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Comparative virulence characterization of the Shiga toxin phage-cured *Escherichia coli* O104:H4 and enteroaggregative *Escherichia coli*

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ABSTRACT

Escherichia coli O104:H4 (*E. coli* O104:H4), which caused in 2011 a massive foodborne outbreak in Germany, is characterized by an unusual combination of virulence traits. *E. coli* O104:H4 contains a prophage-encoded Shiga toxin (Stx) gene, which is the cardinal virulence factor of enterohemorrhagic *E. coli* (EHEC). However, the outbreak strain shares highest DNA sequence similarity with enteroaggregative *E. coli* (EAEC) and displays the EAEC-characteristic tight adherence to epithelial cells. The virulence potential of the underlying EAEC background has not been investigated and it is therefore not clear whether *E. coli* O104:H4 displays distinct virulence characteristics in comparison to prototypical EAEC. In this study, we performed a detailed comparative phenotypic characterization of the Stx phage-cured *E. coli* O104:H4 strain C227-11φcu, the closely related EAEC strain 55989 and two other well-characterized EAEC strains 042 and 17-2 with focus on virulence traits. C227-11φcu displayed superior aggregative adherence phenotype to cultured HCT-8 epithelial cells, adhering with 3–6 times more bacteria per epithelial cells than the tested EAEC strains. Otherwise, C227-11φcu showed similar virulence characteristics to its closest relative 55989, *i.e.* strong acid resistance, good biofilm formation and cytotoxic culture supernatants. Furthermore, C227-11φcu was characterized by significantly weaker motility and pro-inflammatory properties than 55989 and 042, nevertheless stronger than 17-2. Taken together, C227-11φcu displayed mostly robust, but not outstanding virulence characteristics in comparison to the tested EAEC. Therefore, it appears likely that the combination of Stx production and EAEC characteristics in general, rather than an exceptionally potent EAEC background resulted in the unusual virulence of the *E. coli* O104:H4. Thus, the emergence of such hypervirulent strains in the future might be more likely than previously anticipated.

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) are enteric Shiga toxin (Stx)-producing bacteria, which can cause diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS), (Karch et al., 2005). During the largest EHEC outbreak ever recorded in Germany (May to July 2011) nearly 4000 EHEC gastroenteritis and 855 HUS cases were reported, leading to 54 deaths (Robert-Koch-Institut, 2011). Since the infections were characterized by an unusual high rate of progression to HUS (Frank et al., 2011), the outbreak is also regarded as the highest incidence of EHEC-associated HUS worldwide. *E. coli* O104:H4 carrying Stx2-encoding prophage was identified as the causative agent of the outbreak. Phylogenetic analyses and comparative genomics based on whole genome sequencing data revealed that *E. coli* O104:H4 is a close relative of the enteroaggregative *E. coli* (EAEC) strain 55989 (also of serotype O104:H4) and only distantly related to commonly isolated EHEC strains (Brzuszkiewicz et al., 2011; Mellmann et al., 2011; Rasko et al., 2011; Rohde et al., 2011). EAEC is another type of intestinal-pathogenic *E. coli* that is usually associated with acute and persistent diarrhea and displays a characteristic “stacked-brick” adherence phenotype to cultured human epithelial cells by means of different aggregative adherence fimbria (AAF) types (Jafari et al., 2012; Nataro et al., 1998). Thus, instead of the frequently found *eae* gene coding for the EHEC adhesin intimin, *E. coli* O104:H4 carries a pAA plasmid-encoded AAF/I mediating its tight adherence to epithelial cells, as well as other EAEC-specific pAA- and chromosome-encoded virulence factors (Bielaszewska et al., 2011).

