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Carbohydrate utilization by enterohaemorrhagic *Escherichia coli* O157:H7 in bovine intestinal content

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Summary

The bovine gastrointestinal (GI) tract is the main reservoir for enterohaemorrhagic *Escherichia coli* (EHEC) responsible for food-borne infections. Characterization of nutrients preferentially used by EHEC in the bovine intestine would help to develop ecological strategies to reduce EHEC carriage. However, the carbon sources that support the growth of EHEC in the bovine intestine are poorly documented. In this study, a very low concentration of glucose, the most abundant

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Growth curves of EDL933 incubated in BSIC samples (open circle) and M9-Glc (filled circle). EDL933 was grown at 39°C without shaking. The arrows indicate the time at which the RNA samples were collected. Values are the mean $\pm$ 1 SEM of three independent experiments.

Fig. S2. Relative expression of genes required for the catabolism of mucus-derived carbohydrates during incubation of the commensal *E. coli* strain BG1 in BSIC compared with M9-Glc. The ratio of mRNA level of each gene was measured in BG1 incubated in filtered BSIC in comparison with cells grown in M9-Glc. RNA samples were collected during the exponential growth phase (grey), when the bacteria entered into the stationary phase (white) and during the stationary phase (black). Values are the mean $\pm$ 1 SEM of three independent experiments.

Fig. S3. Growth competition assays between the commensal *E. coli* BG1 and the EHEC strain EDL933 or isogenic mutants of EDL933. The BSIC-LEM samples were inoculated with a 1:1 mixture of the two strains. The EDL933 mutants were defective for the pathway required for the catabolism of fucose (EDL933 Δ *fucAO*), galactose (EDL933 Δ *galk*), GlcNAc (EDL933 Δ *nagE*), mannose (EDL933 Δ *manA*) or Neu5Ac (EDL933 Δ *nanAT*). Bars represent the SEM of three independent experiments. The double asterisk denotes statistical significance, $P < 0.05$ and the triple asterisk denotes statistical significance, $P < 0.01$ as determined by Student's *t*-test for paired samples.

Table S1. Genes involved in catabolism of mucus-derived carbohydrates by the EHEC strain EDL933.

Table S2. Bacterial strains and plasmids used in this study.

Table S3. Primer pairs used for gene cloning. The DNA sequence of restriction enzyme site is underlined. Start and stop codons of the cloned genes are in bold.

Table S4. Sequence of primers used in relative mRNA quantification.

monomer included in the cattle dietary polysaccharides, was detected in bovine small intestine contents (BSIC) collected from healthy cows at the slaughterhouse. Six carbohydrates reported to be included in the mucus layer covering the enterocytes [galactose, *N*-acetyl-glucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), fucose, mannose and *N*-acetyl neuraminic acid (Neu5Ac)] have been quantified for the first time in BSIC and accounted for a total concentration of 4.2 mM carbohydrates. The genes required for enzymatic degradation of the six mucus-derived carbohydrates are highly expressed during the exponential growth of the EHEC strain O157:H7 EDL933 in BSIC and are more strongly induced in EHEC than in bovine commensal *E. coli*. In addition, EDL933 consumed the free monosaccharides present in the BSIC more rapidly than the resident microbiota and commensal *E. coli*, indicating a competitive ability of EHEC to catabolize mucus-derived carbohydrates in the bovine gut. Mutations of EDL933 genes required for the catabolism of each of these sugars have been constructed, and growth competitions of the mutants with the wild-type strain clearly demonstrated that mannose, GlcNAc, Neu5Ac and galactose catabolism confers a high competitive growth advantage to EHEC in BSIC and probably represents an ecological niche for EHEC strains in the bovine small intestine. The utilization of these mucus-derived monosaccharides by EDL933 is apparently required for rapid growth of EHEC in BSIC, and for maintaining a competitive growth rate as compared with that of commensal *E. coli*. The results suggest a strategy for O157:H7 *E. coli* survival in the bovine intestine, whereby EHEC rapidly consumes mucus-derived carbohydrates that are poorly consumed by bacteria belonging to the resident intestinal micro-biota, including commensal *E. coli*.

Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) strains are Shiga-toxin-producing *E. coli* (STEC) that cause human illnesses ranging from uncomplicated diarrhoea to haemorrhagic colitis (HC) and haemolytic-uraemic syndrome (HUS) (Law, 2000). The gastrointestinal (GI) tract of cattle and other ruminants is the principal reservoir of EHEC strains and outbreaks have been associated with direct contact with the farm environment, and with the consumption of meat, dairy products, water and fruits or vegetables contaminated with ruminant manure (Cieslak *et al.*, 1993; O'Brien *et al.*, 2001; Yatsuyanagi *et al.*, 2002; Caprioli *et al.*, 2005; Muniesa *et al.*, 2006). It is important to determine the mechanisms underlying EHEC persistence in the bovine intestine in order to develop nutritional or ecological strategies to reduce EHEC survival in the GI tract and thus limit contamination of food products. Therefore, understanding of EHEC physiology in the ruminant gut is critical for limiting EHEC shedding.

In the rumen, growth of EHEC O157:H7 is limited by the resident microbiota and strictly anaerobic conditions (de Vaux *et al.*, 2002; Chaucheyras-Durand *et al.*, 2006; 2010). However, O157:H7 *E. coli* strains may survive passage through the acid barrier of the abomasum to the small intestine, which constitutes a more favourable environment than rumen contents for bacterial growth (Diez-Gonzalez *et al.*, 1998; de Vaux *et al.*, 2002; Chaucheyras-Durand *et al.*, 2010). Although the recto-anal junction is considered as the primary *E. coli* O157:H7 colonization site for persistent shedding in cattle (Naylor *et al.*, 2005; Lim *et al.*, 2007; Chase-Topping *et al.*, 2008), the small intestine and the proximal colon are also minor sites of EHEC carriage (Baines *et al.*, 2008; Nart *et al.*, 2008). Furthermore, a recent report showed that STEC O157 strains are distributed along the entire GI tract of naturally shedding cattle (Keen *et al.*, 2010). However, little is known about the nutrients preferentially used by EHEC or the metabolic pathways required for persistence and growth in the bovine GI tract. Snider *et al.* demonstrated that fucose is a critical carbon source for maintenance of EHEC in the bovine rectum (Snider *et al.*, 2009) and *in vivo* colonization experiments showed that the genes *agaB* and *dctA* coding for the specific

transport of GlcNAc and C4-dicarboxylic acids respectively influence colonization of the bovine gut by EHEC (Dziva *et al.*, 2004). More recently, we demonstrated that ethanolamine is an important nitrogen source for EHEC in the bovine small intestine content (BSIC) and favours EHEC persistence at this site (Bertin *et al.*, 2011).

