The Highly Virulent 2006 Norwegian EHEC O103:H25 Outbreak Strain Is Related to the 2011 German O104:H4 Outbreak Strain

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Abstract

In 2006, a severe foodborne EHEC outbreak occured in Norway. Seventeen cases were recorded and the HUS frequency was 60%. The causative strain, Escherichia coli O103:H25, is considered to be particularly virulent. Sequencing of the outbreak strain revealed resemblance to the 2011 German outbreak strain E. coli O104:H4, both in genome and Shiga toxin 2-encoding (Stx2) phage sequence. The nucleotide identity between the Stx2 phages from the Norwegian and German outbreak strains was 90%. During the 2006 outbreak, stx2-positive O103:H25 E. coli was isolated from two patients. All the other outbreak associated isolates, including all food isolates, were stx-negative, and carried a different phage replacing the Stx2 phage. This phage was of similar size to the Stx2 phage, but had a distinctive early phage region and no stx gene. The sequence of the early region of this phage was not retrieved from the bacterial host genome, and the origin of the phage is unknown. The contaminated food most likely contained a mixture of E. coli O103:H25 cells with either one of the phages.

Introduction

Enterohaemorrhagic Escherichia coli (EHEC) can cause serious disease in humans. Infection manifests itself as diarrhoea or haemorrhagic colitis. The life threatening haemolytic uraemic syndrome (HUS) is a potential sequela. Previously, EHEC isolates belonging to serogroups O157, O26, O111, O145 and O103 were most frequently isolated in food borne outbreaks [1]. Recently, less common serotypes and pathotypes, have received more attention. This is illustrated by the enteragggregative E. coli (EAEC) O104:H4 causing a large European outbreak in 2011 involving more than 4000 diseased patients, a 22% HUS incidence, and 50 fatalities [2,3].

EHEC virulence is mainly attributed to the production of Shiga toxins (Stx), which are regarded as essential in EHEC disease. Shiga toxins are divided into two major families, Stx1 and Stx2. Toxins belonging to the Stx2 group are the most heterogenic and also include the most potent variants [4]. In E. coli, Stx are usually encoded by temperate, lambdaoid bacteriophages whose genomes are mosaic in structure, and may integrate at several sites in the E. coli chromosome [5–7]. Upon certain stimuli of the bacterial cell, the phage enters a lytic cycle inducing production of Stx and phage particles. This culminates in bacterial cell lysis and release of toxin and infectious phages [8,9]. Released Stx phages may infect new bacterial cells, playing an important role in the evolution of EHEC [10].

During the spring of 2006, Norway experienced a national disease outbreak caused by EHEC O103:H25. The outbreak was characterized by an extraordinary high frequency of HUS. Of seventeen recorded cases sixteen had diarrhoea, ten developed HUS and one case was fatal [11]. Stool cultures for E. coli O103:H25 were positive in 11 of the patients, but only two of the retrieved isolates were stx2-positive while the remaining nine were stx-negative [11]. The absence of stx genes in EHEC serotypes isolated from patients is not uncommon, and such strains are believed to be offspring of EHEC present at an earlier phase of the infection [12]. The more surprising finding in the Norwegian O103:H25 outbreak was that none of the food isolates had stx-positivity [12]. The more surprising finding in the Norwegian O103:H25 outbreak was that none of the food isolates had stx-positivity [12]. The more surprising finding in the Norwegian O103:H25 outbreak was that none of the food isolates had stx-positivity [12]. The more surprising finding in the Norwegian O103:H25 outbreak was that none of the food isolates had stx-positivity [12].

In this study, the genome of the outbreak strain was sequenced to elucidate its high virulence and examine potential relationship to other virulent EHEC strains. In addition, we aimed to characterize the Stx2 phage demonstrated in the stx2-positive isolates.

