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Lysogenic conversion of atypical enteropathogenic *Escherichia coli* (aEPEC) from human, murine, and bovine origin with bacteriophage Φ3538 *Δstx$_2$::cat* proves their enterohemorrhagic *E. coli* (EHEC) progeny

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ABSTRACT

Bacteriophages play an important role in the evolution of bacterial pathogens. A phage-mediated transfer of *stx*-genes to atypical enteropathogenic *E. coli* (aEPEC) which are prevalent in different hosts, would convert them to enterohemorrhagic *E. coli* (EHEC). We decided to confirm this hypothesis experimentally to provide conclusive evidence that aEPEC isolated from different mammalian hosts are indeed progenitors of typical EHEC which gain the ability to produce Shiga-Toxin by lysogeny with *stx*-converting bacteriophages, utilizing the model phage Φ3538 *Δstx$_2$::cat*.

We applied a modified *in vitro* plaque-assay, using a high titer of a bacteriophage carrying a deletion in the *stx$_2$* gene (Φ3538 *Δstx$_2$::cat*) to increase the detection of lysogenic conversion events. Three wild-type aEPEC strains were chosen as acceptor strains: the murine aEPEC-strain IMT14505 (sequence type (ST)28, serotype Ont:H6), isolated from a striped field mouse (*Apodemus agrarius*) in the surrounding of a cattle shed, and the human aEPEC-strain 910#00 (ST28, Ont:H6). The close genomic relationship of both strains implies a high zoonotic potential. A third strain, the bovine aEPEC IMT19981, was of serotype O26:H11 and ST21 (STC29).

All three aEPEC were successfully lysogenized with phage Φ3538 *Δstx$_2$::cat*. Integration of the bacteriophage DNA into the aEPEC host genomes was confirmed by amplification of chloramphenicol transferase (*cat*) marker gene and by Southern-Blot hybridization. Analysis of the whole genome sequence of each of the three lysogens showed that the bacteriophage was integrated into the known tRNA integration site *argW*, which is highly variable among *E. coli*.

In conclusion, the successful lysogenic conversion of aEPEC with a *stx*-phage *in vitro* underlines the important role of aEPEC as progenitors of EHEC. Given the high prevalence and the wide host range of aEPEC acceptors, their high risk of zoonotic transmission should be recognized in infection control measures.

1. Introduction

The bacteriophage encoded Shiga-Toxin genes, *stx$_1$* and/or *stx$_2$*, are the main characteristic features of enterohemorrhagic *Escherichia coli* (EHEC), as well as other Shiga-Toxin producing *E. coli* (STEC). EHEC are the major cause of hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and bloody diarrhea in humans. These toxigenic intestinal bacteria are the cause of several food-borne outbreaks worldwide. While humans develop severe diseases when infected with EHEC, ruminants, the main reservoir of STEC, do not usually show any symptoms while being colonized with the bacteria (La Ragione et al., 2009; Lowe et al., 2009). In calves, however, STEC can be a cause of diarrhea (Wieler et al., 1996). Apart from ruminants, STEC have been isolated from the feces and gut of a variety of animals in the farm

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environment like synanthropic rodents and birds as well as from domestic animals (Ferens and Hovde, 2011; Garcia et al., 2010). This large range of STEC reservoir or infection source respectively of STEC carriers and propagators constitutes a potential risk for humans, due to the fact that EHEC are zoonotic agents. Both STEC and EHEC are transmitted to humans mainly *via* the fecal-oral infection route and therefore cause a wide range of food-borne outbreaks.

Besides EHEC, enteropathogenic *E. coli* (EPEC) are the main cause of persistent diarrhea in children and adults worldwide. An estimated 79,000 deaths in children under 5 years was calculated for 2011 (Lanata et al., 2013). Based on molecular typing methods 5%–10% of the cases of infantile diarrhea in the developing world are caused by EPEC. While atypical EPEC (aEPEC) are more prevalent than typical EPEC, they are associated with a significantly longer duration of diarrhea (Ochoa et al., 2008). In addition, typical EPEC are only isolated from humans, whereas the reservoir of aEPEC has been suggested to include humans as well as animals (Trabulsi et al., 2002; Wieler et al., 2007).

Both typical and atypical EPEC, as well as EHEC, induce attaching and effacing (A/E) lesions on epithelial cells. This common ability of aEPEC and EHEC to cause A/E lesions is conferred by a pathogenicity island (PAI), the locus of enterocyte effacement (LEE) that encodes for a Type Three Secretion System (TTSS), effector proteins, and chaperons. The bacteria attach to the intestinal epithelial cells and rearrange the cytoskeleton leading to the formation of pedestal like structures directly beneath the adherent bacteria (Gomes et al., 2016).