The gastrointestinal epithelium is covered by a mucus gel layer (MGL) synthesized and secreted by host goblet cells. The MGL is an integral structural component of the mammal intestine, acting as a medium for protection and transport between the luminal content and the epithelium lining (Deplancke and Gaskins, 2001). The major function of the MGL is to lubricate and to protect the intestinal epithelium from damage caused by food and digestive secretions (Deplancke and Gaskins, 2001). The MGL also acts as a trap for microorganisms, including pathogens, preventing their access to the epithelia (Johansson *et al.*, 2011). Mucin, the main constituent of mucus, is a filamentous glycoprotein constituted of high-molecular-weight subunits, each subunit being rich in oligosaccharides linked to serine or threonine residues. In human, *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), fucose and galactose are the four primary mucin oligosaccharides that are often terminated with sialic acid or sulfate groups (Deplancke and Gaskins, 2001). Interestingly, in the intestine, mucin can constitute a direct source of carbohydrates constantly released in the luminal content, and can offer numerous ecological advantages to intestinal bacteria (Deplancke and Gaskins, 2001). *E. coli* is limited to growth on mono- or disaccharides and cannot degrade the complex polysaccharides constituting mucin (Hoskins *et al.*, 1985). However, resident anaerobes in the bovine gut are able to degrade mucin and to release carbohydrate monosaccharides into the luminal content (Png *et al.*, 2010).

Infection of streptomycin-treated mice is a useful animal model for studying colonization of the mammal gut by enteric bacteria (Wadolowski *et al.*, 1990). This model has been extensively used to study EHEC metabolism during gut colonization (Miranda *et al.*, 2004; Fabich *et al.*, 2008; Leatham *et al.*, 2009). EHEC O157:H7 has been shown to be present both in the mucus layer that overlies the mouse intestinal epithelium and closely associated with epithelial cells (Miranda *et al.*, 2004). Bacterial competition experiments have shown that the EHEC strain EDL933 uses seven sugars known to be present in the mouse caecal mucus (arabinose, fucose, GlcNAc, galactose, hexuronates, mannose and ribose) (Fabich *et al.*, 2008). Importantly, *E. coli* EDL933 appears to consume four sugars (galactose, hexuronates, mannose and ribose) that are not used by the non-pathogenic *E. coli* MG1655, suggesting a strategy for EHEC to invade the mouse intestine and colonize in the presence of commensal *E. coli* (Fabich *et al.*, 2008). In contrast, the utilization of mucus-derived carbohydrates by EHEC in the bovine intestine is poorly documented. The composition of the mucin covering the epithelium of the bovine small intestine has been described: proteins and carbohydrates represent 53% and 47% of the bovine mucin respectively, and the main fermentable monosaccharides constituting the mucin carbohydrate fraction are galactose, GlcNAc, GalNAc, fucose, mannose and *N*-acetyl neuraminic acid (Neu5Ac) (Montagne *et al.*, 2000).

In this report, we hypothesize that mucus-derived carbohydrates can be released in the bovine small intestine from the mucus layer and used as substrates for EHEC. To test this hypothesis, we compared the consumption of carbohydrates in the BSIC by endogenous microbiota, EHEC, and commensal *E. coli*, and we performed *in vitro* bacterial competitive experiments using a wild-type EHEC and isogenic mutants deficient in carbohydrate catabolic pathways. We demonstrated that utilization of mucus-derived carbohydrates confers a competitive growth advantage to EHEC in the BSIC and that EHEC consumes free carbohydrate monosaccharides more rapidly than the bacteria belonging to the resident intestinal microbiota.

Results

Carbohydrate degradation by *E. coli* O157:H7

To degrade oligosaccharides, bacteria must produce glycosidases with the appropriate specificities for each of the glycoside linkages within the oligosaccharide chains. In order to analyse the genetic information encoding carbohydrate degradation carried by the EDL933 genome, we performed *in silico* analyses using the CAZy (Carbohydrates Active enZYme) database describing the families of glycoside hydrolases (GHs) (EC 3.2.1.-) that break down or modify glycosidic bonds. Fifty-two open reading frames predicted to encode hydrolases included in 19 families of GHs were present in the bacterial genome. Most of the sequences encoded family-24, family-13 and family-23 GHs comprising essentially lysozyme (EC 3.2.1.17) and amylase (EC 3.2.1.1) enzymes. However, genes coding for the hydrolysis of dimers were also present in the genome of EDL933: *bglA* and *ascB* encode a 6-phospho- β -glucosidase (GH1), *ebgA* and *lacZ* a β -D-galactosidase (GH2), *iudA* a β -D-glucuronidase (GH2), *bglX* a β -D-glucoside-glucohydrolase (GH3), *celF* a 6-phospho- β -glucosidase, and *mela* an α -galactosidase (GH4). In summary, *E. coli* EDL933 possesses the genetic information required to hydrolyse disaccharides into monosaccharides (β -D-galactosidase, phospho- β -glucosidase and β -D-glucuronidase) and to hydrolyse the terminal residues from oligosaccharides (α -galactosidase and β -D-glucoside-glucohydrolase). In agreement with previous studies concerning *E. coli* (Hoskins *et al.*, 1985), DNA sequence analysis strongly suggests that *E. coli* EDL933 is limited to grow on mono- or disaccharides.