Results

Genome sequencing and phylogeny

The sequence achieved through genome sequencing using 454 technology was assembled into 554 contigs and spanned altogether
Serotype O103:H25 is a rare cause of EHEC disease, and has not previously been associated with outbreaks [11], only with
EHEC O103:H25 NOS is Related to EHEC O104:H4 GOS

A

B

C

O103_H25
O104_H4
O103_H2
EAEC_55989
O111:H4
O157_H7
O26_H11

100% identity
70% identity
50% identity

5000 kbp
500 kbp
4500 kbp
4000 kbp
3500 kbp
3000 kbp
2500 kbp
2000 kbp
1500 kbp
1000 kbp

0.001

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sporadic disease cases [21,22]. A few E. coli O103:H25 strains have been characterized and found to carry stx2, but they have not been associated with severe disease [18]. The Norwegian 2006 outbreak caused by EHEC O103:H25 had a 60% HUS frequency, and the strain is considered to be particularly virulent [11].

We have shown that the genome of EHEC O103:H25 NOS resemble the EAEC O104:H4 GOS and EHEC O103:H2 str 12009. This is supported in a pan genomic study of 61 sequenced E. coli genomes, showing that the E. coli O103 Oslo (O103:H25 NOS) clusters closely together with the EHEC O103:H2 str 12009 [23], and a more recent study shows the similarities between the EHEC O103:H2 str 12009 and the EAEC O104:H4 GOS genomes [2]. EAEC O104:H4 GOS caused diarrhoea in approximately 4000 individuals, 22% of which developed HUS, and the strain is notably more virulent than most EHEC [2]. The E. coli O104:H4 outbreak began in Germany in May 2011, but was later identified in other European countries [2,24]. Due to phenotypic and genotypic characteristics, the German O104:H4 outbreak strain is not classified as an EHEC, but rather as a Shiga toxin producing enteroaggregative E. coli (EAEC) [2,3,25]. Despite the lack of genes characteristic of EAEC in O103:H25, the O104:H4 outbreak strain is classified as an EHEC, but rather as a Shiga toxin producing enteroaggregative E. coli (EAEC) [2,3,25].

Despite the lack of genes characteristic of EAEC in EHEC O103:H25 NOS, and thus differing in both pathotype and serotype, the genomes of the Norwegian and the German outbreak strains are highly similar, as illustrated in Figures 1A and 1B. The close relationship between the two strains is supported by MLST analysis (Figure 1C). Also the Stx2 phages in these two strains show a striking homology with a DNA sequence identity of 90% (Figure 4) [2]. The identity includes a 1 bp silent nucleotide mutation in the stx2A gene [3,26] which is rare in other stx genes. This indicates a common origin for the two phages. The finding of closely related Stx2 phages and genomes in two outbreak strains of different serotypes and pathotypes, but with a high HUS incidence in common, is remarkable and will be investigated further.

Other strains with related Stx2 phages include EHEC O103:H2 str 12009 from a sporadic case of diarrhoea in Japan in 2001, and E. coli O111:H- str 11128. Similar Stx2 phages to the Norwegian outbreak strain are thus present in E. coli strains of serogroups O103, O104 and O111, and the phage seems to be rather promiscuous in nature. The similarity between the Stx2 phage of EHEC O103:H25 NOS and the reference Stx2 phage 933W from O157:H7 EDL933 is high in the 38 kb late region of the phages where the stx genes are located (95%), however, the early regions of these phages differ in composition (Figure 4). In EHEC O103:H25 NOS the Stx2 phage is inserted into wrbA, a previously described integration site of Stx2 phages, e.g. in EHEC O157:H7 EDL933 and Sakai strains [27,28]. The wrbA had not been observed as an integration site in serogroup O103 prior to the Norwegian outbreak [26]. The closely related Stx2 phage in O104:H4 GOS is also inserted in wrbA, while the Stx2 phage in EHEC O103:H2 str 12009 is located within the argW gene.

The only observed feature distinguishing between the Norwegian outbreak strain and stx-negative isolates from the 2006 outbreak is the presence of either the stx2A-positive or the stx-negative phage, respectively. These two phages are related and share parts of their sequences and insertion site. While the 30 kb late regions are similar in the two phages, the early regions are completely different. Interestingly, the shift between the similar and dissimilar parts is abrupt and in proximity of the stx genes (Figure 3). As Stx2 phages are mosaic by nature and rearrangements are not uncommon [6], the stx-negative phage could have developed from the Stx2 phage by acquiring its distinctive sequence from the chromosome of the E. coli O103:H25 host.