Several studies have shed light on the determinants involved in *E. coli* O104:H4 pathogenicity. The outbreak strain caused weight loss and mortality in germ-free mice and Stx2 was found to be the key virulence factor responsible for the observed pathogenesis (Zangari et al., 2013). Moreover, loss of the pAA plasmid was sporadically observed during the course of the outbreak and correlated with a significantly reduced probability of HUS development in patients, which speaks for an attenuated virulence of the pAA-negative strain (Zhang et al., 2013). Indeed, it was shown that the presence of pAA in *E. coli* O104:H4, in

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particular the AAF/I, promote the translocation of Stx2 across an epithelial cell monolayer and that both Stx2 production and expression of certain EAEC virulence factors contribute to intestinal inflammation (Boisen et al., 2014). Thus, a synergistic interplay between characteristic EHEC and EAEC factors apparently plays a crucial role in *E. coli* O104:H4 pathogenicity. However, the virulence potential of the underlying EAEC background has not been studied and it is therefore unclear if it specifically contributes to *E. coli* O104:H4 exceptional virulence.

Here, we addressed the question whether the 2011 *E. coli* O104:H4 outbreak strain displays distinct virulence characteristics in comparison to prototypical EAEC. We therefore performed a detailed comparative phenotypic characterization of the Stx phage–cured *E. coli* O104:H4 strain C227-11φcu, the closely related EAEC strain 55989 and two other well-characterized EAEC strains 042 and 17-2 with focus on virulence. We evaluated the acid resistance, adherence, biofilm, motility, cytotoxicity and inflammatory properties of the strains.

2. Materials and methods

2.1. Bacterial strains and cultivation

C227-11φcu is a Stx2a phage-cured but isogenic derivative of the 2011 *E. coli* O104:H4 outbreak strain (Kunsmann et al., 2015; Zangari et al., 2013). EAEC strain 55989 (O104:H4) was isolated from a HIV-positive adult with persistent watery diarrhea in Central Africa and is closely related to the 2011 outbreak strain (Mellmann et al., 2011; Touchon et al., 2009). The other prototype EAEC strains used in this study were strains 042 (O44:H18) (Nataro et al., 1985) and 17-2 (O3:H2) (Vial et al., 1988). As a negative control served the *E. coli* K-12 strain MG1655 (Bachmann, 1996). Glycerol stocks of the strains were plated on Lysogenic broth (LB) agar (Lennox with 5 g/L NaCl; Roth; #X965.3) and incubated overnight at 37 °C. The following liquid culture media were used: LB medium (Lennox; Roth; #X964.4), LB supplemented with 4 g/L bile salts (50% sodium cholate, 50% deoxycholate, Fluka, #F 48305 02500), Dulbecco's modified eagle medium (DMEM) with 4.5 g/L glucose (Gibco; #21063029), simulated colonic environment medium (SCEM; 6.25 g/L bacto tryptone (BD, #211705), 2.6 g/L D-glucose (Roth, #X997.1), 0.88 g/L NaCl (Roth, #3957.1), 0.43 g/L KH_2PO_4 (Roth, #3904.2), 1.7 g/L $NaHCO_3$ (Roth, #6885.2), 2.7 g/L $KHCO_3$ (Roth, #P748.1), 4 g/L bile salts) (Müsken et al., 2008) and Roswell Park Memorial Institute 1640 (RPMI 1640; serum free, without glutamine, Lonza, #880175-12). The exact experimental-specific growth conditions are described in the corresponding Materials and Methods chapters.

2.2. Growth kinetics

Growth kinetics measurements were performed in a TECAN Infinite F200 instrument for 24 h at 37 °C with orbital shaking with an amplitude of 2 mm. Optical density (OD) at 595 nm was recorded automatically every 20 min. The doubling time was calculated using the linear region of the obtained growth curves, while the final OD values were determined after a 1:10 dilution. Measurements were performed with three biological replicates per strain.

2.3. Acid resistance assay

Acid resistance assays were performed as described elsewhere with some minor modifications (Coldewey et al., 2007). Briefly, overnight LB cultures were diluted 1:100 in LB pH 2.5 (adjusted with 25% HCl) and incubated for 2 h at 37 °C, 180 rpm. A sample of 1 mL of each culture before and after the treatment was centrifuged at 2200 *g* and the pellet was resuspended in PBS (Lonza). Next, 10-fold serial dilutions were plated on LB agar, incubated overnight and the colony forming units (CFU) per mL were determined. The assay was performed with three biological replicates per strain and the results are depicted as percent survival after acid exposure in comparison to the untreated samples.