The EDL933 genome contains genes encoding the transport and the degradation of the six main monosaccharides included in bovine mucin: fucose, galactose, GalNAc, GlcNAc, mannose and Neu5Ac (Table S1). To determine whether EDL933 was able to utilize these sugars, the bacterial strain was incubated in M9 medium supplemented with each carbohydrate (10 mM) as the sole carbon source. Growth of EDL933 on glucose, reported to be the most suitable fermentation substrate for *E. coli*, was monitored as a control. The bacterial growth curves showed that EDL933 could use each of the six monosaccharides as the sole carbon source (Fig. 1). However, differences in growth patterns were observed: (i) the use of GlcNAc or Neu5Ac resulted in growth patterns similar to that of EHEC grown on glucose (high growth yield and high rate), (ii) EHEC could use mannose, galactose and GalNAc but exhibited lower growth efficiency (extended lag phase, lower growth rate and lower yield) and (iii) incubation of EHEC with fucose resulted in a much lower cell yield, which is to be expected since fucose catabolism requires excretion of propanediol as a by-product. These results indicate that these sugars were not equivalent in providing carbon and energy to the bacterial cell.

Bovine small intestinal content and mucus-derived carbohydrate quantification

BSIC samples with a live endogenous microbiota (BSIC-LEM) were collected at the slaughterhouse and stored at -80°C to keep most of the endogenous microbiota viable (see the *Experimental procedure* section). Bacterial counts performed using freshly collected BSIC-LEM samples revealed the presence of 9×10^4 to $1.4 \times 10^5 \text{ ml}^{-1}$ of strict anaerobes and 3×10^5 to $9.5 \times 10^5 \text{ ml}^{-1}$ of facultative anaerobes. Because the mammalian small intestine is neither fully aerobic nor anaerobic (Jones *et al.*, 2007; Fabich *et al.*, 2008), strict anaerobic conditions were not physiologically relevant for *in vitro* incubation of BSIC. Consequently, BSIC-LEM samples were incubated at 39°C (internal bovine temperature) without shaking (to minimize oxygen availability) to mimic the physiological conditions of the bovine gut. After 7 h of incubation, the endogenous bacterial population reached 5.6×10^7 to $7.8 \times 10^7 \text{ ml}^{-1}$ of strict anaerobes and 8.5×10^6 to $1.2 \times 10^7 \text{ ml}^{-1}$ of facultative anaerobes. As previously described (Bertin *et al.*, 2011), the endogenous bacterial

population initially present in BSIC was able to grow under the culture conditions defined in this report.

The presence of free mucus-derived carbohydrates in the BSIC was investigated by gas chromatography (GC). BSIC samples contained 1.43 mM (± 0.028) galactose, 0.89 mM (± 0.016) GlcNAc, 0.72 mM (± 0.014) GalNAc, 0.64 mM (± 0.013) fucose, 0.50 mM (± 0.014) mannose and 0.09 mM (± 0.0018) Neu5Ac resulting in a total concentration of 4.2 mM (± 0.08) carbohydrates (BSIC samples also contained 0.35 mM glucose). In agreement with the glycoprotein composition of mucin previously purified from bovine small intestine by Montagne and colleagues (2000), galactose and GlcNAc were the most abundant (34.2 mol per cent and 19.3 mol per cent, respectively, of the total mucosal carbohydrates), whereas Neu5Ac was low (only 2.4 mol per cent of the total mucosal carbohydrates). These results strongly suggest that the six carbohydrates were released into the bovine luminal content from the mucosal surface covering the enterocytes.

Expression of genes required for carbohydrate catabolism

To investigate the ability of EDL933 to degrade the mucus-derived carbohydrates present in the BSIC, relative expression of the genes *galK*, *agaF*, *nagE*, *fucA*, *manA* and *nanA* encoding the specific catabolism of galactose, GalNAc, GlcNAc, fucose, mannose and Neu5Ac respectively (Table S1), was investigated by real-time PCR. RNA samples were collected during the exponential growth phase, when the bacteria entered into the stationary phase, and during the stationary phase (Fig. S1). The ratio of mRNA levels was calculated for EDL933 incubated in BSIC relative to cells grown in M9 minimal medium supplemented with glucose (M9-Glc) and normalized using the *tufA* gene.

Maximal expression was observed for all the genes except *manA* during the exponential growth of EDL933. Particularly, a high induction of *nanA* (≈ 370 -fold increase), *fucA* (≈ 130 -fold increase) and *agaF* (≈ 125 -fold increase) was observed (Fig. 2). In contrast, a maximal level of *manA* transcripts was found when EDL933 incubated in BSIC entered into the stationary phase, whereas the remaining genes appeared to be weakly activated or showed an expression ratio close to 1 (Fig. 2). During the stationary growth phase, all the genes were poorly expressed or were downregulated.

Utilization of mucus-derived carbohydrates by endogenous microbiota and EHEC

The utilization of mucus-derived carbohydrates by the endogenous microbiota and by EHEC was next analysed. BSIC-LEM samples [$\approx 7.5 \times 10^5$ anaerobes (strict and facultative) ml^{-1}] and BSIC-LEM samples inoculated with EDL933 ($\approx 5 \times 10^3$ bacteria ml^{-1}) were incubated at 39°C without shaking, and at each time point the mucus-derived carbohydrates were quantified. As shown in Fig. 3A, the intestinal endogenous microbiota was able to consume mucus-derived carbohydrates from the first hour of incubation. However, despite the differences in initial bacterial populations (endogenous microbiota vs EHEC), the concentration of total mucus-derived carbohydrates decreased more rapidly when BSIC-LEM samples were inoculated with EHEC ($P < 0.01$, BSIC samples inoculated with EDL933 vs BSIC samples from 1 to 3 h of incubation) (Fig. 3A). During the first 4 h of incubation, the disappearance of 25–45% of each carbohydrate was associated with the incubation of EDL933, suggesting a high efficiency of EHEC in fermenting mucus-derived carbohydrates. Determination of the concentration of each carbohydrate after 3 h of incubation of BSIC inoculated with or without EHEC confirmed that EDL933 contributed significantly to degrading mucus-derived carbohydrates (Fig. 3B). In addition, the monosaccharides were not consumed in a sequential order since the concentration of each sugar decreased simultaneously during the incubation of BSIC-LEM samples (results not shown) or BSIC-LEM samples inoculated with EHEC (Fig. 4). It is also of interest to note

that carbohydrates did not completely disappear from BSIC-LEM samples after 6 h of incubation [as 20–25% of the total mucus-derived carbohydrates initially present in BSIC-LEM were still present (Figs 3A and 4)]. This suggests that the concentration of carbohydrate quantified in BSIC samples constitutes the balance between the carbohydrates degraded by intestinal bacteria and the release of carbohydrate monomers from mucus-derived polysaccharides.

