequencing and comparative genomics provides insights on the evolutionary dynamics and pathogenic potential of different H-serotypes of Shiga toxin-producing *Escherichia coli* O104

Xianghe Yan1,3*, Pina M Fratamico1, James L Bono2, Gian Marco Baranzoni1 and Chin-Yi Chen1

**Abstract**

**Background:** Various H-serotypes of the Shiga toxin-producing *Escherichia coli* (STEC) O104, including H4, H7, H21, and H−, have been associated with sporadic cases of illness and have caused food-borne outbreaks globally. In the U.S., STEC O104:H21 caused an outbreak associated with milk in 1994. However, there is little known on the evolutionary origins of STEC O104 strains, and how genotypic diversity contributes to pathogenic potential of various O104 H-antigen serotypes isolated from different ecological niches and/or geographical regions.

**Results:** Two STEC O104:H21 (milk outbreak strain) and O104:H7 (cattle isolate) strains were shot-gun sequenced, and the genomes were closed. The intimin (eae) gene, involved in the attaching-effacing phenotype of diarrheagenic *E. coli*, was not found in either strain. Examining various O104 genome sequences, we found that two “complete” left and right end portions of the locus of enterocyte effacement (LEE) pathogenicity island were present in 13 O104 strains; however, the central portion of LEE was missing, where the eae gene is located. In O104:H4 strains, the missing central portion of the LEE locus was replaced by a pathogenicity island carrying the aidA (adhesin involved in diffuse adherence) gene and antibiotic resistance genes commonly carried on plasmids. Enteroaggregative *E. coli*-specific virulence genes and European outbreak O104:H4-specific stx2-encoding *Escherichia* P13374 or *Escherichia* TL-2011c bacteriophages were missing in some of the O104:H4 genome sequences available from public databases. Most of the genomic variations in the strains examined were due to the presence of different mobile genetic elements, including prophages and genomic island regions. The presence of plasmids carrying virulence-associated genes may play a role in the pathogenic potential of O104 strains.

**Conclusions:** The two strains sequenced in this study (O104:H21 and O104:H7) are genetically more similar to each other than to the O104:H4 strains that caused an outbreak in Germany in 2011 and strains found in Central Africa. A hypothesis on strain evolution and pathogenic potential of various H-serotypes of *E. coli* O104 strains is proposed.

**Keywords:** STEC serogroup O104, Virulence, Plasmids, Genotyping, Comparative genomics, Next generation sequencing
**Background**

The occurrence of a large outbreak involving enteroaggregative hemorrhagic *E. coli* (EAHEC) O104:H4 infections in Europe and North America in 2011 with prior reporting of similar strains in patients with severe infections and hemolytic uremic syndrome (HUS) in Asia, Norway, and the Republic of Georgia raises questions about the origin, evolution, genetic diversity, and virulence of these pathogens [1-7]. The German outbreak O104:H4 strain 2011C-3493 had the characteristics of an enteroaggregative *E. coli* (EAEC); however, it carried the gene that encodes Shiga toxin 2a (*stx*₂ᵃ), and thus can also be classified as a Shiga toxin-producing *E. coli* (STEC). Bielaszewska et al. [8] analyzed 80 isolates from this outbreak and found that they belonged to ST (sequence type) 678, similar to the HUSEC041 strain isolated from a patient with HUS in Germany in 2001. Moreover, the outbreak strains possessed a plasmid carrying CTX-M-15, encoding an extended-spectrum-β-lactamase, which is absent in HUSEC041. *E. coli* O104:H4 strains and O104 strains with other H-types showed genetic and phenotypic differences, including the presence of specific virulence and antibiotic resistance genes, the types of Shiga toxin genes, and their pulsed-field gel electrophoresis profiles [7,9,10].

Some of the most common flagellar H antigens associated with STEC strains include H₁, H₂, H₇, H₈, H₁₁, H₁₂, H₁₆, H₁₉, H₂₁, H₂₅, and H₂₈ [3,11]. Shiga toxin-producing strains belonging to serogroup O104 with the H₇ or H₂₁ flagellar antigens could be considered as more pathogenic [10]. According to the European Food Safety Authority, serogroup O104 has been reported from studies related to surveillance of animals and food by the EU Member States [12]. *E. coli* O104:H₁₁ and O104:H2₁ were isolated from young cattle in Austria in 2009, O104:H7 from sheep and wild boar (O104/O127) in Spain, O104: H₇ from sheep in India, from young cattle in Argentina, and from unspecified meat and sheep meat in New Zealand [13-19]. To date, other O104 serotypes attributed to human cases have been identified as O104:H₂₁ [3], O104:H₂ [20], and O104:H [21]. In the U.S., there was an outbreak in Montana in 1994 caused by milk contaminated with STEC O104:H₂₁ [22].

Differences in virulence and antibiotic resistance genes linked to disease severity and HUS among various O104 serotype strains, including O104:H₁₁, O104:H₂₁, O104:H₇, O104:H₂, O104:H₁₂, and O104:H₁₆ have been observed [10,23]. The differential phenotypic properties of O104 strains with H₂, H₄, H₁₁, H₁₂, and H₂₁ antigens on selective media, such as Brilliance ESBL (extended-spectrum beta-lactamases), CHROMagar STEC, and CHROMagar O104 were also reported [24,25]. However, there is little information regarding the genetic basis of virulence of non-O104:H₄ pathogens and how they might have emerged or were transmitted. Therefore, these serotypes should be fully investigated at the genomic level to identify genes important for virulence and for growth and survival in food. Moreover, besides EAEC-STEC O104:H₄, STEC O104:H₇, and STEC O104: H₂₁, several *stx*-negative O104:H₄ strains have been isolated from human patients in Central Africa [4,26,27] at different locations and at different times. Thus far, humans are considered as the only natural reservoir of EAEC strains [28], and the search for the source of the O104:H₄ outbreak strain revealed that there was no animal reservoir for EAEC/EHEC/STEC O104:H₄ isolates [29-32]. In contrast, STEC O104:H₇ and STEC O104:H₂₁ are reported to be associated with contaminated milk [22], feces of cattle, and/or from food of bovine origin [33]. Facilitated by next generation sequencing (NGS) technologies, the identification of mobile genetic elements, including plasmids, transposons, prophages, genomic islands, and chromosomal pathogenicity islands (PAIs) encoding virulence- and antibiotic resistance-associated genes, has provided insight into the genomic differences among environmental, animal, and clinical strains isolated from geographically dispersed areas. Unlike other STEC that cause serious human illness, STEC O104 strains lack the *eae* gene; however, they can attach to intestinal cells. The EAHEC O104:H₄ outbreak strain 2011C-3493 also lacked *eae*; however, this strain possessed a plasmid (pAA) that encodes for adherence fimbriae [9]. Studies found that not all O104 serotypes possess *aggR*, which encodes a regulator of virulence plasmid and chromosomal genes including aggregative adherence fimbriae and *aaiA*-P (*aggR*-activated island encoding a type VI secretion system), a characteristic of EAEC. EAHEC O104:H₄ colonizes the human bowel through aggregative adherence fimbriae encoded by the EAEC plasmid (pAA). In the European O104:H₄ outbreak strains, the intimin (*eae*) function was substituted by the plasmid-encoded aggregative adherence fimbrial colonization mechanism, and once attached, the cells were able to produce and deliver Shiga toxin 2a, resulting in severe illness and HUS. Recently, Beutin et al. [1] studied the possible uptake of the chromosome-encoded *stx*₂-phage P13374 (from the German EAHEC O104:H₄ strain) by EAEC strains of different serotypes. In this study, the authors concluded that the *stx*₂-phage P13374 had a restricted host range, and its spread was dose-sensitive. One of the bovine-specific phages (P13803) was found to be capable of lysogenizing a *stx*-negative EAEC O104:H₄ strain and converted it into an EAEC-STEC producing *Stx*₂ [1,34].

Previously published sequence data on O104:H₄ isolates revealed variations in gene content among strains from different hosts and geographical sources [2,4,6,9,35]. Differences were observed in virulence- and resistance-
associated genes among various H-serotypes of O104 strains. A better understanding of the genomic differences among strains belonging to different O104:H serotypes will help to reveal the basis for their pathogenicity and will provide information to understand how they evolved. The aim of this study was to sequence the genomes of the O104:H21 milk outbreak strain and a STEC O104:H7 cattle isolate, which had not been associated with human illness, and perform comparative analyses with the genomes of several E. coli O104:H4 strains isolated from different geographical locations. The resultant sequence analysis revealed notable differences among O104 strains belonging to H-serotypes H21, H7, and H4, including the presence of different plasmids and other mobile genetic elements.

Results and discussion
General genomic features of O104:H21 and O104:H7
The genomes of STEC O104:H21 strain 94–3024 that caused an outbreak of hemorrhagic colitis in Montana in 1994 linked to contaminated milk and strain O104:H7 RM9387, isolated from cattle feces, were sequenced. The genome size of O104:H21 strain 94–3024 was 4,902,583 bp, while that of O104:H7 RM9387 was 4,827,630 bp. O104:H7 RM9387 carried 4 plasmids, 3,173, 6,673, 6,819, and 169,634 bp in size; on the other hand, O104:H21 strain 94–3024 had only one large 161,447-bp plasmid. The G + C content for strains O104:H7 RM9387 and O104:H21 94–3024 were 50.8% and 50.7%, respectively, similar to that of other E. coli O104 strains. Information on all strains analyzed in this study, including genome size, GC content, and number of plasmids, is shown in (Additional file 1: Table S1).