2.4. Quantitative aggregative adherence assay

Aggregative adherence assay was performed as previously described (Aldick et al., 2007). The human intestinal epithelial cells HCT-8 (ATCC CCL-244 (Tompkins et al., 1974)) were maintained in RPMI 1640 supplemented with 10% fetal calf serum (PAA), 2 mM ultra-glutamine (Lonza) and 1 mM sodium pyruvate (Lonza) at 37 °C with 5% CO_2. HCT-8 cells (1×10^5 cells/mL) were seeded in 4-well plates and grown overnight to semi-confluence. Bacterial infection mixtures were prepared adjusting 100 µL of static stationary overnight cultures grown in DMEM to an OD_{600} of 0.4. Bacteria and cells were incubated in serum-free RPMI 1640 for 3 h in the presence of 0.5% D-Mannose (inhibiting type I fimbria). Afterwards, cell were washed 3x with serum-free RPMI 1640 and 3x with Dulbeccos's PBS (containing Ca^{2+} and Mg^{2+}, PAA). Cells were then fixed with 70% Ethanol for five minutes, stained with 10% Giemsa for 30 min in the dark and washed with ddH_2O. After drying, cells were examined with phase-contrast microscopy using the 100x magnification (AxioImagerA1; Zeiss). The experiment was performed with three biological replicates of each bacterial strain and for each replicate the number of bacteria adhering per epithelial cell was counted manually in 10 microscopy images with the area of 0.014 mm^2.

2.5. Biofilm assay

Biofilm assays were performed as described elsewhere (Reisner et al., 2006) with some minor modifications. In summary, overnight LB or DMEM cultures grown without shaking were diluted 1:10 in LB, SCEM, DMEM and RPMI 1640 medium in 96-well plate. The plates were incubated at 37 °C for 48 h without shaking. Medium was discarded, biofilms were washed 3x with PBS and stained with crystal violet (1:1 dilution in ddH_2O) for 30 min in the dark. Afterwards, plates were washed 3x with ddH_2O and destained in 150 µL of 80% Ethanol: 20% Aceton. Absorbance at 570 nm was measured on a BMG FLUOstar OPTIMA microplate reader. Three biological replicates were analyzed per bacterial strain.

2.6. Motility assay

The motility assay was carried out as previously described (Nachin et al., 2005). Briefly, one colony was used to inoculate 15 cm swimming-agar plates (LB with 0.3% agar). The plates were incubated at 37 °C for 12 h and the diameter in cm of the swimming zones was measured. The assay was performed with three biological replicates per strain.

2.7. Preparation of protein samples

Overnight cultures of three biological replicates per strain were diluted 1:1000 in 10 mL of culture medium and grown for 16 h at 37 °C, 180 rpm. Total protein samples were prepared as follows: 1 mL from each sample was centrifuged at 2200 *g*, 4 °C, 5 min, the supernatant discarded and the pellet was resuspended with Laemmli buffer (Bio-Rad) to an OD = 0.01. The samples were incubated at 99 °C for 10 min, put on ice and stored at −20 °C. For proteins secreted in the supernatant, 8 mL of the overnight culture were centrifuged (2200 *g*, 4 °C, 30 min) and sterile filtered (0.2 µm, Corning). The samples were then concentrated (20-400x) with Vivaspin (MWCO 5000 Da, GE Healthcare), washed 5x with PBS (Lonza) and used in cytotoxicity assays or mixed with Laemmli buffer, cooked and used for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Prior SDS-PAGE, the protein samples from culture supernatant were additionally normalized based on the OD_{600} values of the overnight cultures. The same SCEM supernatants were analyzed by Western blot analysis (see