Enterohaemorrhagic *Escherichia coli* gains a competitive advantage by using ethanolamine as a nitrogen source in the bovine intestinal content

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Summary

The bovine gastrointestinal tract is the main reservoir for enterohaemorrhagic *Escherichia coli* (EHEC) responsible for food-borne infections. Characterization of nutrients that promote the carriage of these pathogens by the ruminant would help to develop ecological strategies to reduce their survival in the bovine gastrointestinal tract. In this study, we show for the first time that free ethanolamine (EA) constitutes a nitrogen source for the O157:H7 EHEC strain EDL933 in the bovine intestinal content because of induction of the *eut* (ethanolamine utilization) gene cluster. In contrast, the *eut* gene cluster is absent in the genome of most species constituting the mammalian gut microbiota. Furthermore, the *eutB* gene (encoding a subunit of the enzyme that catalyses the release of ammonia from EA) is poorly expressed in non-pathogenic *E. coli*. Accordingly, EA is consumed by EHEC but is poorly metabolized by endogenous microbiota of the bovine small intestine, including commensal *E. coli*. Interestingly, the capacity to utilize EA as a nitrogen source confers a growth advantage to *E. coli* O157:H7 when the bacteria enter the stationary growth phase. These data demonstrate that EHEC strains take advantage of a nitrogen source that is not consumed by the resident microbiota, and suggest that EA represents an ecological niche favouring EHEC persistence in the bovine intestine.

Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) are Shiga toxin-producing *E. coli* that cause human illnesses ranging from uncomplicated diarrhoea to haemorrhagic colitis and haemolytic-uremic syndrome (Law, 2000). *E. coli* O157:H7 is the most prevalent serotype associated with large outbreaks. Epidemiological investigations reveal that cattle and other ruminants are the principal reservoir of EHEC strains (for reviews see Caprioli et al., 2005; Naylor et al., 2005a,b; Muniesa et al., 2006). Outbreaks have been associated with direct contact with the farm environment, and with the consumption of meat, raw milk and dairy products, water, and fruits or vegetables contaminated with ruminant manure (Cieslak et al., 1993; O’Brien et al., 2001; Yatsuyanagi et al., 2002). Using a simulation model, Jordan and colleagues have predicted that a reduction in the carriage of EHEC in ruminants will lead to a decrease of carcass contamination at slaughter (Jordan et al., 1999). Understanding of EHEC ecology and physiology in the ruminant gastrointestinal tract (GIT) is critical for limiting EHEC shedding and consequently the incidence of human EHEC infections.

In the rumen, growth of *E. coli* O157:H7 is limited or even suppressed because of the presence of the resident microbiota (de Vaux et al., 2002; Chaucheyras-Durand et al., 2006; 2010). However, O157:H7 EHEC strains may survive to their transit through the acid barrier of the abomasum, and the intestinal luminal content constitutes a more favourable environment than rumen fluid for bacterial growth (Diez-Gonzalez et al., 1998; de Vaux et al., 2002; Chaucheyras-Durand et al., 2006; 2010). Little is known about the nutrients preferentially used by EHEC for growth in the bovine intestine or the metabolic pathways required in this digestive compartment. It is important to
determine the mechanisms underlying Shiga toxin-producing E. coli persistence in the bovine intestine to be able to develop nutritional or ecological strategies to reduce EHEC survival in the animal GIT and thus limit the risk for food contamination.

The intestinal content is a complex ecosystem with a high diversity of bacterial species. Proliferation of EHEC strains in the intestine of ruminants may be mediated through competition with the resident microbiota for solvvariety of carbon, nitrogen and energy sources. According to Freter’s nutrient niche theory, bacteria present in the mammalian intestine can coexist as long as each member of the microbiota is able to utilize one or a few limiting nutrients (ingested foods, epithelial and bacterial cell debris) better than the others (Freter et al., 1983a).

The constant renewal of the intestinal epithelium essential for maintaining tissue homeostasis results in the daily release of 25% of enterocytes into the gut (Snoeck et al., 2005). This high epithelial turnover results in a large release of nutrients into the luminal content. Phosphatidylethanolamine (PE) is the most abundant phospholipid in microbial cell membranes (80% of the total membrane phospholipids of E. coli) and the most abundant after phosphatidylcholine in animal and plant cell membranes (Dowhan, 1997a,b; Bakovic et al., 2007). Interestingly, O157:H7 E. coli strains specifically bind to PE but are unable to bind to phosphatidylserine or phosphatidylcholine, the two other major phospholipids present in the leaflet of host epithelial cell membranes (Barnett Foster et al., 1999; 2000). This observation suggests a specific binding of EHEC strains to ethanolamine (EA) included in the hydrophilic head of PE. Therefore, Forster and colleagues suggested that specific recognition of PE may help EHEC to colonize the ruminant GIT (Barnett Foster et al., 2000).