Table 1. Bacterial isolates included in the study.

| Strain     | Synonym               | Year | Stx | Source                        | Origin     |
|------------|-----------------------|------|-----|-------------------------------|------------|
| NVH-734    | NIPH-11060424         | 2006 | stx2| Patient no 2                  | Norway     |
| NVH-847    | NIPH-11060708         | 2006 | stx2| Patient no 9                  | Norway     |
| NVH-848    | NIPH-11060707         | 2006 | -   | Patient no 9                  | Norway     |
| NVH-849    | NIPH-11060747         | 2006 | -   | Patient no 10                 | Norway     |
| NVH-760    | 625/06                | 2006 | -   | Fermented sausage, home of patient 7 | Norway     |
| NVH-737    | 2006                  | -    | Fermented sausage              | Norway     |
| NVH-763    | 2006                  | -    | Food                            | Norway     |
| NVH-661    | NIPH-10306923         | 2003 | stx2| Patient                       | Norway     |
| NVH-731    | NIPH-11051601         | 2005 | stx2| Patient                       | Norway     |
| cdc-08-201 | Not known             |      |     |                               | Maine, USA |
| cdc-08-202 | Not known             |      |     |                               | Virginia, USA |

All isolates are E. coli O103:H25.

*Referred to as Norwegian outbreak strain (NOS).

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However, the distinctive stx-negative phage sequence has not been identified in the EHEC O103:H25 NOS genome neither by in silico analysis nor by PCR. This indicates that this phage, or at least part of it, has another origin. The distinctive sequence of the stx-negative phage shows some similarities to phage related sequences in a BLAST search, but it seems to have a rather unique construction.

The 2006 EHEC O103:H25 outbreak is remarkable because all food isolates were stx-negative, and only two of 11 isolates from patients were stx-positive [11,13]. The lack of stx genes in EHEC serotypes isolated from patients is not uncommon and has been reported in both O157 and non-O157 isolates [12,29]. In studies where the mechanism of stx gene loss has been investigated, it has been shown that the stx-negative strains lack the entire stx-encoding bacteriophage. This has been demonstrated by the presence or reappearance of an intact integration site for the stx phage and an altered PFGE pattern [29–32]. Such stx-negative isolates from patients are believed to be progenies of an EHEC that lost the stx genes during the course of illness, and might be referred to as EHEC-LST [lost Shiga toxin] [12]. The EHEC-LST model is supported by the finding that EHEC are difficult to isolate from patients late in illness [33], and the theory is further confirmed by Mellmann and Karch who demonstrated the presence of stx-negative strains of O26:H11/NM and sorbitol fermenting (SF) O157:NM subsequent to stx-positive isogenic isolates in the same patients [12,34,35].

In contrast to the EHEC-LST phenomenon, we find that the integration site of the Stx2 phage is occupied in the stx-negative isolates by a partly related phage but without stx genes. The stx-negative isolates thus differ from the stx-positive isolates not only by the lack of the Stx2 phage but also by the presence of this stx-negative phage. The similar size and the lack of an XhoI restriction site in both the Stx2 phage and the stx-negative phage explain the finding of identical XhoI digested PFGE profiles of the stx-positive and stx-negative isolates shown by Sekse et al. [13]. Digestion with AscII, however, revealed a difference in PFGE pattern, and sequencing of the two phage genomes identified an AscII restriction site in the Stx2 phage which is not present in the stx-negative phage and that most likely explains the observed difference.

Only two stx2-positive patient isolates were retrieved in 2006, and as no stx2-positive isolates were retrieved from food, it could be speculated that the stx-negative E. coli acquired the Stx2 phage in the patients’ gut. However, the Stx2 phages from the two patient isolates have identical RFLP pattern and a rare silent nucleotide mutation in stx2, which both are found in two EHEC O103:H25 isolates from sporadic cases in Norway in 2003 and 2005 [26]. This strongly indicates that the Stx2 phages in these four isolates are epidemiologically linked and that the stx2-positive isolates from 2006 originates in the same source.