Comparison at the genomic level
In order to gain insight on the genomic differences among environmental, animal, and clinical strains isolated from geographically dispersed areas, the whole genome sequence of various EAEC/STEC O104 strains were analyzed. Whole-genome comparison showed that these 13 O104 strains shared a conserved core chromosomal backbone but contained various mobile genomic elements reflected in the differences in their genome sizes. Details will be further discussed in the next three sections.

A comparison of seven O104 genomes, as visualized by the program MAUVE [36], including three O104:H21 strains isolated during an outbreak of hemorrhagic colitis in Montana in 1994 [37], is shown in Figure 1. Homologous regions that are conserved are shown in the same colors in Figure 1; gaps within a unique genomic region are in white. In this Figure, considerable conservation in the 7 genomes is revealed, although some serotype-specific regions were observed. For example, all three O104:H4 isolates possessed 3 prominent regions either completely missing (region #1, PAI carrying aidA and various type VI secretion-associated proteins; region #2, hypothetical proteins), or partially missing (region #7, telurite resistance proteins and transporters) in O104:H7 and O104:H21 strains. Region #3 (phage_Yersin_413C; Additional file 2: Table S2) was present in all analyzed O104:H4 and O104:H21 strains, but missing in the sequenced O104:H7 strain. Other regions of significant genomic dissimilarity in O104:H7 include region #4 (various transporters and ATPase), #5 (transporters, etc.), and #6 (the adhesin gene, iha, Table 1).

Each O104 strain carried 1–4 plasmids of different sizes, ranging from 3,173 to 169,634 bp (Table 2). Interestingly,
| Serotype | Strain name       | Gene accession | Virulence, IS, and other biomarker genes |
|----------|-------------------|----------------|------------------------------------------|
|          |                   | fliCH4 | fliCH7 | fltCH21 | wzxO104 | wzyO104 | stx2 | tehB | iha | lpfα | ent | tir | stx1 | eae | Efa1 | lpfα | tccp | tccp2 | IS629 | CRISPR2 |
| O104:H7  | RM9387            | CP009104 | -      | +       | -       | +       | +     | +    | -    | -    | -    | -    | -     | -    | -    | -    | -    | -    | -     | -    | -  |
| O104:H21 | 94-3024           | CP009106 | -      | -       | +       | +       | +     | +    | -    | -    | -    | -    | -     | -    | -    | -    | -    | -    | -     | -    | -  |
| O104:H21 | BAA-178 (CDC 1994-3024) (CFSAN002236) | SRX319158 | -      | -       | +       | +       | +     | +    | -    | -    | -    | -    | -     | -    | -    | -    | -    | -    | -     | -    | -  |
| O104:H21 | BAA-182 (CDC 1994-3023) (CFSAN002237) | SRX319159 | -      | -       | +       | +       | +     | +    | -    | -    | -    | -    | -     | -    | -    | -    | -    | -    | -     | -    | -  |
| O104:H4  | 2009EL-2050       | NC_018650.1 | +      | -       | -       | +       | +     | +    | +    | -    | -    | -    | -     | -    | -    | -    | -    | -    | -     | -    | +  |
| O104:H4  | 2009EL-2071       | NC_018661.1 | +      | -       | -       | +       | +     | +    | +    | -    | -    | -    | -     | -    | -    | -    | -    | -    | -     | -    | +  |
| O104:H4  | 2011C-3493        | NC_018658.1 | +      | -       | -       | +       | +     | +    | +    | -    | -    | -    | -     | -    | -    | -    | -    | -    | -     | -    | +  |
| O104:H4  | LB226692          | SRR254209 | +      | -       | -       | +       | +     | +    | -    | -    | -    | -    | -     | -    | -    | -    | -    | -    | -     | -    | +  |
| O104:H4  | TY-2482           | SRX067313 | +      | -       | -       | +       | +     | +    | +    | -    | -    | -    | -     | -    | -    | -    | -    | -    | -     | -    | +  |
| O104:H4  | C760-09           | SRX078303 | +      | -       | -       | +       | +     | -    | +    | -    | -    | -    | -     | -    | -    | -    | -    | -    | -     | -    | +  |
| O104:H4  | C777-09           | SRX078304 | +      | -       | -       | +       | +     | +    | +    | -    | -    | -    | -     | -    | -    | -    | -    | -    | -     | -    | +  |
| O104:H4  | C734-09           | SRX078308 | +      | -       | -       | +       | +     | -    | +    | -    | -    | -    | -     | -    | -    | -    | -    | -    | -     | -    | +  |
| O104:H4  | C754-09           | SRX078302 | +      | -       | -       | +       | +     | -    | +    | -    | -    | -    | -     | -    | -    | -    | -    | -    | -     | -    | +  |
| O104:H4  | 4281-1            | FM872416.1 | na      | na      | na      | na      | na    | na   | na   | na   | na   | na   | na     | na   | na   | na   | na   | na   | na     | na   | na  |
| O91:H21  | B2F1              | AGT01000001-AGT01000023 | -      | -       | +       | -       | +     | +    | -    | -    | -    | -    | -     | -    | -    | -    | -    | -    | -     | -    | -  |
| O113:H21 | CL-3              | NZ_AGTH01000001-NZ_AGTH01000002 | -      | -       | +       | -       | +     | +    | -    | -    | -    | -    | -     | -    | -    | -    | -    | -    | -     | -    | +  |
| O157:H7  | Sakai             | NC_002695 | -      | +       | -       | -       | +     | +    | +    | +    | +    | +    | +     | +    | +    | +    | +    | +    | +     | +    | +  |
| O145:H28 | 4865/96           | AGTL01000001-AGTL01000005 | -      | -       | -       | -       | -     | +    | +    | +    | +    | +    | +     | +    | +    | +    | +    | +    | +     | +    | +  |
| O45:H2   | 03-EN-705         | AGTK01000001-AGTK01000043 | -      | -       | -       | -       | -     | -    | +    | +    | +    | +    | +     | +    | +    | +    | +    | +    | +     | +    | +  |
| O26:H11  | Str. 11368        | NC_013361 | -      | -       | -       | -       | -     | +    | +    | +    | +    | +    | +     | -    | -    | -    | -    | -    | -     | -    | +  |
| O121:H19 | MT#2              | AGTJ01000001-AGTJ01000005 | -      | -       | -       | -       | -     | +    | -    | -    | +    | +    | +     | +    | +    | +    | +    | +    | +     | +    | +  |
| O103:H2  | Str. 12009        | NC_013353 | -      | -       | -       | -       | -     | +    | +    | +    | -    | -    | -     | -    | -    | -    | -    | -    | -     | -    | +  |
| O111:H   | 11128             | NC_013364 | -      | -       | -       | -       | -     | -    | -    | -    | -    | -    | -     | -    | -    | -    | -    | -    | -     | -    | +  |
| O111:H21 | Str. 226          | SRX160797 | -      | -       | +       | -       | -     | +    | -    | -    | -    | -    | -     | -    | -    | -    | -    | -    | -     | -    | +  |