As previously described (Chaucheyras-Durand *et al.*, 2010), the EHEC strain appeared to be well adapted for growth on BSIC-LEM since the initial bacterial population ($\approx 5 \times 10^3$ bacteria ml^{-1}) reached $\approx 10^6$ and $\approx 5 \times 10^8$ bacteria ml^{-1} after 3 and 7 h of incubation respectively. Taken together, these results suggested that the rapid growth of EDL933 in BSIC-LEM could be due, at least partially, to its effectiveness in degrading mucus-derived monosaccharides.

Bacterial growth competition in BSIC-LEM

EDL933 was able to grow in undiluted BSIC-LEM samples without any supplementation [≈ 5 log increase in colony-forming units (cfu) ml^{-1} after 7 h of incubation]. Considering the effectiveness of EDL933 in degrading mucus-derived monosaccharides, we hypothesized that utilization of these carbohydrates gives it a growth advantage in BSIC. To test this hypothesis, we used the isogenic mutants of EDL933 described by Fabich and colleagues (2008) with mutations that directly impact the first steps of galactose, GlcNAc, fucose, mannose and Neu5Ac catabolism and we constructed the EDL933 Δ *agaF* mutant with a specific defect in GalNAc degradation. Growth competition assays are usually performed to compare the growth pattern of a wild-type strain and its isogenic mutant co-incubated in biological fluids or liquid growth medium (Farrell and Finkel, 2003; Palchevskiy and Finkel, 2006; Pradhan *et al.*, 2010; Bertin *et al.*, 2011). A mutant strain that does not compete efficiently for nutrients fails to reach the same population density as the parent strain, whereas similar growth curves indicate that both strains are able to use limiting nutrients equally well or do not compete for the same limiting nutrient.

EDL933 and each mutant strain were co-incubated in BSIC-LEM samples at the same concentration (5×10^3 cfu ml^{-1}), and at each time point the wild type and the mutant strains were enumerated. The competitive index (CI) was calculated as described in the experimental procedure section. As shown in Fig. 5, an important growth defect of *E. coli* strains impaired in the mannose, GlcNAc, Neu5Ac or galactose catabolic pathway was observed during co-incubation in BSIC-LEM samples. The CI values of EDL933 Δ *manA*, EDL933 Δ *nagE*, EDL933 Δ *nanAT* and EDL933 Δ *galK* versus EDL933 were 0.0018, 0.0028, 0.0035 and 0.016 respectively, at 7 h of co-incubation. These results clearly demonstrate that the catabolism of mannose, GlcNAc, Neu5Ac and galactose confers a competitive growth advantage to EHEC in BSIC-LEM. Mutation of the fucose catabolic pathway has a weaker but statistically significant impact on bacterial growth during co-incubation in BSIC-LEM (Fig. 5). The CI value of EDL933 Δ *fucAO* versus EDL933 was 0.0923 at 7 h of incubation. Interestingly, the wild-type strain significantly outcompeted Δ *manA* and Δ *galK* mutants from 2 and 3 h respectively, of co-incubation, whereas a significant growth defect was only observed after 4 h of co-incubation of EDL933 with mutant strains defective for GlcNAc, Neu5Ac or fucose catabolism (Fig. 5). In contrast, similar growth phenotype and CI value close to 1 were observed when EDL933 was co-incubated with the Δ *agaF* mutant, demonstrating that GalNAc was not required for the growth of EDL933 in BSIC-LEM samples.

The growth curves of Δ *manA*, Δ *nagE*, Δ *nanAT*, Δ *galK* and Δ *fucAO* mutants individually incubated in BSIC-LEM samples were closely similar to that of EDL933 (data not shown), indicating that (i) the growth defects observed during co-incubation did not result from an

inability of the mutants to grow in BSIC but from an inability to compete with the wild-type strain and (ii) in the absence of a strain competing for the same sugars, the EHEC mutant strains grow at the same rate as the wild-type EDL933.

In summary, growth competition assays suggest that the capacity of EDL933 to degrade mannose, GlcNAc, Neu5Ac and galactose is required for maximal growth of EHEC in the bovine small intestine.

Utilization of mucus-derived carbohydrates by commensal bovine *E. coli*

BLAST analysis from bacterial genome libraries showed that *galK*, *nagE*, *agaF*, *nanA*, *fucA* and *manA* are present in the genome of numerous pathogenic and commensal *E. coli* strains present in the 'microbes genomic' BLAST database. However, *agaF* is absent in the genome of the K12 *E. coli* MG1655 resulting in its inability to utilize GalNAc as a nutrient (Fabich *et al.*, 2008; Mukherjee *et al.*, 2008). The presence of *agaF* was investigated by PCR amplification among commensal *E. coli* strains from our laboratory collection. In contrast to *E. coli* MG1655, a specific *agaF* amplification product was obtained from all the bovine strains tested ($n = 30$) (data not shown), indicating the presence of the genetic information required for GalNAc utilization in the genome of bovine commensal *E. coli*.