The peculiar circumstance is that both stx2-positive and stx-negative O103:H25 E. coli cells must have been present in the contaminated fermented sausage in 2006. Which of the two variants is the ancestor is difficult to predict, but there is reason to believe that the stx2-positive clone preceded the stx-negative clone because the related Stx2 phage was identified in the same E. coli serotype three years prior to the 2006 outbreak. On the other hand, stx-negative isolates could have been overlooked in earlier cases, and the origin and history of this clone is difficult to evaluate. The hypothesis that bacterial cells with stx-negative or

### Table 2. Characteristics of EHEC O103:H25 NOS, the related strains O104:H4 GOS and O103:H2 str 12009, and the EHEC reference strain O157:H7 EDL933.

| Characteristics          | O157:H7 EDL933 | O103:H25 NOS | O104:H4 GOS | O103:H2 str 12009 |
|--------------------------|----------------|--------------|-------------|-------------------|
| **Outbreak**             |                |              |             |                   |
| Year of isolation        | 1982           | 2006         | 2011        | 2001              |
| Country                  | USA            | Norway       | Germany     | Japan             |
| Pathotype                | EHEC           | EHEC         | EAEc        | EHEC              |
| No of diseased           | 47             | 16           | 4075        | 1                 |
| No of HUS (%)            | 0              | 10 (62.5%)   | 908 (22%)   | 0                 |
| No of deaths             | 1              | 50           |             | 0                 |
| **Stx2 phage**           |                |              |             |                   |
| Insertion site           | wrbA           | wrbA         | wrbA        | argW              |
| Stx2A, nucleotide position 867 | T         | C            | C           | T                 |
| **LEE**                  |                |              |             |                   |
| LEE operons              | five           | five         | none        | five              |
| Intimin type             | gamma          | theta        | not present | epsilon           |
| **O1122**                |                |              |             |                   |
| sen (Shet2)              | yes            | yes          | no          | yes               |
| pagC                     | yes            | yes          | no          | yes               |
| efa1/lifA                | 2.1 kb         | 9.7 kb       | not present | 9.7 kb            |
| **Accessory virulence**  |                |              |             |                   |
| set1A (shet1)            | no             | no           | yes         | no                |
| Colicin                  | E2             |              |             |                   |
| ehsA                     | yes            | yes          | not present | yes               |

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negative isolates. Patients have been shown to be the same isolates from food, and not offspring from the bacterial cells, the two clones may not have been present in equal numbers. As only the genomic island present in the 2006 outbreak strain is the putative LEE operons are present in EHEC O103:H25 NOS, however, (Tir) and a type III secretion system (TTSS). These are all involved encoding the intimin gene (eae), the translocated intimin receptor (Tir) and a type III secretion system (TTSS). These are all involved in the intimate attachment of EHEC to enterocytes [39]. All five LEE operons are present in EHEC O103:H25 NOS, however, LEE is not part of the EAEC O104:H4 GOS genome. Another genomic island present in the 2006 outbreak strain is the putative pathogenicity island OI122 with the genes xun, pagC and efa1 which have been strongly correlated with virulence and disease severity [18,40]. The complete efa1/lifA gene is 9672 kb and is a bifunctional protein for adherence and inhibition of lymphocyte activation [19]. The distribution of this large toxin is limited to less than 30 strains of heterologous serogroups (BLAST search), only one of the sequenced O157:H7 strains and two O157:NM strains exhibit it [41,42]. EAEC O104:H4 GOS does not exhibit any version of efa/lifA, while O103:H2 str 12009 exhibits the large version of efa/lifA. Enterohemolysin (Ehx) is regarded a virulence factor of EHEC and the genes are located on large plasmids like pO157 [43–45]. One large plasmid is detected in O103:H25 NOS, in contrast to O104:H4 GOS which carries two large plasmids [25].