na: there is no whole genome sequence but partial fragment available.
| Serotype | Strain name | Gene accession | Size (~Kb) | %GC | Virulence or other biomarker genes |
|----------|-------------|----------------|-----------|-----|-----------------------------------|
|          |             |                |           |     | *AggR* | *AggB*-C-D | *saa* | *Ecf* cluster | *Hly* cluster | *excA* | *subA* | *SubB* | *espP* | *type IV pilus gene cluster* | *katP* | *espA* | *Etp cluster* (type II) | *msbB* | *stcE* | *toxB* |
| O104:H7  | RM9387      | CP009104       | 169,634   | 49.5 | -       | -             | -     | -             | +             | +       | +       | +       | +       | +                   | -       | -       | -             | -       | -       | -       |
| O104:H21 | 94-3024     | CP009106       | 161,447   | 49.5 | -       | -             | -     | -             | +             | +       | +       | +       | +       | +                   | -       | -       | -             | -       | -       | -       |
| O104:H21 | BAA-178     | (CDC 1994-3024) | (CFSAN002236) | SRX319158 | na    | na             | -     | -             | +             | +       | +       | +       | +       | +                   | -       | -       | -             | -       | -       | -       |
| O104:H21 | BAA-182     | (CDC 1994-3023) | (CFSAN002237) | SRX319159 | na    | na             | -     | -             | +             | +       | +       | +       | +       | +                   | -       | -       | -             | -       | -       | -       |
| O104:H4  | 2009EL-2050 | NC_018651      | 109,274   | 45.3 | -       | -             | -     | -             | -             | -       | -       | -       | -       | -                   | -       | -       | -             | -       | -       | -       |
| O104:H4  | 2009EL-2071 | NC_018662.1    | 75,573    | 47.2 | +       | +             | -     | -             | -             | -       | -       | -       | -       | -                   | -       | -       | -             | -       | -       | -       |
| O104:H4  | 2011C-3493  | NC_018659      | 88,544    | 49.7 | -       | -             | -     | -             | -             | -       | -       | -       | -       | -                   | -       | -       | -             | -       | -       | -       |
| O104:H4  | LB226692    | SRR254209      | na        | na    | +       | +             | -     | -             | -             | -       | -       | -       | -       | -                   | -       | -       | -             | -       | -       | -       |
| O104:H4  | TY-2482     | SRX067313      | na        | na    | na      | +             | -     | -             | -             | -       | -       | -       | -       | -                   | -       | -       | -             | -       | -       | -       |
| O104:H4  | C76-09      | SRX078303      | na        | na    | -       | -             | -     | -             | -             | -       | -       | -       | -       | -                   | -       | -       | -             | -       | -       | -       |
| O104:H4  | C77-09      | SRX078304      | na        | na    | -       | -             | -     | -             | -             | -       | -       | -       | -       | -                   | -       | -       | -             | -       | -       | -       |
| O104:H4  | C734-09     | SRX078308      | na        | na    | -       | -             | -     | -             | -             | -       | -       | -       | -       | -                   | -       | -       | -             | -       | -       | -       |
| O104:H4  | C754-09     | SRX078302      | na        | na    | -       | -             | -     | -             | -             | -       | -       | -       | -       | -                   | -       | -       | -             | -       | -       | -       |
| O157:H7  | Sakai       | NC_002128.1    | 92,721    | 47.6 | -       | -             | -     | -             | -             | -       | -       | -       | -       | -                   | +       | +       | +             | +       | +       | +       |
| O157:H7  | 3072/96     | NC_009620.1    | 121,239   | 49.7 | -       | -             | -     | -             | -             | -       | -       | -       | -       | -                   | -       | -       | -             | -       | -       | -       |
| O113:H21 | EH41        | NC_007365.1    | 165,548   | 49.6 | -       | -             | -     | -             | -             | -       | -       | -       | -       | -                   | -       | -       | -             | -       | -       | -       |
| O26:H11  | Str. 11368  | NC_013369.1    | 85,167    | 47.5 | -       | -             | -     | +             | +             | +       | -       | -       | -       | -                   | -       | -       | +             | +       | +       | -       |
|          | H30         | FJS86569.1     | 168,100   | 50.2 | -       | -             | +     | -             | -             | -       | -       | -       | -       | -                   | +       | -       | +             | +       | +       | +       |
| O145     | RM13516     | CP006028       | 87,120    | 47.6 | -       | -             | +     | -             | +             | +       | -       | -       | -       | -                   | -       | -       | +             | +       | +       | -       |
| O121:H19 | MT#2        | AGTJ010000001-AGTJ010000389 | na        | na    | -       | -             | -     | -             | -             | -       | -       | -       | -       | -                   | -       | -       | -             | +       | +       | -       |
| O103     | APEC O103   | NC_011964.1    | 124,705   | 50.8 | -       | -             | -     | -             | -             | -       | -       | -       | -       | -                   | -       | -       | -             | -       | -       | -       |
| O103     | Str. 12009  | NC_013354.1    | 75,546    | 49.1 | -       | -             | +     | -             | -             | -       | -       | -       | -       | -                   | -       | -       | +             | +       | +       | -       |
Table 2 Comparison of large plasmids among STEC strains using BLASTN (coverage >90%, identity >90%) (Continued)

| Strain | Accession | Size (bp) | Coverage (%) | Identity (%) | Feature 1 | Feature 2 | Feature 3 | Feature 4 | Feature 5 | Feature 6 | Feature 7 | Feature 8 | Feature 9 |
|--------|-----------|-----------|--------------|--------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| O45:K1:H7 S88 | CU928146 | 133,853 | 49.3 | - | - | - | - | - | - | - | - | - | - |
| O111-aEPEC D275 | KC340959.1 | 115,452 | 53.1 | - | - | - | - | + | - | - | - | + | - |
| O111:H21 Str. 226 | SRX160797 | na | na | + | - | - | - | + | - | - | - | + | - |
| O91:H21 B2F1 | AGTI01000001-AGTI01000233 | na | na | - | - | - | - | + | + | - | - | + | - | - | - |

Yan et al. BMC Microbiology (2015) 15:83
the plasmids from the newly sequenced O104:H21 strain 94–3024, (161,447 kb, GenBank accession CP009107) and O104:H7 strain RM9387 (169,634 kb, GenBank accession CP009105) shared a high degree of overall sequence similarity to an STEC O113:H21 plasmid (strain EH41, GenBank accession NC_007365.1), less sequence similarity to plasmids from serotypes O157:H7 (NC_002128.1), and O26:H30 (FJ386569.1), and no sequence similarity to plasmids from the German outbreak O104:H4 strain (2009EL-2050, GenBank accession NC_018651.1, Figure 2A and B).

**Whole genome phylogenetic comparisons among various O104 H type strains and other LEE-negative STEC**

In this study, we first inferred a whole-genome phylogeny of strains based on an alignment (Additional file 1: Table S1) based on an alignment of whole-genome contigs or complete full genome sequence mapped onto the complete closed genome sequence of the O104:H4 outbreak strain 2009EL-2050 (NC_018650.1) as the reference. A phylogenetic tree of O104 strains of different H-types from different sources revealed overall similar clustering of

---

**Figure 2** Pairwise comparison of plasmids from different H-types of O104 isolates (A) and other STEC strains (B). The plasmid sequences from various STEC strains were aligned and then visualized using the Artemis Comparison Tool (ACT). Plasmid sequence with similarity is shown by red (Homologous blocks) and blue lines (sequence block inversion) between the plasmids.
strains belonging to the H4 type, and that H7 and H21 had a closer relationship to each other than to O104:H4 (Figure 3A). The similarity of the profiles of the mobile genetic elements and the phylogenetic tree analysis indicated that O104:H7 and O104:H21 might share a similar evolutionary path since they are more closely related to each other than to O104:H4 strains. Phylogenetic comparisons among various LEE-negative STEC serotypes and different O104 strains showed that LEE-negative STEC serotypes O91:H21 and O113:H21 were genetically closer to O104:H21 and O104:H7 than to O104:H4 (Figure 3B). Our phylogenetic analyses (Figures 3) demonstrated that O104 strains with the same H-type clustered together (i.e. monophyletic) but their corresponding serogroups were cladded into multiple independent lineages (i.e. polyphyletic). Scattered distribution patterns of virulence factors and resistance genes, as well as phylogenetic analysis of these different O104 strains may suggest that strains from individual lineages might have acquired virulence genes and antibiotic resistance genes independently in parallel evolutionary processes.

**stx and eae genotypes**

Shiga toxins Stx₁ and Stx₂ are recognized as the most important phage-encoded virulence factors in all disease-associated STEC strains, and the genes encoding for these toxins are located in the genome of mobile bacteriophages [38,39]. The stx phages are genetically distinct groups of

---

**Figure 3** Phylogenetic comparisons of *E. coli* O104 strains with various H-types from different sources (A) and to other LEE-negative STEC serotypes (B) by using whole genome sequences based on a MAUVE progressive alignment.
temperate phages that can be identified in their prophage states inserted in the STEC chromosome, but also can be detected as phages released from the bacteria into the environment and/or animal hosts. stx phages can exist in polluted waters, feces, food, in humans, and the environment [40,41]. Generally speaking, phages could be used to detect and to derive lineage/evolution of newly emerged STEC strains from different hosts and/or environments [34,42-44]. The diagram in Figure 4A demonstrates significant diversity in gene sequence and structure to the predicted stx prophage region among serotypes O104:H7 (strain RM9387), O104:H21 (strain 94–3024), O104:H4 (strain 2009EL-2050, data not shown for other European outbreak strains), and O104:H4 (Africa strain C734-09, data not shown for other Africa strains). Sequence alignment and the linkage pattern analysis (Figure 4B) among O104 strains confirm that the region of predicted stx prophage shares weak sequence similarity among O104:H4, O104:H7, and O104:H21 strains. There was high sequence similarity among German outbreak strains (strain 2009EL-

**Figure 4** Comparison of predicted stx prophage region in various H-types of O104 strains. Top panel (A): general gene features of the predicted stx prophage region among O104 strains; color and number codes for various function categories are below the main figure (Box). Bottom panel (B): sequence alignment of the predicted stx prophage region visualized using ACT.
phage modular genetic structure in various O104 strains is illustrated on the diagram of Figure 4A. Genetic sequence analysis of the European outbreak O104:H4 strains showed that all isolates possessed stx subtypes, and the combination of stx2c, stx2d, and stx2e genes (data not shown). STEC strains that carry specific stx2 subtypes, especially stx2, stx2c, and stx2d tend to be more virulent [45]. In this study, activatable stx2d was found to be the sole stx gene in our sequenced O104:H21 and O104:H7 strains. Sequence analysis also revealed that none of the O104 strains harbored the stx2c, stx2d or stx2e alleles.

*eaе* is another important virulence gene of EPEC, EHEC, and STEC strains. The three prominent LEE integration sites selenocystyl-tRNA (selC), pheII, and pheV were previously described [46]. The different insertion sites of the LEE in various STEC suggest that the acquisition of LEE may depend on the strain genetic background, the environmental conditions to which the strain is exposed, and the mechanisms of genetic recombination. Our analysis showed that the O104:H7, O104:H21, and O104:H4 do not possess *eaе* genes. It has been reported that serotype O104:H7 carried an *eaе*-TAU (GenBank Accession: FM872416.1) variant. Strains expressing *eaе*-TAU were generally restricted to and efficiently colonized Peyer’s patches in human intestinal mucosa [47]; so far, serotype O104:H4 has been found only in humans [21]. Schmidt and colleagues [48] reported a PAI in *E. coli* O91:H7 that occurs exclusively in a subgroup of STEC strains that are *eaе* negative and contain the variant stx2d gene, similar to O104:H7 and O104:H21 in this study.