The pathotype EPEC is subdivided into typical and aEPEC based on the presence of the EAF plasmid in typical EPEC, which encodes *bfp* facilitating the expression of bundle forming pili. The EAF plasmid is found neither in aEPEC, nor STEC (Kaper et al., 2004). Typical and atypical EPEC are two distinct groups of pathogens, with aEPEC being more closely related to LEE-positive STEC, regarding virulence properties, genetic features, serotypes, and reservoirs – with the notable exception that aEPEC are not capable of Stx production. The lack of Stx and the shared characteristics of aEPEC and STEC strains suggest that aEPEC can be lysogenized with *stx*-converting bacteriophages. Stx-converting bacteriophages are lambdoid phages which are able to integrate into the genome of bacteria and hence confer the ability to produce Stx *via* lysogenic conversion. Karch and colleagues have shown that these prophages can be either lost or taken up by *E. coli* in the human intestine. Therefore aEPEC were initially EHEC that have lost the *stx*-converting bacteriophage during human infections, and hence they are called EHEC-LST (Bielaszewska et al., 2007a; Mellmann et al., 2005). Further, it was shown that STEC could emerge in the rumen of cattle due to natural occurring lysogenization with *stx*-converting bacteriophage (Sekse et al., 2008).

EHEC and aEPEC therefore seem to represent a dynamic system of bidirectional conversion, a scenario which we have recently corroborated by phylogenetic analyses of aEPEC and EHEC isolates belonging to MLST sequence type complex 29 (STC29) (Eichhorn et al., 2015). Nevertheless, the possibility of lysogenic conversion of wild-type aEPEC with *stx*-converting bacteriophages remains to be proven *in vitro* to underline the importance of this pathotype as progenitors of EHEC. Consequently, aEPEC would play a major role for the spread of EHEC and hence should be considered in the development of EHEC reduction strategies.

As rodents and shrews have been shown to be the reservoir for various pathogens (Bordes et al., 2015; Ulrich et al., 2008), in this study we first aimed to get insights into the prevalence of STEC and aEPEC in rodents and shrews, by analyzing the occurrence of these pathogens in fecal specimens of small mammal origin. A study concerning the prevalence and molecular typing of murine isolates was conducted in 2007 and embedded into the German network program Food-Borne Zoonotic Infections of Humans (FBI-Zoo)". The second aim was to examine the hypothesis that aEPEC are progenitors of EHEC, utilizing aEPEC from different animal sources. By utilizing *stx*-gene depleted bacteriophage C600 Φ3538Δstx_2::*cat* (Schmidt et al., 1999), we indeed were able to obtain lysogens of three wild-type aEPEC strains of human, murine and bovine origin. Each time the phages´ integration site was the *argW* locus, the arginine-specific tRNA gene, which is a known integration site of lambdoid bacteriophages (Ogura et al., 2007, 2009; Shringi et al., 2012).

2. Material and methods

2.1. Bacterial strains

Between the years 2005 and 2007, the Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health (FLI), collected a total of 1400 wild living rodents and shrews in six different German federal states (Mecklenburg-Western Pomerania (n = 565), Brandenburg (n = 229), Lower Saxony (n = 221), Saxony –Anhalt (n = 146), Baden-Wuerttemberg (n = 131), Bavaria (n = 96) and North Rhine-Westphalia (n = 12)) within the research network „Rodent-borne pathogens". Trapping of the rodents and shrews, removal of the intestinal tract and sample preparation was performed as described before (Guenther et al., 2013; Ulrich et al., 2009). The intestinal tract of all trapped rodents/shrews was sent for microbiological diagnostic purposes to the Institute for Microbiology and Epizootics (IMT), where further investigations were made and *E. coli* was identified using standard biochemical methods after enrichment in 2 ml EC Broth (Merck, Darmstadt, Germany) and incubation overnight at 37 °C (Guenther et al., 2010).

The three additionally used human aEPEC strains of ST28 had previously been isolated and described by Bielaszewska et al. in patients suffering from diarrhoea (Bielaszewska et al., 2008) within the "FBI-Zoo" research consortium (www.fbi-zoo.de).

The bovine aEPEC *E. coli* strain IMT19981 was also sampled within an exploratory clinical study of the "FBI-Zoo" research consortium as described in Eichhorn et al. (Eichhorn et al., 2015).

The donor strain used in this study was C600 Φ3538Δstx_2::*cat* which contained a recombinant stx_2 encoding temperate phage with a *cat* gene encoding a chloramphenicol resistance marker was kindly provided by Herbert Schmidt (Hohenheim University, Germany) (Schmidt et al., 1999).

2.2. Serotyping

Serotyping was performed using a microtiter method with antisera against *E. coli* O antigens 1–182 and H antigens 1–56 according to the method of Prager et al. (Prager et al., 2003).

2.3. Pathotyping

Pathotypes of the strains were determined based on the presence or absence of the virulence genes stx_1, stx_2, *escV*, and *bfpB*, which were identified by PCR using published primer pairs and multiplex PCR protocols (Muller et al., 2007). LEE-positive strains were identified by the detection of *escV*, a LEE-located translocator gene. In addition, we also screened for the intimin-encoding gene *eae* to confirm the presence of the LEE (Friedrich et al., 2003). aEPEC strains do not harbour the EAF plasmid and therefore are *bfpB* negative, as well as negative for all subtypes of stx_1 and stx_2 (Scheutz et al., 2012).

2.4. Multilocus sequence typing (MLST)

MLST was performed by analysing internal fragments of seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA, recA*) (Wirth et al., 2006). The alleles and sequence types (STs) were assigned in accordance with the *E. coli* MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).