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Growth advantage of *Escherichia coli* O104:H4 strains on 5-*N*-acetyl-9-*O*-acetyl neuraminic acid as a carbon source is dependent on heterogeneous phage-Borne nanS-p esterases

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ABSTRACT

Enterohemorrhagic *E. coli* (EHEC) are serious bacterial pathogens which are able to cause a hemorrhagic colitis or the life-threatening hemolytic-uremic syndrome (HUS) in humans. EHEC strains can carry different numbers of phage-borne *nanS*-p alleles that are responsible for acetic acid release from mucin from bovine submaxillary gland and 5-*N*-acetyl-9-*O*-acetyl neuraminic acid (Neu5,9Ac$_2$), a carbohydrate present in mucin. Thus, Neu5,9Ac$_2$ can be transformed to 5-*N*-acetyl neuraminic acid, an energy source used by *E. coli* strains. We hypothesize that these NanS-p proteins are involved in competitive growth of EHEC in the gastrointestinal tract of humans and animals. The aim of the current study was to demonstrate and characterize the *nanS-p* alleles of the 2011 *E. coli* O104:H4 outbreak strain LB226692 and analyze whether the presence of multiple *nanS*-p alleles in the LB226692 genome causes a competitive growth advantage over a commensal *E. coli* strain.

We detected and characterized five heterogeneous phage-borne *nanS*-p alleles in the genome of *E. coli* O104:H4 outbreak strain LB226692 by *in silico* analysis of its genome. Furthermore, successive deletion of all *nanS*-p alleles, subsequent complementation with recombinant NanS-p13-His, and *in vitro* co-culturing experiments with the commensal *E. coli* strain AMC 198 were conducted. We could show that *nanS*-p genes of *E. coli* O104:H4 are responsible for growth inhibition of strain AMC 198, when Neu5,9Ac$_2$ was used as sole carbon source in co-culture. The results of this study let us suggest that multiple *nanS*-p alleles may confer a growth advantage by outcompeting other *E. coli* strains in Neu5,9Ac$_2$ rich environments, such as mucus in animal and human gut.

1. Introduction

Between May and July 2011, a large outbreak of diarrhea and the hemolytic-uremic syndrome (HUS) was recorded in Germany and Europe, which affected especially middle-aged women after consumption of contaminated fenugreek sprouts (Frank et al., 2011; King et al., 2012; Robert Koch-Institut, 2012). The outbreak was caused by a particular enterohemorrhagic *E. coli* strain of serotype O104:H4. Before that outbreak, only sporadic cases of *E. coli* O104:H4 infections had been described (Bae et al., 2006; Mellmann et al., 2008; Rauw et al., 2014; Scheutz et al., 2011).

Classical "EHEC" can be described as a group of pathogenic *E. coli* that produces one or more phage-encoded Shiga toxins (Stx) and contain the pathogenicity island "locus of enterocyte effacement (LEE)" (Kaper, 1998). The O104:H4 outbreak strain produced Stx2a and lacked the LEE (Bielaszewska et al., 2011; Brzuszkiewicz et al., 2011). Moreover, its genetical backbone was found to be closely related to those of enteroaggregative *E. coli* (EAEC), with an additional *stx*$_{2a}$-containing prophage in the chromosomal integration site *wrbA* (Brzuszkiewicz et al., 2011; Mellmann et al., 2011; Rasko et al., 2011). The genome of the O104:H4 outbreak strain showed high DNA sequence identity with the Stx-negative EAEC strain 55989, which was isolated from an HIV-positive adult in Africa in 1990 (Brzuszkiewicz et al., 2011; Mossoro et al., 2002). In culture, the strain was able to build the EAEC-typical aggregative stacked-brick pattern on HEp-2 cells (Bielaszewska et al., 2011; Tietze et al., 2015). Therefore, Brzuszkiewicz et al. (2011) described it as a new pathotype, namely entero-aggregative-haemorrhagic *Escherichia coli* (EAHEC). Further virulence factors, that have been

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Table 1
E. coli strains and plasmids used in this study.

E. coli strains	Relevant characteristic	Reference
LB226692	serotype O104:H4	Mellmann et al. (2011)
C227-11ϕcu	serotype O104:H4, cured from the stx_{2a}- phage	Zangari et al. (2013)
C227-11ϕcuΔ*nanS*-p11	serotype O104:H4, deletion of *nanS*-p11	This study
C227-11ϕcuΔ*nanS*-p14	serotype O104:H4, deletion of *nanS*-p14	This study
C227-11ϕcuΔ*nanS*-p11,12	serotype O104:H4, deletion of *nanS*-p11 and *nanS*-p12	This study
C227-11ϕcuΔ*nanS*-p11,12,13	serotype O104:H4, deletion of *nanS*-p11, *nanS*-p12 and *nanS*-p13	This study
C227-11ϕcuΔ*nanS*-p11,12,13,14	serotype O104:H4, deletion of *nanS*-p11, *nanS*-p12, *nanS*-p13 and *nanS*-p14	This study
AMC 198		German Collection of Microorganisms and Cell Cultures (DSMZ) (DSM No. 787) (ATCC 11229)
BW25113/pKD46	Donor for plasmid pKD46	Datsenko and Wanner (2000)
BW25141/pKD4	Donor for plasmid pKD4	Datsenko and Wanner (2000)
BT340	*E. coli* DH5α carrying plasmid pCP20	Cherepanov and Wackernagel (1995)
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q} *ZΔM15* Tn*10* (Tet^r)]	Stratagene
XL1-Blue/pKEC1.5	Donor for pKEC1.5 (derivative of plasmid pKD46 containing a chloramphenicol resistance gene instead of the original ampicillin resistance gene)	This study
DH5α/pK18	Donor for plasmid pK18	Pridmore (1987)
BL21(DE3)	F–*ompT hsdSB*(rB−,mB−) *gal dcm* (DE3)	Studier and Moffatt (1986)
BL21(DE3)/pET22b(+)	BL21 (DE3) carring plasmid pET-22b(+) (Novagen, Merck)	Nübling et al. (2014)
BL21(DE3)/pET-*nanS*-p13	pET-22b(+), *nanS*-p13	This study