The colicin production of E. coli O103:H25 may have provided an advantage for the bacterial cells in the harsh competition of the gut, making the colonization more efficient. In addition, the colicin E2 is a DNAse colicin which has been found to increase the in vitro production of Stx in EHEC cells exposed to it [46]. It is however unlikely that colicin E2 can affect the production of Stx when the genes coexist in the same cell, but it has been shown that intestinal E. coli cells can act as chaperones and contribute to the production of Stx [47] and the colicin could possibly play a role here. Similar colicin plasmids are found in EHEC O26:H11 and EHEC O111:H− [7], and in a study by Karama et al. [48], 38.4% of E. coli O103:H2 strains were found to have a colicin producing phenotype.

The phi-like phage isolated from the wild-type EHEC O103:H25 NOS has an unknown function in the E. coli O103:H25 host. However, the number of the phi-like phage particles released from EHEC O103:H25 NOS after induction with Mitomycin C is estimated to be approximately ten times the number of the phi-like phage particles released (data not shown), and hence this abundant phi-like phage is a practical challenge as it complicates the isolation of the Stx2 phage and stx-negative phage directly from wild types.

Conclusion
Both the Stx2 phage and the bacterial genome from EHEC O103:H25 NOS are related to the Shiga toxin producing EAEC O104:H4 that caused a large European E. coli outbreak in 2011. Two patient isolates from the Norwegian O103:H25 outbreak carry an Stx2 phage, while other outbreak associated isolates carry a related phage in the same insertion site. The two variants, E. coli O103:H25 with the Stx2 phage or the stx-negative phage, have probably both been present in the contaminated food which caused the Norwegian outbreak.

Materials and Methods

Bacterial isolates
The E. coli O103:H25 isolates included in the study are presented in Table 1. NVH-734 is the outbreak reference strain and is also referred to as the Norwegian outbreak strain (NOS). E. coli DH5α was used as recipient strain in the plaque assay. E. coli strains were cultured in Luria-Bertani (LB) broth or on LB agar plates (LB containing 1% agar).

Whole genome sequencing, alignments and Multilocus sequence typing (MLST)
Isolate NVH-734 (EHEC O103:H25 NOS) was sequenced using 454 technology (454 Life Sciences, Branford, Connecticut, USA). Initial genome analysis revealed similarity to the E.coli O103:H2 str 12009 (accession: AP010958.1), and this strain was
used as template for the alignment of contigs from the EHEC O103:H25 Norwegian Outbreak Strain (NOS) using Mauve [15]. Subsequently, genome alignment of EHEC O103:H25 NOS and the EAEC O104:H4 str German Outbreak Strain (GOS)1 (distributed on 208 contigs, accessions: AFWO0100001.1-AFWO01000208.1) was also carried out with Mauve, using the progressive alignment option. Whole genome BLAST-comparison was performed using the BRIG software package [49] on the following genomes: EHEC O103:H25 NOS, EAEC O104:H4 GOS1, EHEC O103:H2 str 12009, EAEC O104:H4 str 55989, EHEC O111:H- str 11128, EHEC O157:H7 EDL933 and EHEC O26:H11 str 11368. MLST was performed according to Wirth et al. [50], using the seven housekeeping genes *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*. A maximum likelihood test using PhyML [51] was carried out to assess the best nucleotide substitution matrix in R [http://www.R-project.org/] with the package ‘ape’ [52]. Based on this, Tamura-Nei with invariant sites was shown to be the best model, and subsequently MEGA 5 [53] was used to generate a maximum likelihood tree, which was bootstrapped 500 times. Sequence type (ST) of EHEC O103:H25 NOS was obtained from MLST Databases at the ERI, University College Cork (http://mlst.ucc.ie). The O103:H25 NOS, O104:H4 str C227-11 (originating from the German outbreak), O103:H2 str 12009 and O157:H7 EDL933 phages were BLASTed against each other and compared using the ACT tool [54].