In this report, we used the *E. coli* strain BG1 isolated from the small intestine of a cow at slaughter (Bertin *et al.*, 2001) to analyse the growth pattern of a commensal *E. coli*. Incubation of BG1 in M9 medium supplemented with carbohydrate demonstrated that the commensal *E. coli* was able to use each of the mucus-derived carbohydrates as the sole carbon source. The relative expression of genes required for the catabolism of mucus-derived carbohydrates was then quantified during growth of BG1 in filtered BSIC in comparison with growth in M9-Glc. The highest induction rates were observed for the *agaF* and *fucA* genes (≈ 25 -fold increase) during the exponential growth phase, whereas *nanA*, *galK*, *manA* and *nagE* were poorly induced or were repressed during the exponential or stationary phases (Fig. S2). More importantly, genes required for the catabolism of carbohydrates appeared to be less induced in BG1 than in EDL933 (Fig. 6). Indeed, the level of *nanA*, *fucA*, *agaF*, *galK* and *nagE* transcripts in BSIC was six- to 97-fold lower in BG1 than in EDL933 during the exponential growth phase, and the level of *manA* transcripts was $\approx$ sevenfold lower during the stationary growth phase (Fig. 6). The commensal *E. coli* strain BG1 was then tested for its ability to catabolize mucus-derived carbohydrates. As described above, BSIC samples were inoculated with BG1 (5×10^3 bacteria ml^{-1}) and disappearance of the carbohydrates was monitored during incubation at 39°C. The patterns of carbohydrate disappearance showed no significant difference between BSIC-LEM samples and BSIC-LEM samples inoculated with BG1 (Fig. 3A and B). In addition, the rate of carbohydrate disappearance in BSIC-LEM samples inoculated with EDL933 is greater than that of BSIC-LEM samples inoculated with BG1 (Fig. 3A and B) suggesting that EDL933 uses sugars at a more rapid rate than BG1.

Bacterial growth competition experiments including BG1 were also performed. Indistinguishable growth curves and CI value close to 1 were obtained when BG1 and EDL933 were co-incubated in BSIC (Fig. S3). In contrast, co-incubation of BG1 and EDL933 Δ *gal*, EDL933 Δ *fucAO*, EDL933 Δ *nagE*, EDL933 Δ *manA* or EDL933 Δ *nanAT* showed that the commensal *E. coli* significantly outcompeted each of the mutant strains (Fig. S3). These results indicated that EHEC needs to consume galactose, GlcNAc, mannose, Neu5Ac or fucose to maintain its growth rate at the same level as that of the commensal *E. coli* strain in the BSIC. Therefore, since in the absence of a strain competing for the same sugars, the EDL933 sugar mutants grow at the same rate as the wild-type EDL933 in BSIC-LEM (Fig. S3), BG1 and EDL933 are competing for the same sugars.

Discussion

Although *E. coli* strains possess the genetic information to synthesize sugars *de novo*, it is clearly in their interest to scavenge sugars from the surrounding environment. Carbohydrates are the largest component of the cow's diet (60–70%) and consist essentially in polysaccharides embedded in plant cell walls. Dietary plant cell wall polysaccharides are mainly cellulose, consisting in linear chains of glucose units (up to 40% of total plant biomass), and hemicelluloses that are composed of more complex heteropolymers including glucose, arabinose, xylose, mannose, galactose or fucose (Dashtban *et al.*, 2010; Schadel *et al.*, 2010) (but not GalNAc, GlcNAc or Neu5Ac). These polysaccharides are extensively degraded in the bovine rumen and the resulting monosaccharides are rapidly and almost completely fermented by the vast microbial population of the rumen (for a review, see Russell *et al.*, 2009; Wilson, 2011). A low proportion of undegraded plant cell wall polysaccharides can only transit through the small intestine and reaches the large intestine where their efficient breakdown occurs thanks to the presence of a diversified microbial fibrolytic activity (Michalet-Doreau *et al.*, 2002). Although a part of dietary starch can be degraded in the small intestine, glucose monomers are generally rapidly absorbed or directly utilized by the intestinal cell wall (Owens *et al.*, 1986) resulting in the presence of a very few or even no soluble carbohydrates of dietary origin in the ileum. Additionally, the six main monosaccharides reported to be included in the mucin glycoprotein fraction were found in the BSIC in similar proportions to those of the mucus layer (Montagne *et al.*, 2000). Taken together, these observations strongly suggest that the six monosaccharides are not provided by dietary polysaccharide degradation but are released from the mucus layer covering the enterocytes.

Bacterial growth patterns clearly showed that Neu5Ac and GlcNAc are the most efficient carbon sources for *in vitro* multiplication of EDL933 in minimal medium. Previous studies have shown that EHEC grown in minimal medium supplemented with a complex mixture of sugars consumes the mucus-derived carbohydrates in a precise order with cascading carbon source utilization and overlapping metabolism (Chang *et al.*, 2004; Fabich *et al.*, 2008). For example, the catabolite-repressing sugars GlcNAc and galactose are first consumed by EDL933, whereas fucose and GalNAc disappear from the media when all the other sugars are completely exhausted (Fabich *et al.*, 2008). In this report, we demonstrated that EDL933 simultaneously consumed the six mucus-derived sugars present in the BSIC, suggesting that the intestinal environment must affect the selection of carbohydrates by EHEC, perhaps owing to the presence of amino acids and other growth factors present in BSIC that were absent in the minimal medium used in the previous study.

Based on competitive growth experiments, we demonstrated that mannose, GlcNAc, Neu5Ac and galactose were the preferred mucus-derived carbohydrates used by EDL933 for its growth in BSIC-LEM. To a lesser extent, fucose also contributed to the growth of EHEC. In agreement with our results, *in vivo* colonization experiments in cattle have shown that specific transport of GlcNAc and utilization of fucose (but not GalNAc) are required for colonization of the bovine gut or the persistence of EHEC in the bovine rectum (Dziva *et al.*, 2004; Snider *et al.*, 2009). Competitive advantage due to mucus-derived carbohydrates in the gut of mammals is also observed for other pathogenic bacteria. For example, Neu5Ac and fucose catabolism confers a competitive advantage to pathogenic *Vibrio cholerae* and *Campylobacter jejuni* in the mouse intestine and in chick caecum respectively, whereas a Δgal mutant is dramatically impaired in EHEC colonization of the infant rabbit intestine (Ho and Waldor, 2007; Sheng *et al.*, 2008; Almagro-Moreno and Boyd, 2009; Muraoka and Zhang, 2011).

Interestingly, all the carbohydrates disappeared from BSIC-LEM samples during the first hour of EDL933 incubation, whereas each of the $\Delta nagE$, $\Delta nanAT$, $\Delta galK$ and $\Delta fucAO$ mutants showed a significant growth defect after 3–4 h of co-incubation. This suggested that during the first hours of co-incubation the wild-type strain probably consumed a part of GlcNAc, Neu5Ac, galactose or fucose initially present in BSIC-LEM, whereas the mutant strains defective for the catabolism of the corresponding carbohydrate could use other carbon sources for growth. We hypothesized that insufficient carbon sources were available in BSIC-LEM for optimal growth of the mutant strains after 3 or 4 h of co-incubation, whereas the wild-type strain could continue to degrade the mucus-derived carbohydrates constantly released by the resident anaerobes from mucus-derived polysaccharides.