Also interestingly, a DNA fragment containing the LEE-encoded translocated-intimin receptor (*tir*) effector gene from serotype O104:H12 strain 4051–6 was sequenced (GenBank Accession# AB288103.1); however *tir* was missing in all O104:H4, O104:H7, and O104:H21 strains examined in the current study (Table 1). The *tir* gene is typically encoded by the LEE, flanked by ORF19 (upstream) and *eaе* (downstream) although the whole genome sequence of O104:H12 strain 4051–6 is not available currently. The functional *Tir* protein is translocated into the epithelial cell and is integrated into the cell membrane where it functions as a receptor for *eaе*. Oswald and coworkers [49] showed that allelic differences in *eaе* were associated with differences in tissue and host specificity. Thus, it is possible that O104 strains belonging to H7 or H21 serotype possess the EAEC or EHEC genetic background to acquire functional genes of various PAIs or *eaе* variants (Figure S1). A detailed description of Figure 5 is presented in next section.

**Identification and analysis of prophages, genomic islands, and/or pathogenicity islands**

The number of predicted prophages varied greatly among various O104 H-type strains (Additional file 2: Table S2). Our analyses showed the distribution of different types of bacteriophages, particularly the *stx*-carrying prophages, in the genomes of O104:H7, O104:H21, and O104:H4. Supporting the hypothesis that the different H-serotypes of O104 may acquire diverse types of bacteriophages due to possible genome adaptation to niches [50,51]. For instance, the similarity between bacteriophage types in the genomes of O104:H7 and O104:H21 shown in (Additional file 2: Table S2) suggested that they may come from the same evolutionary path or evolved in a similar environment.

An integrated comparative genomic program known as IslandViewer was used to identify genomic islands (GIs) and/or PAIs that may have been introduced into the genome by horizontal gene transfer (HGT). By directly applying IslandViewer for the identification of GIs in the genomes of O104:H21, O104:H7, and the O104:H4 reference strain (NC_018650), we found that the percentage of horizontally transferred genomic islands varied from 9.3%, 7.6%, and 12.3% among O104:H21, O104:H7, and the O104:H4 reference strain. The conserved GIs are identified by aligning all GIs in the reference strain O104:H4 to the GIs in other O104 strains. We found the conserved GI pools represented 70.9%, 65.7%, 65.5%, 49.1%, 44.3%, 19.3%, 15.3%, and 13.6% of the reference genomic islands from NC_018650 in O104:H4 strains LB226692, TY2482, C734-09, C754-09, C777-09, C760-09, O104:H21 94–3024, and O104:H7 RM9387, respectively. O104:H7 and O104:H21 appeared to be closer to each other and the O104:H4 C760-09 strain from Africa than to other O104:H4 strains, which is consistent with the phylogenetic tree in Figure 3A.

PAIs belong to a specialized class of GIs. They carry multiple virulence loci, may take up a large chromosomal region (>10 kb) of pathogens, are absent from non-pathogens, and usually have a different GC content from that of the core genome. A PAI from an O103:H strain which is inserted into the *selC* site between *yicI* and *nlpA* (shown in Figure 5) contains 40 ORFs encoding proteins with ≥50 deduced amino acids and one *selC* site (numbered sequentially from 1 to 41). The proteins encoded in this island include a novel serine protease EspI (ORF#15); an adherence-associated locus, similar to *iha* of *E. coli* O157:H7 (ORF#18); an *E. coli* vitamin B12 receptor (ORF #20); an *araC*-type regulatory module (ORF #22-26); and several other important and/or unknown function proteins. The remaining sequence consists largely of complete and incomplete
insertion sequences, prophage sequences, and an intact phage integrase gene that is located directly downstream of the chromosomal selC [48]. A pairwise alignment of the selC-tRNA loci between yicI and nlpA to this typical O103 PAI shows some interesting genomic structural differences among O104 H-serotypes, O103:H^−, O157:H7, non-O157 STEC, and other serotypes including E. coli K-12 (Figure 6). All of these strains shown in Figures 5 and 6 share the same gene structure at the N-terminal (yicI, yicJ, and selC) and C-terminal (yicL and nlpA), but none of these serotypes are genetically similar in the central portion of this typical PAI structure.

The genomic region of the selC site in O104:H7 and O104:H21 is 7775 bp and it encodes no known functional proteins. Here we propose the concept of “pseudo PAI”, a genomic region less than 10 kb in size that lacks key functional gene products typically found in O157:H7 and O103:H^− (Figure 5). Interestingly, strains carrying “pseudo PAI” in this selC site normally have smaller genome sizes and they could have lower pathogenicity if they also lack plasmids carrying virulence genes. It is important to note that the eae gene has at least 27 different variants and one of the subtypes, eae-TAU, has been isolated from patients with hemolytic-uremic syndrome in O104:H^− [Additional file 3: Figure S1] [52]. As shown in Figure 6, the presence and size of the PAI in the selC tRNA locus also shows no correlation with serotype. A “pseudo PAI” (7775 bp between genes yicI to nlpA) was found in strains of serotypes O104:H7, O104:H21, O111: H^+, O113:H21, O121:H19, O91:H21, O103:H25, O103:H2, O26:H11, and even non-pathogenic E. coli K12. These differences in the size of the functional PAIs (serotypes O26, O145, O157, O104:H4, and strain 4797/97 O103:H^−) or “pseudo PAI” among the same serogroup was also observed for O104 and O103:H^−. These observations are suggestive of the acquisition of the different PAIs by the same serotype, including O104:H4 strains due to their different ecological niches and/or the geographical regions where they were found. The selC site can be targeted by bacteriophage carrying LEE or LEE-like PAIs (e.g., O157, O26, etc.), bacteriophage harboring resistance genes (e.g. O104:H4) (shown in Figures 5 and 6) through horizontal genetic transfer (HGT) and adaptation to new ecological niches, or it can remain empty (e.g., O104:H7 and O104:H21).

As stated above, within different types of PAI inserted into the selC site, genes that may encode antibiotic resistance, are associated with altered metabolism, or that express virulence factors are located in the central portion of the island and are surrounded by mobility-associated genes (ORF with red color, Figure 5). Mobile genetic elements (ORF 28–30, 33, 34–36, and 38) are
extensively interspersed throughout the genome of serotype O104:H4, but less so in O104:H21 (ORF 28, 29, 38, 19, 30, 33) and O104:H7 (no ORFs, except the pseudo PAI portion), particularly O104:H7 (Figure 5). However, Schmidt and coworkers [46] indicated that LEE in O91:H− was flanked by prophage sequences and was devoid of IS-related sequences, which were only shown in O157:H7 in Figure 5. Our data may indicate that O104 H-serotypes could acquire PAI with or without an intact or a portion of LEE by phage transduction or HGT, which potentially could make O104 H-serotypes more pathogenic.

At present, it is not known if the PAIs in different O104 H-serotypes were in the process of stepwise insertion of genes into the region, or stepwise deletion of genes from the "original" PAI, similar to those in O103 and O91:H-serotypes via complex mechanisms of horizontal transfer and/or recombination. A novel PAI, originally identified in O103:H− and O91:H−, was also partially found in one O104 H− serotype but showed some rearrangement (Additional file 3: Figure S1).

Based on our analyses, we propose an evolutionary model (Additional file 3: Figure S1) in the selC-tRNA site

---

**Figure 6** Pairwise alignment of the selC-tRNA loci between yicI and nlpA among various *E. coli* strains using MAUVE.
of various H-serotypes of *E. coli* O104 strains in a stepwise insertion fashion. This model suggests that O104:H\(^+\) and O104:H4 may derive from the ancestral O104:H7 or O104:H21 by the acquisition of antibiotic resistance genes (O104:H4) or eae-TAU carried by a prophage/PAI (O104: H\(^+\)) and other genetic components, such as the plasmid carrying *aggR* gene. Various O104 strains were also shown to carry *stx*\(_2\) [53] (Table 2), implicating that within the O104 serogroup there are multiple acquisition events of prophage, GI, and/or PAI. PAs can excise at different frequencies depending on growth conditions [54-56]. This suggests that genetic versatility is needed for the survival of *E. coli* in diverse environments, which may be relevant regarding its host specificity, as well.

