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Shiga toxin-producing *Escherichia coli* strains from cattle as a source of the Stx2a bacteriophages present in enteroaggregative *Escherichia coli* O104:H4 strains

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ABSTRACT

Enteroaggregative, Shiga toxin-producing *E. coli* (EAEC-STEC) O104:H4 strains are emerging pathogens causing life threatening diseases in humans. EAEC-STEC O104:H4 strains isolated between 2001 and 2011 were found to harbor a distinct type of Shiga toxin 2a- (Stx2a) encoding prophage. This phage type shows only <65% genetic similarity to so far described viable Stx phages due to differences in the modules for DNA replication, metabolism, regulation and host specificity. Stx production in EAEC is rarely observed and the source of the Stx2a phage in the EAEC-STEC O104:H4 strains is not known. We identified two DNA segments derived from *orf15* and the *cI* gene of the O104:H4 Stx2a phage P13374 that are characteristic for Stx2a prophages present in EAEC-STEC O104:H4 strains. By PCR, these sequences were detected in 14 (5.8%) of 241 Stx2-positive STEC from animals and food. Infectious Stx2a phages could be isolated from four bovine STEC strains. These were found highly similar to P13374 for *orf15*, *cI* and *stx*2a sequences, the chromosomal integration site (*wrbA*), for phage DNA restriction profiles, virion morphology and superinfection immunity. Stx2a phages of the four bovine STEC strains formed lysogens on the *E. coli* K-12 strain C600. Phage P13374 from an EAEC-STEC O104:H4 outbreak strain and one of the bovine STEC phages (P13803) lysogenized the Stx-negative EAEC O104:H4 strain CB14647 by integrating in the *wrbA* gene of CB14647 and converted it into a Stx2a producer. Our findings provide experimental evidence that EAEC-STEC O104:H4 strains have evolved by uptake of Stx2a phages from the bovine reservoir.

Introduction

In summer 2011, Shiga toxin-producing *Escherichia coli* (STEC) O104:H4 strains caused an outbreak affecting nearly 4000 patients in Germany. The outbreak spread to sixteen countries inside and outside Europe (European Food Safety Authority, 2011a; Muniesa et al., 2012). An unusual high incidence of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) was noted in the infected patients (Beutin and Martin, 2012). The incriminated food vehicles of the outbreak were sprouts grown from fenugreek seeds (European Center for Disease Prevention and Control and European Food Safety Authority, 2011c). Trace back epidemiological investigations pointed to contaminated fenugreek seeds imported from Egypt as the possible source of STEC O104:H4 outbreak strain in Europe (European Food Safety Authority, 2011b).

STEC O104:H4 infections in humans were rarely observed before the 2011 outbreak. The first reported human STEC O104:H4 infections occurred in 2001 in Cologne, Germany. Two siblings in a family were affected with HC and subsequently HUS while the infection source remained unknown (Beutin et al., 2012; Bielaszewska et al., 2011). Sporadic cases of human infections with STEC O104:H4 were reported for the time period between 2001 and 2011 from different countries in Europe and Asia. None of these cases was linked to a food vehicle (European Center for Disease Prevention and Control and European Food Safety Authority, 2011c).

Investigation of phenotypes and virulence genes of STEC O104:H4 strains isolated from sporadic cases and outbreaks in 2001 until 2011 revealed fundamental differences to other STEC and enterohemorrhagic *E. coli* (EHEC) strains. STEC O104:H4 isolates share virulence properties with enteroaggregative *E. coli* (EAEC), including the intestinal colonization mechanism which is mediated by aggregative adherence fimbriae (AAF) (Bielaszewska et al., 2011). All STEC O104:H4 strains isolated between 2001 and 2011 were found to produce Stx2a; a toxin type which is associated with severe clinical outcome in infected patients (Persson et al., 2007;

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Scheutz et al., 2012). Stx2a was shown to be encoded by temperate bacteriophages integrated in the *wrbA* locus of the STEC O104:H4 chromosome (Beutin et al., 2012).

Initial analyses showed that the Stx2a-encoding bacteriophages of epidemiologically unrelated STEC O104:H4 strains are very similar in morphology, restriction pattern, integration locus and superinfection immunity but exhibit significant differences to previously described Stx phages (Beutin et al., 2012). The properties of a typical O104:H4 Stx2a bacteriophage P13374 (GenBank HE664024.1) have been characterized in detail (Beutin et al., 2012). Sequence analyses showed that the P13374 genome is closely related to Stx2a prophages of published EAEC-STEC O104:H4 genomes isolated in 2011 (JQ011318.1) with >99% similarity and 2009 (CP003297.1, CP003301.1) (97% similarity) (Ahmed et al., 2012). It is less related (91% similarity) to the prophage TL-2011c (JQ011318.1) of a STEC O103:H25 strain (L'abee-Lund et al., 2012). Compared to other Stx1 and Stx2 phages, P13374 and the Stx2a prophages of STEC O104:H4 strains have only <65% similarity, showing characteristic differences in the modules for DNA replication and metabolism, in regulation (immunity) and in the tail fiber protein (Ahmed et al., 2012; Beutin et al., 2012).

For reasons so far unknown, EAEC strains are rarely found as STEC (Iyoda et al., 2000; Morabito et al., 1998). According to published data, Stx phages are not randomly spread in strains of *E. coli* and it appears likely that the different types of Stx phages encoding different Shiga toxin variants show specificities to particular *E. coli* strains and serotypes (Beutin et al., 2008; Recktenwald and Schmidt, 2002; Steyert et al., 2012; Strauch et al., 2008). We assume that P13374 and closely related Stx2a phages are specifically able to infect EAEC O104:H4 strains generating the more virulent EAEC-STEC O104:H4 type strains. So far, nothing is known about the evolution and spread of this new Stx2a phage variant (Muniesa et al., 2012).