In the bovine gut, EHEC is present at a low concentration as compared with the high density of the resident microbiota, and must compete for carbon sources to persist and grow. In our experiments, BSIC-LEM contained approximately 8×10^5 anaerobic bacteria ml^{-1} and was inoculated at a low population by EHEC ($\approx 5 \times 10^3$ bacteria ml^{-1}). In the bovine intestine, anaerobes of the endogenous microbiota have been adapted for the breakdown and fermentation of complex polysaccharides that escape hydrolysis in the rumen. In contrast to *E. coli*, which is limited to growth on mono- or disaccharides, the endogenous microbiota can also use oligosaccharides, and even in some cases preferentially uses oligosaccharides rather than the corresponding monomers (Amaretti *et al.*, 2006). Moreover, EDL933 grew more rapidly in BSIC-LEM than the resident strict and facultative anaerobic bacteria. Consequently, EDL933 can probably consume free monosaccharides more rapidly, suggesting a competitive ability of EHEC to catabolize mucus-derived carbohydrates. This is consistent with the nutrient-niche theory postulating that a microorganism can coexist in the mammal intestine by utilizing limiting nutrients better than the other bacterial species (Freter *et al.*, 1983). In addition to the resident microbiota, the commensal *E. coli* BG1 also consumes the mucus-derived sugars present in BSIC-LEM more slowly than EHEC. However, based on competitive growth experiments, we demonstrated that EHEC and commensal *E. coli* grow together at the same rate in BSIC-LEM and compete for mucus-derived sugars. Therefore, it appears that the commensal *E. coli* strain is more efficient than EHEC in using sugars for growth in BSIC-LEM, i.e. same rate of growth metabolizing fewer sugars. Interestingly, these results suggested that commensal strains may be better adapted to limiting nutrients in the intestine. This may help to explain why O157 infections run their course in a week or two, while commensals colonize their host for months.

The metabolism of EHEC during gut colonization of mice has been extensively explored (Miranda *et al.*, 2004; Fabich *et al.*, 2008; Leatham *et al.*, 2009). The strain EDL933 is present both in the mucus layer and closely associated with epithelial cells, but the bacterial strain is unable to grow *in vitro* on intestinal luminal contents, suggesting that EHEC colonizes the mouse intestine by growing in the mucus layer (Miranda *et al.*, 2004; Fabich *et al.*, 2008). Moreover, colonization of streptomycin-treated mice by EDL933 is supported by the catabolism of several carbohydrates including galactose, fucose, mannose and GlcNAc, but not Neu5Ac or GalNAc (Fabich *et al.*, 2008). In particular, inactivation of the galactose and fucose pathways has the largest impact on mouse colonization fitness (Fabich *et al.*, 2008). In contrast, we showed that EDL933 grows rapidly in BSIC-LEM and we found that mannose and GlcNAc catabolism conferred the greatest competitive growth advantage to EHEC in BSIC. Furthermore, we highlight the considerable growth advantage conferred by Neu5Ac to EHEC despite its low abundance. In fact, diet, digestive system and intestinal microbiota of ruminant and monogastric animals resulted in different status of nutrient limitation and, consequently, different strategies may be adopted by EHEC to persist in the gut according to the infected host.

Freter's nutrient-niche theory postulates that colonization of the intestine by a particular bacterium is defined by its ability to occupy nutrient-defined ecological niches that differ from the other species present (Freter *et al.*, 1983). According to this hypothesis, the population density of a particular bacterium is determined by the available concentration of its preferred nutrient, for which its affinity likely is the highest. Our data indicate a competitive ability of EHEC compared with the resident microbiota or commensal *E. coli* to catabolize mucus-derived carbohydrates (in particular mannose, GlcNAc, Neu5Ac and galactose). These carbon sources probably constitute an ecological niche for EHEC strains in the bovine small intestine. In-depth knowledge of the physiology of EHEC in the digestive tract of the ruminant will help to select nutritional or ecological strategies (for example probiotics) in order to reduce EHEC carriage prior to slaughter and to limit the dissemination of EHEC into the human food chain.

Experimental procedures

Bacterial strains and growth conditions

Escherichia coli strains used in this study are listed in Table S2. The spontaneous nalidixic acid- and rifampicin-resistant mutants EDL933 Nal^R and EDL933 Rif^R respectively showed growth curves similar to that of EDL933 when cultured in Luria–Bertani (LB) broth at 37°C. Similarly, the nalidixic acid-resistant mutant BG1 Nal^R showed growth curves similar to that of BG1. The capacity of EDL933 to use a specific mucus-derived carbohydrate as a sole carbon source was assessed by using M9 minimal medium (DIFCO) supplemented with glucose, galactose, GlcNAc, GalNAc, fucose, mannose or Neu5Ac (10 mM) (Sigma-Aldrich). Broth cultures were started from a single colony in LB medium and grown overnight at 37°C with aeration. Cells were then pelleted by centrifugation, resuspended in sterile PBS and diluted 75-fold in M9 minimal medium supplemented with the corresponding sugar. The cultures were then incubated at 37°C with shaking and the growth was monitored spectrophotometrically at an optical density of 600 nm (OD₆₀₀).

Preparation of bovine intestinal contents and microbial enumeration

BSIC with a live endogenous microbiota (LEM) were collected and stored as previously described (Bertin *et al.*, 2011). Briefly, three beef cattle were slaughtered in accordance with the guidelines of the local Ethics Committee in the experimental facility of Unit of Research on Herbivores, INRA, Saint-Genès Champanelle, France. The jejunum and the ileum were removed as a single piece and the total luminal contents were collected in O₂-free N₂ saturated sterile flasks. The BSIC-LEM samples were then pooled, rapidly filtered through four layers of cheesecloth and immediately frozen at –80°C until use.