**Chromosome- and/or plasmid- encoded virulence genes, CRISPR, and IS elements among O104 strains**

The most noticeable difference in the genomes of these O104 serogroups was the presence of various types of mobile genetic elements, and the variations in the numbers of chromosome-encoded virulence genes. The H7, H21, and H4-specific *flgC*, *wzx*, *wzy*, *eae*, *lpfA* (plasmid-based), *stx*, *stx*, *efA1*, *ent*, *espL2*, *tir*, *nleB* and *nleE* (O1-122 specific), *espK* (prophage CP-933 N), *tehB*, *iha*, and O104- and O157-specific *lpfa*, *tccp*, *tccp2*, IS629 integrate, and the CRISPR2 sequence were used to define serogroup and pathogroups of O104:H7, O104:H21, and other O104:H4 strains. Based on the genotypic differences, *E. coli* pathotypes can be categorized as atypical EPEC (eae only), typical EPEC (*eae* and *lpfA*), STEC (*stx* and/or *stx* with or without *eae*), EHEC (*eae* and *stx* and/or *stx*), *aggR*-positive EAHEC (absence of *eae* but with *stx* and/or *stx* and *aggR*), and *aggR*-negative EAHEC/STEC (absence of *eae* and *aggR* but with *stx* and/or *stx*). These virulence factors and O- and H-antigen specific genes are useful for molecular characterization of EPEC, STEC, EHEC and EAHEC strains at the genomic level (Table 1). The *E. coli* O104:H4 European outbreak strain contains a *stx*-encoding phage similar to 933 W with only one nucleotide polymorphism in each of the subunits (*stx*\(_{20}\) and *stx*\(_{20}\)) compared to 933 W. The German outbreak O104:H4 strains (Additional file 1: Table S1, see the origin of strain) also contain genes encoding several important serine protease autotransporter toxins, including *sepA* (Shigella extracellular protein A), *sigA* (Shigella IgA protease-like homologue), *pic* (protein involved in intestinal colonization), *aap* (dispersin), *aatPABCD* (dispersin transporter), and others; however, none of these were found in the sequenced O104:H7 and O104:H21 strains (data not shown). Aside from C760-09, O104:H4 strains from Africa did not carry noteworthy virulence factors other than the *iha* gene; however, C777-09 possessed the *stx*\(_2\) gene, thus indicating potential virulence (Table 1).

Strain O104:H7 RM9387 and all O104:H21 isolates carried virulence genes that included *lpf* (long polar fimbriae) and *iha* (IrgA homologue adhesin) that are associated with EHEC and colonization of the gut, but lacked the *aggR*, *aggB-C-D* modular genes (Table 2).

The *stx*\(_1\)- and *stx*\(_2\)- prophages are normally flanked by integrative element IS629 at both ends in almost all O157 and non-O157 STEC, including O26, O45, O111, O121, and O145. Our analysis of the *stx* prophages indicated that the transposable insertion sequence element IS629 may be a driver of *stx* and *stx*, prophage evolution among O104:H7, O104:H21, and other LEE-negative STEC strains. We have hypothesized that *stx* genes may excise from African strains after infection, owing to a functional change of a putative integrase near the *yggA* (inner membrane protein) gene due to frameshift- and nonsense-mutations. After transposition, there is only one IS629 left in O104:H4. IS629 insertion is highly biased toward prophages and prophage-like integrative elements. Both IS629 and IS602 are not present in O104:H7 and O104:H21 but exist in all O104:H4 strains (Table 1). IS602 also appears to be part of kanamycin (and related aminoglycosides) resistance transposons [57]. In addition, O104: H4-specific CRISPR2 was not found in any of the other STEC strains examined in this study (Table 1), including O104:H7 and O104:H21. The presence and the functions of CRISPR2 [58] may play a significant role in O104:H4 for maintaining a high degree of genomic plasticity and flexibility for adapting to host and environmental changes. Analysis of IS629 and CRISPR distribution among O104 strains might be useful for identification and detection of specific O104 strains and population genetic analysis, as well as molecular epidemiology studies. The presence of IS629, CRISPR, and prophage regions (data not shown) may potentially contribute to the larger genome size of O104:H4 (Additional file 1: Table S1 and Figure 1).

There was no apparent correlation between serotypes and plasmid profiles among O104:H4, O104:H7, and O104:H21 strains, as well as other non-O104 STEC strains (Table 2, Figure 2). There was marked variability in the numbers and types of genes carried by the plasmids in the O104 strains (Tables 2 and 3); none of the 13 *E. coli* O104 isolates carried the same set of plasmids. STEC O104:H7 RM9387 had four plasmids, while most other O104 strains carried only 1 or 2 plasmids. The following genes used to define EAEC, STEC, and EHEC strains were located on some plasmids: *aggR*, *aggB-C-D* cluster (encoding major fimbrial subunits), *saa*, *ecf* cluster, *ehx* cluster, *etp* cluster (type II secretion system apparatus), *toxB* (putative adhesion), *katP* (catalase-peroxidase), *espP*, *espA*, *sepA* (serine protease *sepA* precursor), *subAB*, typeV pilus gene cluster, *mabB*, *stcE* (zinc metalloprotease), and *excA* (exclusion-determining protein), in agreement with other previous reports [10,59-62]. These genes are useful
| Serotype | Strain name       | Chromosome- and/or plasmid-encoded antibiotic resistance genes |
|----------|-------------------|---------------------------------------------------------------|
|          |                   | mdtL  vancomycin  beta-lactamase  emrE  ksgA  emrB/QacA  mdtG  mdaB  ydeB  dacC |
| O104:H7  | RM9387            | +     +          -           +       +   +     +     -       +     -     +     +     +       |
| O104:H21 | 94-3024           | +     +          -           -       +   +     +     +       +     +     +     +     +       |
| O104:H21 | BAA-178 (CDC 1994-3024) (CFSAN002236) | +     +          -           -       +   +     +     +       +     +     +     +     +       |
| O104:H21 | BAA-182 (CDC 1994-3023) (CFSAN002237) | +     +          -           -       +   +     +     +       +     +     +     +     +       |
| O104:H4  | 2009EL-2050       | +     +          +           +       +   +     +     +       +     +     +     +     +       |
| O104:H4  | 2009EL-2071       | +     +          +           +       +   +     +     +       +     +     +     +     +       |
| O104:H4  | 2011C-3493        | +     +          +           +       +   +     +     +       +     +     +     +     +       |
| O104:H4  | LB226692          | +     +          +           +       +   +     +     +       +     +     +     +     +       |
| O104:H4  | TY-2482           | +     +          +           +       +   +     +     +       +     +     +     +     +       |
| O104:H4  | C760-09           | +     +          +           -       +   +     +     +       +     +     +     +     +       |
| O104:H4  | C777-09           | +     +          +           -       +   +     +     +       +     +     +     +     +       |
| O104:H4  | C734-09           | +     +          +           -       +   +     +     +       +     +     +     +     +       |
| O104:H4  | C754-09           | -     -          +           -       +   +     +     +       +     +     +     +     +       |
| O157:H7  | Sakai             | +     +          -           -       +   +     +     +       +     +     +     +     +       |
| O145:H28 | 4865/96           | +     +          -           -       +   +     +     +       +     +     +     +     +       |
| O45:H2   | 03-EN-705         | +     +          -           -       +   +     +     +       +     +     +     +     +       |
| O113:H21 | CL-3              | +     +          -           -       +   +     +     +       +     +     +     +     +       |
| O26:H11  | Str. 11368        | +     +          -           -       +   +     +     +       +     +     +     +     +       |
| O121:H19 | MT#2              | +     +          -           -       +   +     +     +       +     +     +     +     +       |
| O103:H2  | Str. 12009        | +     +          -           -       +   +     +     +       +     +     +     +     +       |
| O111:H4  | 11128             | +     +          +           +       +   +     +     +       +     +     +     +     +       |
| O111:H21 | Str. 226          | +     +          -           -       +   +     +     +       +     +     +     +     +       |
| O91:H21  | B2F1              | +     +          -           -       +   +     +     +       +     +     +     +     +       |
| Serotype  | Strain name           | Chromosome- and/or plasmid-encoded antibiotic resistance genes |  |
|-----------|----------------------|---------------------------------------------------------------|---|
|           |                      | folA-O157  | gentamicin resistance protein* | streptomycin 3'-kinase* | tetA (class A) * | tetA (class D) * | folA | beta-lactamase2* | aph* | erythromycin resistance repressor* | floR* |
| O104:H7   | RM9387               | +         | -                               | -                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O104:H21  | 94-3024              | +         | -                               | -                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O104:H21  | BAA-178 (CDC 1994-3024) (CFSAN002236) | +         | -                               | -                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O104:H21  | BAA-182 (CDC 1994-3023) (CFSAN002237) | +         | -                               | -                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O104:H4   | 2009EL-2050          | +         | -                               | +                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O104:H4   | 2009EL-2071          | +         | -                               | +                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O104:H4   | 2011C-3493           | +         | -                               | +                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O104:H4   | LB226662             | +         | -                               | +                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O104:H4   | TY-2482              | +         | -                               | +                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O104:H4   | C760-09              | -         | -                               | +                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O104:H4   | C777-09              | +         | -                               | +                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O104:H4   | C734-09              | -         | -                               | +                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O104:H4   | C754-09              | -         | -                               | +                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O104:H7   | Sakai                | +         | -                               | -                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O157:H7   | 4865/96              | -         | -                               | +                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O145:H28  | 03-EN-705            | +         | -                               | -                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O1113:H21 | CL-3                 | +         | -                               | -                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O26:H11   | Str. 11368           | +         | -                               | -                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O121:H19  | MT#2                 | +         | -                               | -                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O103:H2   | Str. 12009           | +         | -                               | -                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O111:H1   | 11128                | -         | -                               | -                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O111:H21  | Str. 226             | +         | -                               | -                          | -                         | -                      | -     | -                         | -     | -                           | -         |
| O91:H21   | B2F1                 | +         | -                               | -                          | -                         | -                      | -     | -                         | -     | -                           | -         |

*: plasmid-encoded.
for the molecular detection and characterization of STEC, EAEC, and EHEC strains. A comparison of the various O104 strains revealed that subAB (a potent toxin involved in inducing cell death), the ehx cluster, espP, and excA were found in O104:H7, O104:H21, and LEE-negative O113:H21 strains, but not in O104:H4 strains. Therefore, it is suggested that virulence of the O104:H21 outbreak strain may be due in part to plasmid-associated virulence genes, including subAB, the ehx cluster, and espP (Table 2). In Table 2, only O104:H4 strains and an O111:H21 strain carried the transcriptional activator aggR and/or the aggregate B-C-D gene cluster (Table 2). subAB was more prevalent in LEE-negative than in LEE-positive STEC strains and likely contributes to the progression to severe disease [63], since the O113:H21 strain was involved in an outbreak with cases of hemolytic uremic syndrome (Table 2).