found in the outbreak strain, are the IrgA homologue adhesin encoding gene (*iha*), the aggregative adherence fimbriae type I (AAF/I), the *rpoS* gene, encoding a regulator for stress response, including high acid tolerance, the occurrence of plasmid-encoded TEM-1 and CTX-M-15 beta-lactamases, responsible for the ESBL phenotype, and the existence of an unusual combination of the three serine protease autotransporters of *Enterobacteriaceae* (SPATE) genes, *sepA*, *sigA* and *pic* (Brzuszkiewicz et al., 2011; Mellmann et al., 2011; Rasko et al., 2011). SPATE proteins seem to be involved in mucosal colonization and damage (Boisen et al., 2009; Dautin, 2010; Harrington et al., 2009).

Infections with EAEC belong to the most frequent *E. coli*-associated illnesses occurring in developing countries especially concerning children, immunosuppressed individuals or travellers (Harrington et al., 2005; Mossoro et al., 2002). The reservoir of typical EAEC (expressing the AggR regulon), such as the 2011 outbreak strain, seems to be the human gastrointestinal tract, different from STEC or atypical EAEC (not expressing the AggR regulon) that are preferentially found in ruminants (Uber et al., 2006; Wieler et al., 2011). Therefore, it is expected that typical EAEC are well adapted to humans and persist in the human gut microbiota.

Recently, we reported that EHEC O157:H7 strain EDL933 express several prophage-encoded 5-*N*-acetyl-9-*O*-acetyl neuraminic acid esterases (NanS-p), which are able to cleave acetyl residues from bovine submaxillary gland mucin and 5-*N*-acetyl-9-*O*-acetyl neuraminic acid (Neu5,9Ac_2) (Nübling et al., 2014; Saile et al., 2016). As a result of this cleavage, Neu5,9Ac_2 is metabolized to *N*-acetyl neuraminic acid (Neu5Ac). Neu5Ac inactivates the transcriptional repressor NanR of *E. coli* and therefore induces the *nan*-operons *nanATEK-yhcH*, *nanCMS* and *yjhBC* generating *N*-acetylmannosamine and pyruvate, which can be further degraded by the tricarboxylic acid cycle and/or glycolysis (Vimr, 2013).

The molecular structure of NanS-p proteins are deposited by domains of unknown functions 1737 and 303 (SASA) and comprise amino acid sequence blocks typical of the SGNH enzyme family of hydrolases (Rangarajan et al., 2011; Rangel et al., 2016; Saile et al., 2016). A special feature of the *nanS*-p genes in *E. coli* O157:H7 is their localization in prophages between the antiterminator Q and the lysis gene S, which is in contrast to the chromosomally located *nanS* of *E. coli* (as part of the *nanCMS* operon), that encodes also a Neu5,9Ac_2-esterase (Rangarajan et al., 2011; Steenbergen et al., 2009). Independently from each other, Rangel et al. (2016) and our group could recently state, that NanS-p can compensate the function of NanS in the Neu5,9Ac_2 catabolism in *E. coli* O157:H7 strain EDL933 (Rangel et al., 2016; Saile et al., 2016). But in contrast to *nanS* the *nanS*-p genes are not under the control of NanR (Rangel et al., 2016). Different from the situation in *nanS*, the *nanS*-p genes are frequently found in pathogenic *E. coli*, and in particular in clinical relevant EHEC serotypes (Vimr, 2013). In a former study of Herold et al. (2005), we could show that several *nanS*-p alleles were transcribed after norfloxacin induction. The data of this study confirmed also the cotransduction of *nanS*-p1 (z1466) and the Shiga toxin 2a gene together with the late phase prophage genes in O157:H7 strain EDL933 (Herold et al., 2005). In a later study, Polzin et al. (2013) demonstrated by differential 2D gel electrophoresis that NanS-p proteins (933Wp42) were 40-fold overexpressed in simulated colonic environmental medium (SCEM) under aerobic conditions in strain EDL933. These data show already evidence that *nanS*-p genes are functional and can be expressed under various conditions, especially under such conditions, where the SOS response is induced.

It is our hypothesis, that EHEC could have an advantage towards commensal *E. coli* and other *Enterobacteriaceae* with respect to substrate utilization in the human colon due to the presence of multiple *nanS*-p genes (Saile et al., 2016).

In the current study, we demonstrate that the O104:H4 outbreak strain LB226692 carries five prophage-located *nanS*-p genes. We characterized their location, analyzed their heterogeneous sequences and found that the presence of the *nanS*-p genes leads to a growth advantage on Neu5,9Ac_2 compared to the commensal *E. coli* strain AMC 198. The presence of multiple functional *nanS*-p alleles therefore increase the virulence of some pathogenic *E. coli* strains by providing growth advantages in the gut and facilitating colonization.

2. Materials and methods

2.1. E. coli strains and plasmids

E. coli strains used in this study are listed in Table 1. Strains were routinely grown in LB-broth (10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl). The pH was adjusted to 7.0 with 1 N NaOH. The antibiotics ampicillin (Roth), kanamycin (Sigma-Aldrich) or chloramphenicol (Roth) were added in final concentrations of 100, 50 or 25 μg/mL, respectively, when needed. Plasmids were prepared from overnight cultures of the respective *E. coli* strain using the QlAprep Spin Miniprep Kit (Qiagen).