To enumerate total facultative anaerobes, 10-fold serial dilutions of each sample were performed in the mineral solution described by Bryant and Burkey (1953). The bacterial dilutions were plated on Petri dishes containing G20 complex medium (Chassard *et al.*, 2008) and incubated at 39°C for 48 h. To enumerate the total viable counts of strictly anaerobic bacteria, 10-fold serial dilutions of BSIC samples were performed in mineral solution (Bryant and Burkey, 1953) under CO₂ flow. Each dilution was inoculated into O₂-free CO₂ saturated roll tubes (Hungate *et al.*, 1966) using the complete CC medium (Leedle and Hespell, 1980). Three roll tubes were inoculated per dilution and the colony counts were determined after 2–3 days at 39°C.

To obtain sterile BSIC, the intestinal contents were centrifuged twice at 2000 g for 20 min and the resulting supernatants were filtered through a 0.22 µm nylon filter. The efficiency of the filtration step was verified by adding BSIC samples on Petri dishes of G20 complex medium and in CC anaerobic broth. The absence of either colonies on agar plates or

bacterial growth in CC medium after 48–72 h at 39°C confirmed that BSIC was sterile. Resulting pooled aliquots were then stored at –80°C until use.

Mutant construction

The mutant strain defective for the gene *agaF* (required for a GalNAc-positive phenotype) (Mukherjee *et al.*, 2008) was constructed by the replacement of *agaF* by the gene conferring resistance to kanamycin using a one-step PCR-based method (Datsenko and Wanner, 2000). The primers used to construct the EDL933Δ*agaF* mutant (*agaF*_Km_F: GCAAAT TCTCGGTGAGCAATCGCAGTTTATCGCCATCGATTTTCC GGAAACAGCCACGTTGTGTCTCAAAATC and *agaF*_Km_R: TGCTCGATGAGTTTTTCTAATAACTCGTCCACCAGACT GGTGAGCCACGATGTCCACACTCCAGCGCCTG) were designed according to the EDL933 genome sequence. Gene knockouts were confirmed by PCR analysis and DNA sequencing. The phenotype of EDL933Δ*agaF* was controlled by incubating the mutant strain in M9 minimal medium supplemented with GalNAc (10 mM) or glucose (10 mM) and monitoring bacterial growth spectrophotometrically (OD₆₀₀).

Complementation of the mutant strains

To generate the complemented mutant strains, PCR amplifications were performed using the high-fidelity Pfx50™ DNA polymerase (Invitrogen). The primers used consist of the first or last nucleotides of the corresponding ORF and of the restriction sequence of the enzymes HindIII, EcoRI or NcoI (Table S3). DNA sequences corresponding to the *agaF*, *fucAO*, *galK*, *manA*, *nagE*, and *nanAT* genes were designed according to the EDL933 genome sequence (Z4488, Z4117 and Z416, Z0927, Z2616, Z0826, Z4583 and Z4582 respectively). The PCR products were purified using the Qiaquick Purification PCR kit (Qiagen) and digested with the relevant enzymes. The PCR fragments were then ligated into the expression vector pBAD24 (conferring resistance to ampicillin, under the control of the *araC*-PBAD promoter) using T4 DNA ligase (Invitrogen) as previously described (Guzman *et al.*, 1995). Each of the resulting recombinant plasmids was then electroporated into the corresponding mutant and selected on an LB agar plate containing ampicillin (50 µg ml⁻¹) and the relevant antibiotic [kanamycin or chloramphenicol (50 µg ml⁻¹ each)]. Gene complementation was checked by PCR analysis and DNA sequencing. In addition, the pBAD24 vector was electroporated into the wild-type strain EDL933 to create a vectoronly control strain. To check the restoration of the phenotype, the resulting strains were inoculated in M9 minimal medium supplemented with ampicillin (50 µg ml⁻¹), L-arabinose (0.5 mM) and the corresponding carbohydrates (10 mM). The bacterial suspensions were then incubated at 37°C with shaking and growth was monitored spectrophotometrically (OD₆₀₀).

RNA extraction and relative mRNA quantification by real-time PCR (q-PCR)

The strain EDL933 was incubated at 39°C without aeration in filtered BSIC samples and in M9 minimal media supplemented with glucose (10 mM) as the sole carbon source (M9-Glc). Bacterial suspensions were collected during the exponential growth phase (2.5 h and 5 h in filtered BSIC and M9-Glc respectively), when the bacteria entered into the stationary phase (4.25 h and 7.25 h in filtered BSIC and M9-Glc respectively), or during the stationary phase (6.5 h and 9.5 h in filtered BSIC and M9-Glc respectively) (Fig. S1). The bacterial suspensions were then centrifuged at 10 000 g for 15 min and the supernatants were stored at –20°C for further investigations. Total RNAs were isolated from bacterial pellets by using TRIzol as previously described (Vareille *et al.*, 2007; de Sablet *et al.*, 2008). RNA quantification was performed using a NanoDrop spectrophotometer and RNA integrity was electrophoretically verified by ethidium bromide staining. One microgram of each RNA sample was then reverse transcribed using the SuperScript II Reverse Transcriptase kit

(Invitrogen) with 3 µg of random primer and 100 units of SuperScript II Rnase H. Real-time PCR runs were carried out using the Mastercycler ep realplex apparatus (Eppendorf) with 20 ng of cDNA, 0.5 µM of each primer, 3 mM of MgCl₂, 10 µl of SYBR[®] Premix Ex Taq[™] mix (Takara Bio) in a final volume of 20 µl. Amplification conditions were as follows: 95°C for 15 s, 55°C for 15 s and 72°C for 20 s. The *tufA* mRNA was used for normalization of mRNA quantification. The relative mRNA quantification was performed using primers designed to specifically amplify fragments of 90–200 bp (Table S4). Control samples lacking the reverse transcriptase were included to assess DNA contamination and triplicate samples were amplified in each case. Results were calculated using the comparative cycle threshold method.