### Antibiotic resistance gene profiling

There were a number of genes related to antibiotic resistance in O104:H4. The O104:H4 outbreak strains were resistant to at least 14 different antibiotics including ampicillin, sulfonamide, cefotaxime, cefazidime, streptomycin, sulfamethoxazole, trimethoprim, cotrimoxazole, tetracycline, and nalidixic acid, but were susceptible to imipenem, kanamycin, gentamicin, chloramphenicol, and ciprofloxacin [62]. The O104:H4 outbreak strain carried a plasmid, pESBL, that encodes for extended spectrum β-lactamase CTX-M-15 [64,65]. Although the actual plasmid sequences were not available, genomic analysis showed that the four O104:H4 strains isolated from Africa carried some resistance genes (both chromosomal and plasmid-borne), but not as many as the O104:H4 strains of European origin (Table 3). On the other hand, O104:H7 and O104:H21 strains did not carry the plasmid-borne antibiotic resistance genes found in the German outbreak O104:H4 strains (Table 3). These antibiotic resistance genes listed in Table 3 may have been acquired through horizontal gene transfer by transposon-based plasmid integration (Table 3, Additional file 3: Figure S1). The analysis of the plasmid profiles of various O104 H-types and other STEC serotypes (Table 2) is consistent with the observation that EHEC plasmid-encoded genes (Table 2, GenBank accession CP009105 and CP009107), including lhl cluster, excA, subA/B, espP, were only found in STEC O104:H7 and O104:H21 strains, but not in O104:H4 strains [10].

### Conclusions

The milk-associated outbreak STEC O104:H21 strain 94–3024 and the O104:H7 cattle isolate RM9387 were shotgun sequenced, analyzed, and compared to elucidate the potential relationship, origin, and evolution of bacterial pathogenesis in various O104 H-serotypes. The observed variation between different O104 H-serotype genomes demonstrated that genome-wide divergence likely occurred via acquisition and loss of genomic islands, prophages, and plasmids. The genetic diversity in O104:H7, O104:H21, and O104:H4 serotypes was reflected in differences in virulence and resistance genes carried on the chromosome and on plasmids, suggesting their independent evolution demonstrated by different distribution and types of genetic mobile elements.

Genetic diversity of stx2 bacteriophages among O104: H4, O104:H7, and O104:H21 is illustrated in Figure 4. Further studies are needed to define the sources (cattle and other animals, fresh produce, the environment, humans) of these stx phages and to determine the frequency of lysogenization of E. coli O104:H7 and O104: H21 and phage origin by comparing to stx2-carrying phages from E. coli O104:H4 and other non-O104 EAEC/ STEC strains. Their roles in dynamic bacterial genome evolution have been increasingly highlighted by the fact that many sequenced bacterial genomes contain multiple prophages carrying a wide range of genes, such as stx and antimicrobial resistance genes.

Bacteriophages are major genetic factors promoting horizontal gene transfer (HGT) between bacteria. In this study, a "pseudo selC-tRNA site PAI" was defined containing perfect direct repeats and transposons, but was less than 10-kb in length (Figure 5, Additional file 3: Figure S1). The results in Figure 5 indicated that O104: H4 and O104:H21 may represent various insertion intermediates of the O104:H7 strain generated in the course of O104 evolution. Our data provide new insights into the potential activities of the functional prophages embedded in bacterial genomes and may lead to the formulation of a novel concept of inter-prophage interactions in prophage communities. That is to say, strains containing prophage without genetic defects may be potentially more capable of spreading (gaining or losing) important virulence determinants such as eae and other genetic traits to other bacterial strains (Figure 6). Our findings suggest that more research is needed to understand the potential roles of prophage in HGT between bacteria and in the evolution of bacterial pathogens.

A key virulence factor, stx2, was found in all European O104:H4 outbreak strains, but only in one of the strains of African origin (C777-09) (Table 1). This finding supports the contention that similarly virulent O104:H4 isolates could be widespread; however, they could be genetically different due to their adaption to specific niches. It is also suggestive that both O104:H7 and O104:H21 may have evolved into pathogenic STEC strains due to the acquisition of stx, other virulence genes, and antibiotic resistance genes such as mdtL, emrE, ksgA, ydeB, dacC, folA-O157, and others from other STEC. It is reasonable to suggest that genetic variation of STEC O104 may partly be due to
adaptation to local environments and interactions with other bacteria and hosts. Serotypes O104:H7 and O104:H12 have been isolated from humans and associated with HUS, thus it is also important that future studies examine the pathogenic potential of different O104 H-types, other than H4, H7, and H21.

Methods

Strains, genome sequencing, assembly, and annotation

STEC O104:H21 strain 94–3024 and O104:H7 strain RM9387 were obtained from the Centers for Disease Control and Prevention (Atlanta, GA) and from Dr. Robert Mandrell at the USDA Agricultural Research Service, Western Regional Research Center (Albany, CA), respectively. Ion Torrent libraries were prepared following the manufacturer’s recommended library construction procedures. Ion Torrent 316-chips with the 200-bp One-Touch kit was used for the generation of sequencing data on the Ion Torrent Personal Genome Machine (PGM). High molecular weight DNA for PacBio sequencing was extracted using Qiagen Genomic-tip 100/G columns and a modified manufacturer’s protocol as previously described [66]. Ten micrograms of DNA was sheared to a targeted size of 20 kb using a g-TUBE (Corvaris, Woburn, MA) and concentrated using 0.45X volume of AMPure PB magnetic beads (Pacific Biosciences, Menlo Park, CA) following the manufacturer’s protocol. Sequencing libraries were created using 5 micrograms of sheared, concentrated DNA and the PacBio DNA Template Prep Kit 2.0 (3Kb - 10Kb) according to the manufacturer’s protocol. The library was bound with polymerase P5 followed by sequencing on a Pacific BioSciences (PacBio) RS II sequencing platform (with chemistry C3 and the 120-min data collection protocol). A fastq file was generated from the PacBio reads using SMRTAnalysis Version 2.1 and error-corrected reads were created using pacBioToCA with self-correction [67]. The longest 20x of the corrected reads were assembled with Celera Assembler 7.0 [65]. The resulting contigs were polished using Quiver [68] and annotated using a local instance of Do-It-Yourself Annotator (DIYA) [69] for initial gene prediction and frame shift verification. The annotated genome sequence was imported into Geneious (Biomatters LTD., Auckland, New Zealand) and duplicated sequence regions were identified using the NCBI online service Microbial Genome Submission Check [71].

Phage identification and analysis

Prophages and putative phage-like elements in the O104:H4 reference strain 2009EL-2050 (NC_018650.1) genome as the “reference” strain, all FDA draft raw sequences for O104:H21 (Table 2, strains BAA-178 and BAA-182) downloaded from SRA (http://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=search_obj) and our two newly sequenced genomes were re-assembled and ordered based on reference genome. In general, progressive Mauve was applied for whole genome comparison. Homologs and/or orthologous relationship of the nucleotide sequences for profiling of virulence- and resistance-associated genes between genomes were determined using NCBI Basic Local Alignment Search Tool (BLAST) with the following criteria: identity >80%, e-value <1e−10, and coverage >90%. The identified homologs and/or orthologous genes were further manually curated and confirmed.

Identification and comparison of pathogenicity islands and other genomic islands

IslandViewer [74] was used to predict genomic islands and/or pathogenicity islands (PAIs) [75] in the O104:H4 reference strain 2009EL-2050. The predicted genomic islands and/or pathogenicity islands were then used as the “reference genomic islands template” for the identification
of corresponding genomic islands from various H serotypes of O104 isolates and other non-O104 STEC genomes based on 90% identity and 90% coverage (90%, 50 nts). The LEE protein sequences from the top 7 STEC serogroups (O26, O45, O103, O111, O121, O145, and O157) were used as query sequences for large-scale BLAST score ration analysis and BLASTp to detect the presence of LEE islands and its associated genes (homologs). The LEE pathogenicity island insertion site was determined using BLASTN to identify the contig and alignment coordinates of the intimin gene, eae, among all O104:H serotype strains listed in this study. The presence of the LEE carrying eae and its flanking genes was confirmed by manual inspection of the annotation for this region, and/or by detection of the coordinates of genes encoded within the LEE of O157:H7 strain EDL933.

Identification and comparison of virulence factors and antibiotic resistance genes

Only bacterial genes experimentally confirmed to be involved in bacterial pathogenesis and antibiotic resistance were collected through an extensive literature mining in PubMed. This was followed by reference mapping to confirm their existence with corresponding raw sequencing reads. SNP discovery for RpoB (mutations in rpoB, β-subunit of RNA polymerase, can result in antibiotic resistance) was also performed (data not shown).