**PCA and sequencing**

Primers for gap-closure PCR for completing the sequence of the Stx2 phage, and for detection of genes in other isolates than EHEC O103:H25 NOS were designed on the genome sequence. All PCRs were carried out in an Eppendorf Mastercycler gradient (Eppendorf AG, Hamburg, Germany). DyNAzyme II DNA polymerase (supplied with 10× buffer) and dNTP Mix from Finnzymes (Vantaa, Finland) were used as instructed by the

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**Figure 3. Comparison of Stx2 phage and stx-negative phage.** Comparison of Stx2 phage and stx-negative phage from EHEC O103:H25 NOS (A) and *E. coli* O103:H25 NVH-848 (B), respectively. The Stx2 phage is sequenced, while the illustration of the stx-negative phage is based on sequence (23 kb) and RFLP pattern (illustrated by arrow). doi:10.1371/journal.pone.0031413.g003

**Figure 4. Stx2 phage from EHEC O103:H25 NOS compared to Stx2 phages from EAEC O104:H4, EHEC O103:H2 and EHEC O157:H7.** ACT visualization between stx2-positive phages from EHEC O103:H25 NOS, EAEC O104:H4 str C227-11, EHEC O103:H2 str 12009 and reference Stx2 phage 933W from EHEC O157:H7 EDL933. The phage genomes are compared using BLAST and the red regions represent hits. The white regions indicate absent genetic regions, which is especially noticeable in the comparison between the O103:H2 phage and the O157:H7 phage. doi:10.1371/journal.pone.0031413.g004
manufactured. The standard program was as follows: 95°C for 1 min, 30 cycles of 95°C for 1 min, 52°C for 1 min and 72°C for 1 min, and finally 72°C for 5 min. Sequencing of PCR products was performed by Source BioScience geneservice (United Kingdom), and DNA sequences were analyzed using Vector NTI Advance 11 (Invitrogen, Carlsbad, USA) and BLAST. Primers used for PCR detection of other virulence genes are listed in Table 3.

**Plaque assay**

E. coli O103:H25 LB broth cultures were incubated at 37°C with shaking at 200 rpm to an OD$_{600}$ of 0.3–0.5 (~2 h). To induce phage production, Mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 0.5 µg/ml and incubation was continued overnight in the dark. The cultures were centrifuged (2000 × g, 10 min) to remove bacterial cells and debris, and the supernatants (phage lysates) were sterile filtered (0.22 µm; Minisart, Sartorius Stedim Biotech). As the wildtype strains also produced colicin (see below) that lysed the DH5α cell lawn, lysates were treated with 100 µg/ml trypsin (Sigma) at 37°C for 60 minutes prior to use to destroy colicin. The E. coli DH5α recipient was grown in LB broth to OD 0.4–0.6 at 37°C and shaking at 200 rpm. For determination of infectious phage particles, 100 µl of tenfold dilutions of phage lysates were added 900 µl E. coli DH5α. CaCl$_2$ was added to a final concentration of 10 mM. The phage-recipient mixture was incubated for 30 min at 37°C before 2.5 ml molten soft agar (0.7% LB broth) was added and the mixture poured onto LB agar plates with CaCl$_2$ (10 mM). The plates were incubated at 37°C overnight, plaques were counted by visual examination and phage titres were calculated.

**Isolation and RFLP analysis of phage DNA**

To ensure that DNA was isolated from only the Stx2- or Stx-related phage, DNA extraction was performed via the plaque assay. This was necessary because an abundant phi-like phage is also present in the wild type strains, but this phage is not able to infect E. coli DH5α. Phage isolation was done from seven strains from the 2006 outbreak, in which five were stx-negative isolates from either food or patients, and two were stx-positive isolates from patients. The isolates are listed in Table 1. Briefly, 0.1 ml from an overnight starter culture of E. coli DH5α was transferred to 10 ml LB-broth and incubated at 37°C with shaking until bacterial growth reached mid log phase. Approximately 50 plaque was picked from the plaque assay described above, dissolved in 50 µl MQ, and added to 0.5 ml of the E. coli DH5α culture together with 12.5 µl 1 M CaCl$_2$ to facilitate bacteriophage infection. The culture was incubated at 37°C for two hours before 9.5 ml of LB-broth was added and then incubated at 37°C overnight. The overnight culture was centrifuged at 2000 × g for 10 minutes and the supernatant containing phage particles was sterile filtered (0.22 µm; Minisart, Sartorius Stedim Biotech, Aubagne, France), precipitated with 0.18×PEG 8000/NaCl at 0°C for 10 min, and 100 µl was treated with 100 µg/ml pronase (Sigma) at 37°C for 60 min.