Bacterial competition experiments

The spontaneous mutant strains EDL933 Nal^R and BG1 Nal^R were used in competition experiments between wild-type strains and isogenic mutants unable to degrade mucus-derived carbohydrates. Competition experiments between pathogenic and commensal *E. coli* were performed using the EDL933 Rif^R and BG1 Nal^R spontaneous mutants respectively. Precultures of the *E. coli* strains, inoculated from a single colony, were incubated in LB broth with appropriate antibiotic for 8 h at 37°C with aeration. The precultures were then 50-fold diluted in LB broth and grown overnight at 39°C without shaking. The next day, a sample of BSIC-LEM was inoculated with approximately 5×10^3 bacteria ml⁻¹ of each of the two strains tested and incubated at 39°C without shaking. At each time point, the co-culture was 10-fold serially diluted in phosphate buffer (PBS) pH 7.2 and plated on Sorbitol MacConkey (SMAC) agar plates containing kanamycin (50 µg ml⁻¹), nalidixic acid (50 µg ml⁻¹), rifampicin (100 µg ml⁻¹) or chloramphenicol (50 µg ml⁻¹). The plates were then incubated overnight at 37°C and the number of cfu was determined. Each experiment was replicated at least three times. The presented values are the log₁₀ mean number of cfu ml⁻¹ ± the standard error. Statistical analysis was done using Student's *t*-test for paired samples (two-tailed). The CI was calculated as follows: (mutant cfu recovered/wild-type cfu recovered)/(mutant cfu inoculated/wild-type cfu inoculated). CI < 1 indicated that the wild-type strain outcompeted the mutant; CI > 1 indicated that the mutant outcompeted the wild-type strain; CI ≈ 1 indicated that none of the two strains has a competitive advantage with regard to the other one.

Monosaccharide analysis

The molar composition of free mucus-derived carbohydrates was determined by GC on a Shimadzu gas chromatograph equipped with a 25 m × 0.32 mm CPSil5 CB Low bleed/MS capillary column, 0.25 µm film phase (Chrompack France, Les Ulis, France), as previously described (Kamerling *et al.*, 1975; Montreuil *et al.*, 1986). Briefly, 5 mg of mesoinositol (internal standard) was added to 250 µl of each BSIC sample. Freeze-dried samples were then submitted to methanolysis for 24 h at 80°C in 500 µl of 0.5 M HCl-methanol. The acidic solution was neutralized by adding silver carbonate and re-N-acetylated overnight at room temperature by adding 20 µl of acetic anhydride. The methanolic phase was washed twice with 200 µl of heptane and dried under a stream of nitrogen. Monosaccharides were further trimethylsilylated by adding 50 µl of BSTFA (bis-silyltrifluoroacetamide) and 50 µl of pyridine. After 2 h, 0.5 µl of the solution was applied to GC. Standard carbohydrates (1 mg ml⁻¹) (Sigma-Aldrich), co-injected with internal standard (mesoinositol), are used in an independent GC experimentation to determine their retention time as well as their relative response factor. Co-injection of internal standard at a known concentration with BSIC sample allows quantifying the relative molar ratio for each monosaccharide. Statistical analysis was done using the Student's *t*-test for independent samples.

In silico and statistical analyses

In silico analyses were performed using the xBASE (<http://xbase.ac.uk>) and BLAST (<http://www.ncbi.nlm.nih.gov>) servers, and the CAZy (Carbohydrates Active enZYme) (<http://www.cazy.org>) database. The nucleotide sequences were compared with bacterial complete genomes present in the 'Microbes genomic' BLAST database at NCBI and with DNA sequences from gut metagenomes (http://www.ncbi.nlm.nih.gov/sutils/blast_table.cgi?taxid=Environmental&taxidinf=enviro_info&selectall).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Growth curves of EDL933 incubated in minimal media. M9 minimal medium was supplemented with 10 mM of each carbon source. Cultures were incubated at 37°C with aeration. Each time point is the mean of three independent experiments.

Fig. 2.

Relative expression levels of genes required for the catabolism of mucus-derived carbohydrates during incubation of EDL933 in BSIC compared with M9-Glc. The ratio of mRNA level of each gene was measured in EDL933 incubated in filtered BSIC in comparison to cells grown in M9-Glc. RNA samples were collected during the exponential growth phase (grey), when the bacteria entered into the stationary phase (white) and during the stationary phase (black). Values are the mean $\pm$ 1 SEM of three independent experiments.

Fig. 3.

Disappearance rate of mucus-derived carbohydrates.

A. Concentration of total mucus-derived carbohydrates was monitored during incubation of BSIC-LEM samples (○) and BSIC-LEM samples inoculated with the EHEC strain EDL933 (●) or with the commensal *E. coli* strain BG1 (□). The concentration of each carbohydrate was quantified individually.

B. Concentration of each carbohydrate was quantified in bacterial supernatants of BSIC-LEM samples (white) and BSIC-LEM samples inoculated with BG1 (black) or EDL933 (grey) after 3 h of incubation. Bars represent the SEM of three independent experiments. *** $P < 0.01$ and ** $P < 0.05$ vs BSIC-LEM samples as determined by the Student *t*-test for independent samples.

Fig. 4. Concentration of each carbohydrate during incubation of EDL933 in BSIC-LEM samples. Bars represent the SEM of three independent experiments.

Fig. 5. Growth competition assays between EDL933 and its isogenic mutants. The BSIC-LEM samples were inoculated with a 1:1 mixture of the two strains. The mutant strains tested were defective for the pathway required for the catabolism of GalNAc (EDL933 Δ agaF), fucose (EDL933 Δ fucAO), galactose (EDL933 Δ galK), GlcNAc (EDL933 Δ nagE), mannose (EDL933 Δ manA) or Neu5Ac (EDL933 Δ nanAT). Bars represent the SEM of three independent experiments. The double asterisk denotes statistical significance, $P < 0.05$ and the triple asterisk denotes statistical significance, $P < 0.01$ as determined by the Student t -test for paired samples.

Fig. 6.

Fold-change comparison of carbohydrate catabolism gene expression between the EHEC strain EDL933 and the commensal *E. coli* strain BG1. The ratio of mRNA level of each gene was measured in the *E. coli* strains incubated in filtered BSIC. RNA samples were collected during the exponential growth phase (grey), when the bacteria entered into the stationary phase (white) and during the stationary phase (black). Values are the mean $\pm$ 1 SEM of three independent experiments.