Stx-negative EAEC O104:H4 strains have been isolated from human patients in Europe and in several central African states (Germani et al., 1997; Scheutz et al., 2012). Comparison of the genome sequences of the EAEC O104:H4 strain 55989 which was isolated in 1995 from a patient in Central Africa and the EAEC-STEC O104:H4 strains isolated in Europe revealed 99.8% similarity (Germani et al., 1997; Rasko et al., 2011; Rohde et al., 2011). It was therefore suggested that EAEC-STEC O104:H4 has evolved from EAEC O104:H4 by uptake of Stx2a-encoding bacteriophages (Brzuszkiewicz et al., 2011; Rasko et al., 2011). So far, only humans are known as a natural reservoir of EAEC strains (Nataro et al., 1998; Okhuysen and Dupont, 2010; Okeke, 2009). Like other EAEC strains, EAEC-STEC O104:H4 were not associated with animals as possible reservoir but only with humans and with contaminated foodstuff (Auvray et al., 2012; Beutin and Martin, 2012; Cassar et al., 2004; European Center for Disease Prevention and Control and European Food Safety Authority, 2011; Paddock et al., 2013; Uber et al., 2006; Wieler et al., 2011).

It was previously shown, that Stx phages can lysogenize Stx-negative *E. coli* and other *Enterobacteriaceae* in the mammalian gut, in the environment and experimentally (Dopfer et al., 2010; Imamovic et al., 2009; Mellmann et al., 2008; Schmidt et al., 1999; Toth et al., 2003). It is therefore possible that the Stx2a phage found in STEC O104:H4 was acquired from other types of STEC strains which are frequently present in animals, food and in the environment (Mathusa et al., 2010; Muniesa et al., 2012). The presence of highly similar Stx2a phages in epidemiologically unlinked EAEC-STEC O104:H4 strains prompted us to search for related phages in non-O104 STEC strains which may be putative progenitor phages with the ability to convert Stx-negative EAEC O104:H4 to Shiga toxin production.

Materials and methods

Bacterial strains, media and culture conditions

The *E. coli* strains used in this study were from the collection of the National Reference Laboratory for *Escherichia coli* (NRL-*E. coli*, BfR, Berlin, Germany). The origin and relevant properties of *E. coli* O104 strains were published elsewhere (Miko et al., 2013). Serotyping of STEC was performed as described (Martin and Beutin, 2011). Presence or absence of stx_1, stx_2, *ehxA*, *eae*, *aggR*, wzx_{O104} and $CRISPR_{O104:H4}$ genes was investigated by real-time PCR as previously described (Delannoy et al., 2012; Tzschoppe et al., 2012). The nomenclature for Stx and Stx-subtypes was used as described (Scheutz et al., 2012).

The Stx-negative EAEC O104:H4 reference strain C1024-00 (CB14647) which was taken for phage transduction experiments was obtained from F. Scheutz (Statens Seruminstitut Copenhagen, Denmark) (Scheutz et al., 2011). CB14647 and the laboratory *E. coli* K-12 C600 was used for plaque formation assays and for lysogenization experiments with Stx2 phages as described previously (Beutin et al., 2012). Cultivation of bacteria, preparation of phage lysates, and phage susceptibility tests were performed as recently described (Beutin et al., 2012).

Isolation, propagation and purification of Stx2 phages

Stx2 phages of the investigated *E. coli* STEC strains (Table 1) were isolated by mitomycin C induction (0.5 μg/ml) as previously described (Beutin et al., 2012). Supernatants were passed through 0.22 μm pore sized filters (VWR International, Darmstadt, Germany) and analyzed for lytic phage activity (plaque assay) on lawns of *E. coli* K-12 strain C600. High titer lysates (10^9–10^{10} pfu/ml) were obtained from phage lysogenic *E. coli* K-12 (C600 derivates) by mitomycin C induction of 200 ml cultures (OD_{588} = ~0.3). After 16 h, phage lysates were centrifugated for 30 min at 10,000 × *g* to remove cell debris followed by sterile filtration (0.22 μm) and DNaseI/RNaseA treatment (Beutin et al., 2012). Phages were concentrated by PEG precipitation and purified for further analysis by CsCl-density gradient centrifugation as previously described (Sambrook and Russel, 2001).

Isolation of phage DNA and restriction analysis

Isolation of phage DNA was conducted by incubating purified phage particles with proteinase K and SDS followed by phenol chloroform extractions (Sambrook and Russel, 2001). To determine the genetic relationship restriction profiles with EcoNI, BsrBI and PvuI were performed according to the manufacturers' recommendations (Biolabs, Berlin, Germany). For analysis of restriction patterns, endonuclease digested phage DNA was separated on 0.7% agarose gels (Beutin et al., 2012).

Characterization of phages by transmission electron microscopy

Phage lysates were investigated by transmission electron microscopy (TEM) using the negative staining procedure with uranyl acetate. Aliquots of phage lysates were applied to pioloform-carbon-coated, 400-mesh copper grids (Plano GmbH, Wetzlar, Germany), incubated for 10 min and fixed with 2.5% aqueous glutaraldehyde (Taap Laboratories, Aldermaston, United Kingdom) for 1 min. Thereafter, phages were stained with 2% aqueous uranyl acetate (Merck, Darmstadt, Germany) for 1 min. Specimens were examined by TEM using a JEM-1010 (JEOL, Tokyo, Japan) at 80 kV accelerated voltage. Photographs and measurements were performed using a digital camera MegaView II and a computer