**Figure 5. RFLP of phage from stx2-positive and stx-negative isolates.** EcoRI restriction fragment length polymorphism analysis of Stx2 phage and stx-negative phage in EHEC O103:H25 NOS and E. coli O103:H25 NVH-848, respectively. The arrow indicates the EcoRI fragments that are indistinguishable between the strains, arrowhead indicates the EcoRI fragment of E. coli O103:H25 NOS where the stx2 gene is located. doi:10.1371/journal.pone.0031413.g005

**Figure 6. Early region of Stx2 phage compared to early region of stx-negative phage.** Dot matrix view of 24 kb early region of the Stx2 phage from EHEC O103:H25 NOS and the 23 kb early region of the stx-negative phage from E. coli O103:H25 isolate NVH-848. The position of the stx2 gene in EHEC O103:H25 NOS is marked (o). Regions of similarity are based upon the BLAST results. Alignments are shown in the plot as lines. Plus strand and protein matches are slanted from the bottom left to the upper right corner, minus strand matches are slanted from the upper left to the lower right. The number of lines shown in the plot is the same as the number of alignments found by BLAST. doi:10.1371/journal.pone.0031413.g006

**Figure 7. Plasmid profiles of E. coli O103:H25.** Comparison of large plasmids in EHEC O103:H25 NOS (lane 4) and E. coli O103:H25 NVH-848 (lane 5) with EHEC O157:H7 EDL 933 (lane 3). GeneRuler 1 kb ladder (lane 2) and Lambda ladder (lane 1) were used as molecular size marker. doi:10.1371/journal.pone.0031413.g007
4°C for 2 hours, and centrifuged at 10,000×g for 1 hour. The pellet was dissolved in 0.5 ml TE buffer with proteinase K (Sigma-Aldrich, 50 mg/ml) and SDS (final concentration of 0.5%) and incubated for one hour at 56°C. The DNA was extracted using phenol/chloroform/isoamylalcohol (25:24:1), and the DNA was precipitated using equal amounts of isopropanol. Phage DNA was used as template in restriction fragment length polymorphism (RFLP) analysis and in PCR reactions. Phage DNA was digested with restriction enzymes EcoRI (New England BioLabs, Hertfordshire, England) and the restriction fragments separated by 1% agarose gels. A probe for detection of stx2A in RFLP hybridization was made using primers listed in Table 3. To complete the phage genomes of the stx2-positive and stx-negative phages, phage DNA from O103:H25 NOS and NVH-848, respectively, were used as templates in PCR reactions.

Colicin production

Colicin production was detected by observation of a lytic effect of sterile filtered supernatant from overnight culture of EHEC O103:H25 NOS on E. coli DH5 cell lawns. The identification of a colicin E2 encoding gene was performed by in silico analysis of the genome sequence, and gap closure PCR was performed to confirm a plasmid configuration of the contig. Primers colE2F and colE2R (Table 3) and standard PCR conditions were used to generate a probe for plasmid hybridization.

Pulsed-Field Gel Electrophoresis (PFGE) and plasmid isolation

The E. coli isolates associated with the outbreak were analyzed by PFGE as described previously [13] using the restriction enzymes XbaI and AvaII (New England BioLabs). DNA for detection of colicin E2 encoding plasmids was isolated from E. coli cultures. Plasmid DNA was extracted twice with phenol-chloroform (1:1, vol/vol) and 25 μl were applied directly to 0.7% agarose gel. Plasmids were separated by electrophoresis at 120 V for 3 h at 4°C.