Phylogeny tree construction

The phylogenetic relationship of the two sequenced O104: H7 and O104:H21 strains and other O104 strains was visualized and constructed using MAUVE whole-genome alignment and presented with the program Mega6 based on features in the genome or specific regions such as PAIs (Figure 6).

GenBank accession numbers

The complete genome and plasmid sequences of O104: H21 94–3024 strain and O104:H7 RM9387 strain were deposited in NCBI GenBank database under the accession numbers: CP009104 (O104:H7 RM9387 chromosome), CP009015, KM085451, KM085452, KM085453 (O104:H7 plasmids pO104_H7, pO104_H7_s1; pO104_H7_s2, pO1, 04_H7_s3), CP009106 (O104:H21 94–3024 chromosome) and CP009107 (O104:H21 plasmid pO104_H21).

Additional files

Additional file 1: Table S1. Strain background and general gene/genome information of the 14 different O104 strains used in this study.

Additional file 2: Table S2. Distribution and general information of the predicted prophages in various H types of O104 strains.

Additional file 3: Figure S1. Proposed evolutionary model of the selC-rRNA site from various H-types of E. coli O104 strains.

Abbreviations

LEE: Locus of enteroctye effacement; STEC: Shiga toxin-producing Escherichia coli; aidA: Adhesin involved in diffuse adherence; EAHEC: Enteroaggregative hemorrhagic Escherichia coli; HUS: Hemolytic uremic syndrome; PAIs: Pathogenicity islands.

Competing interests

Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Authors’ contributions

PF initiated this project. PF and XY coordinated this project and sequence data collection. PF provided strains. XY, GB, and JB constructed the DNA libraries and conducted the sequencing. XY and CC developed strategies and performed the computational analyses. XY and CC wrote the manuscript. All authors edited the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Dr. George Paoli for reviewing the manuscript.

Author details

1 USDA, Agricultural Research Service, Eastern Regional Research Center, 600 E. Mermaid Lane, 19038 Wyndmoor, PA, USA. 2 USDA, Agricultural Research Service, Meat Animal Research Center, Clay Center, NE 68933, USA. 3 U.S. Department of Agriculture, Eastern Regional Research Center, Agricultural Research Service, 600 East Mermaid Lane, 19038 Wyndmoor, PA, USA.

Received: 2 October 2014 Accepted: 12 March 2015

Published online: 03 April 2015

References

1. Beutin L, Hammerl JA, Reetz J, Strauch E, Shiga toxin-producing Escherichia coli strains from cattle as a source of the Stx2a bacteriophages present in enteroaggregative Escherichia coli O104:H4 strains. Int J Med Microbiol. 2013;303:595–602.

2. Beutin L, Hammerl JA, Strauch E, Reetz J, Dieckmann R, Kelner-Burgos Y, et al. Spread of a distinct Stx2-encoding phage prototype among Escherichia coli O104:H4 strains from outbreaks in Germany, Norway, and Georgia. J Virol. 2012;86:10444–55.

3. Beutin L, Krause G, Zimmermann S, Kauffuss S, Geier K. Characterization of Shiga toxin-producing Escherichia coli strains isolated from human patients in Germany over a 3-year period. J Clin Microbiol. 2004;42:1099–108.

4. Beutin L, Martin A. Outbreak of Shiga toxin-producing Escherichia coli (STEC) O104:H4 infection in Germany causes a paradigm shift with regard to human pathogenicity of STEC strains. J Food Prot. 2012;75:408–18.

5. Bielaszewska M, Friedrich AW, Alidick T, Schurk-Bürglin R, Karch H. Shiga toxin activatable by intestinal mucus in Escherichia coli isolated from humans: predictor for a severe clinical outcome. Clin Infect Dis. 2006;43:1160–7.

6. Rasko DA, Webster DR, Sahl JW, Bashir A, Boisen N, Scheutz F, et al. Origins of the E. coli strain causing an outbreak of hemolytic-uremic syndrome in Germany. N Engl J Med. 2011;365:709–17.

7. Kim J, Oh K, Jeon S, Cho S, Lee D, Hong S, et al. Escherichia coli O104:H4 from, European outbreak and strain from South Korea. Emerg Infect Dis. 2011;2011;17:1755–6.

8. Bielaszewska M, Meillmann A, Zhang W, Kock R, Fruth A, Bauwens A, et al. Characterisation of the Escherichia coli strain associated with an outbreak of haemolytic-uraemic syndrome in Germany, 2011: a microbiological study. Lancet Infect Dis. 2011;11:671–6.

9. Ahmed SA, Awoxisa J, Baldwin C, Bishop-Lilly KA, Biswas B, Broomall S, et al. Genomic comparison of Escherichia coli O104:H4 isolates from 2009 and 2011 reveals plasmid, and prophage heterogeneity, including Shiga toxin encoding phage stx2. PLoS One. 2012;7:e48228.

10. Miko A, Delannoy S, Fach P, Stockbine NA, Linstedt BA, Mariani-Kurkdjian P, et al. Genotypes and virulence characteristics of Shiga toxin-producing Escherichia coli O104 strains from different origins and sources. Int J Med Microbiol. 2013;303:410–21.

11. Gould LH, Mody RK, Ong KL, Clogher P, Cronquist AB, Garman KN, et al. Increased recognition of non-O157 Shiga toxin-producing Escherichia coli...
infections in the United States during 2000–2010: epidemiologic features and comparison with E. coli O157 infections. Foodborne Pathog Dis. 2013;10:453–60.

12. European Food Safety Authority. [http://www.ecdc.europa.eu/en/publications/Publications/106_TER_ECOL_jump_FINAL.pdf]

13. Brett KN, Hornitzy MA, Bettelheim KA, Walker MJ, Jordviejc SP. Bovine non-O157 Shiga toxin 2-containing Escherichia coli isolates commonly possess stx2-EDL933 and/or stx2vrb subtypes. J Clin Microbiol. 2003;41:2716–22.

14. Brett KN, Ramachandran V, Hornitzy MA, Bettelheim KA, Walker MJ, Jordviejc SP. stx1c Is the most common Shiga toxin 1 subtype among Shiga toxin-producing Escherichia coli isolates from sheep but not among isolates from cattle. J Clin Microbiol. 2003;41:926–36.

15. Meichtri L, Milwolsky E, Gioffre A, Chinen J, Baschker L, Chillemi G, et al. Shiga toxin-producing Escherichia coli in healthy young beef steers from Argentina: prevalence and virulence properties. Int J Food Microbiol. 2004;96:189–98.

16. Wani SA, Bhat MA, Samanta I, Ishaq SM, Ashraf MA, Buchh AS. Epidemiology of diarrhoea caused by rotavirus and Escherichia coli in lambs in Kashmir valley. India Small Rumin Res. 2004;52(1–2):145–53.

17. Blanco J, Blanco M, Blanco JE, Mora A, González EA, Bernardes MI, et al. Verotoxin-producing Escherichia coli in Spain: prevalence, serotypes, and virulence genes of O157:H7 and non-O157 VTEC in ruminants, raw beef products, and humans. Exp Biol Med (Maywood). 2003;228:345–51.

18. Blanco M, Blanco JE, Mora A, Rey J, Alonso JM, Hermoso M, et al. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing Escherichia coli isolates from healthy sheep in Spain. J Clin Microbiol. 2003;41:3151–6.

19. Sánchez S, Martínez R, García A, Vidal D, Blanco J, Blanco M, et al. Detection and characterisation of O157:H7 and non-O157 Shiga toxin-producing Escherichia coli in wild boars. Vet Microbiol. 2010;143:420–3.

20. Scotland SM, Rowe B, Smith HR, Willshaw GA, Gross RJ. Vero cytotoxin-producing Escherichia coli strains commonly possess a CRISPR locus as a target for a diagnostic real-time PCR. J Clin Microbiol. 2014;52:1394–403.

21. Bockemühl J, Aleksic S, Karch H. Serological and biochemical properties of the 2011 German outbreak strain or in healthy young beef steers from northern Germany, the hotspot of the 2011 HUS outbreak area. Gut Pathog. 2011;3:17.

22. European Centre for Disease Prevention and Control (ECDC) report. [http://www.ecdc.europa.eu/en/publications/Publications/106_TER_ECOL_jump_FINAL.pdf]

23. Muniesa M, Blanco JE, De Simon M, Serra-Moreno R, Blanch AR, Jofre J. Diversity of stx2 converting bacteriophages induced from Shiga-toxin producing Escherichia coli strains isolated from cattle. Microbiology. 2004;150:2959–71.

24. Guy L, Jernberg C, Iverson S, Hedenström L, Engstrand L, Andersson SG. Genomic diversity of the 2011 European outbreaks of Escherichia coli O104:H4. Proc Natl Acad Sci U S A. 2012;109:E627–8.

25. Darling AC, Mau B, Blattner FR, Perna NT. MAUVE: multiple alignment of conserved genomic sequence with rearrangements. Genome Res. 2004;14(7):1394–403.

26. Gonzalez-Escalona N, McFarland MA, Rump LV, Payne J, Andrzejewski D, Brown EW, et al. Draft genome sequences of two O104:H21 Escherichia coli isolates causing hemorrhagic colitis during a 1994 Montana outbreak provide insight into their pathogenicity. Genome Announc. 2013;1(5):doi:10.1128/genomeA.00805-13.