Southern blotting and hybridization

Plasmid DNA and digested phage DNA were transferred to nylon membranes (Hybond-N, Amersham International plc, Amersham, United Kingdom) by Southern blotting [56]. For detection of the colicin E2 encoding gene and phage genes probes were labelled with digoxigenin (DIG) and hybridized with a DIG DNA labelling and detection kit (Roche Diagnostics, Basel, Switzerland) according to the instructions by the manufacturer.

Cloning of phage DNA

Phage DNA isolated directly from EHEC O103:H25 NOS culture supernatants was digested with EcoRI and cloned in pUC18, and the clones were subsequently sequenced.

GenBank accession numbers

The genome sequence of EHEC O103:H25 NOS has been deposited at DDBJ/EMBL/GenBank under the accession no AGSG00000000. The version described in this paper is the first version, AGSG01000000. The 61 kb Stx2 phage genome has accession no JQ011318 and the 24 kb early region of the stx-negative phage has accession no JQ011316. The 45 kb phi-like phage has accession no JQ011317.

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Author Contributions

Conceived and designed the experiments: TMLL HJJ JB PEG TL. Performed the experiments: TMLL HJJ JB PEG TL. Analyzed the data: TMLL HJJ JB PEG TL. Contributed reagents/materials/analysis tools: TMLL HJJ JB PEG TL. Wrote the paper: TMLL HJJ JB PEG TL.

### Table 3. Primers used in the study.

| Primer | Sequence (5′-3′) | Reference | Probe |
|--------|-----------------|-----------|-------|
| colE2F | ATGACCGGTTCTGGCATGGACGC | This study | Hybridisation of plasmid profiles |
| colE2R | GCCCGGGCATTGGCCACATTCT |
| pagC F | ATGAGTGGGACAGACTGG | [18] |
| pagC R | CCAATCCACAGTAAATCC |
| sen F | GGATGGAAACACTACCTGG | [18] |
| sen R | CGCAATGATGCTAATTGC |
| efa1 F | CTCGCCAGAGATAATTGAGG |
| efa1 R | CAACCTGATGGCAGAATTACTC |
| efa2 F | CTGTCAGAGCAGACATTGG |
| efa2 R | GAAGGATGGCAGATTCTC |
| stx2 F | GCGTTTGGACCATCTCAGATG |
| stx2 R | AAGGAGGACGTTTCAAGACAG |

Plasmid DNA was extracted twice with phenol-chloroform (1:1, vol/vol) and 25 μl were applied directly to 0.7% agarose gel. Plasmids were separated by electrophoresis at 120 V for 3 h at 4°C.

Southern blotting and hybridization

Plasmid DNA and digested phage DNA were transferred to nylon membranes (Hybond-N, Amersham International plc, Amersham, United Kingdom) by Southern blotting [56]. For detection of the colicin E2 encoding gene and phage genes probes were labelled with digoxigenin (DIG) and hybridized with a DIG DNA labelling and detection kit (Roche Diagnostics, Basel, Switzerland) according to the instructions by the manufacturer.

Cloning of phage DNA

Phage DNA isolated directly from EHEC O103:H25 NOS culture supernatants was digested with EcoRI and cloned in pUC18, and the clones were subsequently sequenced.

GenBank accession numbers

The genome sequence of EHEC O103:H25 NOS has been deposited at DDBJ/EMBL/GenBank under the accession no AGSG00000000. The version described in this paper is the first version, AGSG01000000. The 61 kb Stx2 phage genome has accession no JQ011318 and the 24 kb early region of the stx-negative phage has accession no JQ011316. The 45 kb phi-like phage has accession no JQ011317.

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Author Contributions

Conceived and designed the experiments: TMLL HJJ JB PEG TL. Performed the experiments: TMLL HJJ JB PEG TL. Analyzed the data: TMLL HJJ JB PEG TL. Contributed reagents/materials/analysis tools: TMLL HJJ JB PEG TL. Wrote the paper: TMLL HJJ JB PEG TL.
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