27. Huang A, Friesen J, Brunton JL. Characterization of a bacteriophage that carries the genes for production of Shiga-like toxin 1 in Escherichia coli. J Bacteriol. 1987;169:4308–12.

28. O'Brien AD, Marques LR, Kerr CF, Newland JW, Holmes RK. Shiga-like toxin converting phage of enterohemorrhagic Escherichia coli strain 933. Microb Res. 1989;196:381–90.

29. Tozzi R, Grande L, Michelacci V, Fioravanti R, Gally D, Xu X, et al. Identification and characterization of a peculiar vtx2-converting phage frequently present in verocytotoxin-producing Escherichia coli O157 isolated from human infections. Infect Immun. 2014;82:3023–32.

30. Tozzi R, Grande L, Michelacci V, Ranieri P, Maugliani A, Caprioli A, et al. Shiga toxin-converting phages and the emergence of new pathogenic Escherichia coli: a world in motion. Front Cell Infect Microbiol. 2014;4:80.

31. Martinez-Castillo A, Muniesa M. Implications of free Shiga toxin-converting bacteriophages occurring outside bacteria for the evolution and the detection of Shiga toxin-producing Escherichia coli. Front Cell Infect Microbiol. 2014;4:46.

32. Melton-Celsa AR, Kokai-Kun JF, Sullivan K, Bohlin J, Ligard G, Granum PE. Identification and characterization of a novel vtx2 converting phage of Shiga toxin producing Escherichia coli: a world in motion. Front Cell Infect Microbiol. 2014;4:80.

33. Delannoy S, Beutin L, Burgos Y, Fach P. Specific detection of enterohemorrhagic Escherichia coli O104:H4 strains by use of the CRISPR locus as a focus for a diagnostic real-time PCR. J Clin Microbiol. 2012;50:1985–92.

34. Auras P, Prager R, Flieger A. EHEC/EAEC O104:H4 strain linked with the German outbreak of haemolytic uraemic syndrome enters into the viable but non-culturable state in response to various stresses and resuscitates upon stress relief. Environ Microbiol. 2011;13(13):3139–48.

35. Aube-Lund TM, Jorgensen HJ, O’Reilly M, Smith EJ. Diarrhoea and Shiga toxin-producing Escherichia coli infection in sheep but not among isolates from cattle. J Clin Microbiol. 2003;41:926–36.

36. Tozzoli R, Grande L, Michelacci V, Ranieri P, Maugliani A, Caprioli A, et al. Shiga toxin-converting phages and the emergence of new pathogenic Escherichia coli: a world in motion. Front Cell Infect Microbiol. 2014;4:80.

37. Martinez-Castillo A, Muniesa M. Implications of free Shiga toxin-converting bacteriophages occurring outside bacteria for the evolution and the detection of Shiga toxin-producing Escherichia coli. Front Cell Infect Microbiol. 2014;4:46.

38. Melton-Celsa AR, Kokai-Kun JF, Sullivan K, Bohlin J, Ligard G, Granum PE. Identification and characterization of a novel vtx2 converting phage of Shiga toxin producing Escherichia coli: a world in motion. Front Cell Infect Microbiol. 2014;4:80.

39. O’Brien AD, Marques LR, Kerr CF, Newland JW, Holmes RK. Shiga-like toxin converting phage of enterohemorrhagic Escherichia coli strain 933. Microb Res. 1989;196:381–90.

40. Tozzi R, Grande L, Michelacci V, Ranieri P, Maugliani A, Caprioli A, et al. Shiga toxin-converting phages and the emergence of new pathogenic Escherichia coli: a world in motion. Front Cell Infect Microbiol. 2014;4:80.

41. Tozzi R, Grande L, Michelacci V, Ranieri P, Maugliani A, Caprioli A, et al. Shiga toxin-converting phages and the emergence of new pathogenic Escherichia coli: a world in motion. Front Cell Infect Microbiol. 2014;4:80.

42. O’Reilly M, Smith EJ. Diarrhoea and Shiga toxin-producing Escherichia coli infection in sheep but not among isolates from cattle. J Clin Microbiol. 2003;41:926–36.

43. Tozzoli R, Grande L, Michelacci V, Ranieri P, Maugliani A, Caprioli A, et al. Shiga toxin-converting phages and the emergence of new pathogenic Escherichia coli: a world in motion. Front Cell Infect Microbiol. 2014;4:80.

44. Tozzi R, Grande L, Michelacci V, Ranieri P, Maugliani A, Caprioli A, et al. Shiga toxin-converting phages and the emergence of new pathogenic Escherichia coli: a world in motion. Front Cell Infect Microbiol. 2014;4:80.

45. Melton-Celsa AR, Kokai-Kun JF, Sullivan K, Bohlin J, Ligard G, Granum PE. Identification and characterization of a novel vtx2 converting phage of Shiga toxin producing Escherichia coli: a world in motion. Front Cell Infect Microbiol. 2014;4:46.
O104:H4: short-term evolution of an emerging pathogen. MBio. 2013;4:e00452–00412.

52. Yamamoto D, Hernandez RT, Blanco M, Greune L, Schmidt MA, Carneiro SM, et al. Invasiveness as a putative additional virulence mechanism of some atypical enteropathogenic Escherichia coli strains with different uncommon intimin types. BMC Microbiol. 2009;9:146.

53. Feng P, Wening SD, Monday SR. Genetic analysis for virulence factors in Escherichia coli O104:H21 that was implicated in an outbreak of hemorrhagic colitis. J Clin Microbiol. 2001;39:24–8.

54. Hochhut B, Wilde C, Balling G, Middendorf B, Dobrindt U, Brzuszkiewicz E, et al. Role of pathogenicity island-associated integrases in the genome plasticity of uropathogenic Escherichia coli strain 536. Mol Microbiol. 2006;61:84–95.

55. Middendorf B, Hochhut B, Leipoldt K, Dobrindt U, Blum-Oehler G, Hacker J. Instability of pathogenicity islands in uropathogenic Escherichia coli strain 536. J Bacteriol. 2004;186:3086–96.

56. Dong T, Schellhorn HE. Global effect of RpoS on gene expression in pathogenic Escherichia coli O157:H7 strain EDL933. BMC Genomics. 2009;10:349.

57. Stibitz S, Davies IE. Tn602: a naturally occurring relative of Tn903 with direct repeats. Plasmid. 1987;17:202–9.

58. Pougach K, Semenova E, Bogdanova E, Datsenko KA, Djordjevic M, Wanner BL, et al. Transcription, processing and function of CRISPR cassettes in Escherichia coli. Mol Microbiol. 2010;77(1):1367–79.

59. Boisen N, Hansen AM, Melton-Celsa AR, Zangari T, Mortensen NP, Kaper JB, et al. The presence of the pAA plasmid in the German O104:H4 Shiga toxin type 2a (Stx2a)-producing enterohaemorrhagic Escherichia coli strain promotes the translocation of Stx2a across an epithelial cell monolayer. J Infect Dis. 2014;210(12):1909–19.

60. Morin N, Santiago AE, Ernst RK, Guillot SJ, Natario JP. Characterization of the AgrR regulon in enterohaemorrhagic Escherichia coli. Infect Immun. 2013;81:122–32.

61. Fratamico PM, Yan X, Caprioli A, Esposito G, Needleman DS, Pepe T, et al. The complete DNA sequence and analysis of the virulence plasmid and of five additional plasmids carried by Shiga toxin-producing Escherichia coli O26:H11 strain H30. Int J Med Microbiol. 2011;301:192–203.

62. Verstraete K, Der K, Vanw S, Pierard D, Dez L, Herman L, et al. Genetic characteristics of Shiga toxin-producing E. coli O157, O26, O113, O111 and O145 isolates from humans, food, and cattle in Belgium. Epidemiol Infect. 2013;141:2903–15.

63. Yahiro K, Sato Y, Morinaga T, Tsutsui H, Ogura K, Nagaawa S, et al. Identification of subtilase cytotoxin (SubAB) receptors whose signaling, in association with SubAB-induced BIP cleavage, is responsible for apoptosis in HeLa cells. Infect Immun. 2011;79:1667–77.

64. Szych J, Wolkowicz T, La Ragione R, Madajczak G. Impact of antibiotics on the intestinal microbiota and on the treatment of Shiga-toxin-producing Escherichia coli and Salmonella infections. Curr Pharm Des. 2014;20:4533–48.

65. Corogeanu D, Willmes R, Wolke M, Plum G, Utermohlen O, Kronke M. Therapeutic concentrations of antibiotics inhibit Shiga toxin release from enterohaemorrhagic E. coli O104:H4 from the 2011 German outbreak. BMC Microbiol. 2012;12:160.

66. Clawson ML, Keen JE, Smith TP, Durso LM, McDaneld TG, Mandrell RE, et al. Invasiveness as a putative additional virulence mechanism of some atypical enteropathogenic E. coli O104:H4 with Bovine origin using a novel set of nucleotide polymorphisms. Genome Biol. 2010;11:R86.

67. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 2014;30:2868–69.

68. Chin CS, Alexander DH, Marks P, Klammer AA, Drake J, Heiner C, et al. DIYA: a bacterial annotation pipeline for any genomics lab. Bioinformatics. 2009;25:962–3.

69. Stewart AC, Osborne B, Read TD. ACT: the Artemis Comparison Tool. Bioinformatics. 2005;21:3422–3.