Korbel and colleagues have performed genome analysis and literature mining in order to predict genomic determinants and food poisoning associations (Korbel et al., 2005). They predict that the EA degradation pathway is an important genomic determinant of pathogenicity associated with foodborne pathogens involved in gastrointestinal infections such as Listeria monocytogenes, Clostridium perfringens and Salmonella enterica (Korbel et al., 2005). It is well documented that EA can be used by S. enterica grown in minimal laboratory media as sole source of carbon, nitrogen and energy (Stojilkovic et al., 1995; Brinsmade and Escalante-Semerena, 2004; Penrod et al., 2004; Sheppard et al., 2004; Starai et al., 2005; Penrod and Roth, 2006). The genetic information required for the degradation and utilization of EA by S. enterica is encoded by 17 genes included in the eut (ethanolamine utilization) operon (Kofoid et al., 1999). Two enzymatic reactions convert EA into acetyl-CoA (Ac-CoA) (Fig. 1). First, ethanolamine ammonia-lyase (EAL) converts EA to acetaldehyde and free ammonia in the presence of cobalamin. Second, acetaldehyde is converted to Ac-CoA by acetaldehyde dehydrogenase (EutE). Ac-CoA can then enter the tricarboxylic cycle (TCA) providing carbon and energy sources. Under conditions of energy limitation, Ac-CoA is converted to acetyl-phosphate by the EutD phosphotransacetylase (and ultimately to acetate by the acetate kinase Ack). In the case of Ac-CoA accumulation, the EutG alcohol dehydrogenase reduces acetaldehyde to ethanol. EutH is a membrane protein needed to transport EA at low pH or at low external concentration of EA. In addition, EutS, EutM, EutN, EutL and EutK are shell proteins constituting a carboxysome, an organelle required to conserve volatile metabolites (acetaldehyde) and to concentrate the EA catabolic enzymes (Stojilkovic et al., 1995; Brinsmade and Escalante-Semerena, 2004; Penrod et al., 2004; Sheppard et al., 2004; Starai et al., 2005; Penrod and Roth, 2006).

Initial biochemical studies of EA degradation by E. coli strains have been described (Blackwell et al., 1976; Scarlett and Turner, 1976; Jones and Turner, 1984), but little genetic analysis is available. Furthermore, the capacity of E. coli or S. enterica strains to utilize EA in biological fluids remains unknown. In the present study, we demonstrate that: (i) free EA is present in the bovine intestine and

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**Fig. 1.** Model for ethanolamine catabolism. Proposed role for the Eut proteins involved in EA degradation. This scheme is consistent with the studies of EA catabolism by S. enterica (Stojilkovic et al., 1995; Brinsmade and Escalante-Semerena, 2004; Penrod et al., 2004; Sheppard et al., 2004; Starai et al., 2005; Penrod and Roth, 2006). The carboxysome is not shown to scale.
provides a nutrient source for *E. coli* O157:H7 and (ii) utilization of EA as a nitrogen source represents an ecological niche that confers a competitive advantage to *E. coli* O157:H7 strain and probably helps it to persist and develop in the bovine intestinal content.

Results

*E. coli* O157:H7 is able to grow in vitro in bovine intestinal content

The bovine intestinal content (BIC) is a biological fluid composed of a part of mucus (glycoproteins, proteins, glycolipids, lipids and sugars) and luminal content (nutrients from ingested food, host and bacterial cell debris). In this study, we compared the growth of the EHEC O157:H7 strain EDL933 incubated in sterile BIC (sBIC) and in M9 minimal media with glucose as a carbon source (M9-gluc). To mimic the physiological conditions of the GIT, EDL933 was cultured at 39°C (bovine temperature) without aeration (to minimize oxygen availability). The results showed that EDL933 is able to grow in vitro in bovine intestinal fluid without any supplementation of nutrients, and started more rapidly in sBIC than in M9-gluc (Fig. S1).

The eut gene cluster of EDL933 is upregulated in sBIC

In order to determine whether *E. coli* O157:H7 possesses the genetic information required for EA utilization, the genomic sequences of *S. enterica* (LT2 strain) and EDL933 were compared. A high identity was found between the eut gene cluster of LT2 (locus AF093749) (Kofoid *et al.*, 1999) and a 14 769 bp DNA fragment present in the chromosome of the EDL933. This genomic fragment comprised 17 open reading frames (ORFs; accession numbers: Z3702 to Z3718). The identical organization of the eut gene cluster of *S. enterica* and EDL933, with 80–98% of nucleotide identity and 84–99% of amino acid similarity strongly suggests an identical function for the encoded proteins.

Expression of the eut gene cluster was investigated by q-PCR and the ratio of mRNA levels was calculated for EDL933 incubated in sBIC relative to cells grown in M9-gluc. In the early stationary phase of growth (4.5 h and 7.5 h in sBIC and M9-gluc, respectively (Fig. S1)), 16 genes were found to be upregulated in sBIC (Fig. 2). Particularly, a very high induction of the eutS, eutM and eutN genes was observed (320-, 225- and 190-fold increase respectively). Overexpression of the eut gene cluster strongly suggested the presence of cobalamin and EA in the bovine intestinal fluid and its possible utilization by EDL933.

EDL933 can use EA as a nitrogen source but not as a carbon source

The capacity of EHEC to use EA as a nutrient source was next investigated by incubating EDL933 on a modified M9 minimal medium supplemented with EA as the sole carbon or nitrogen source. EDL933 failed to utilize EA as a carbon source but was able to use EA as the sole nitrogen source, although a long lag period (approximately 24 h) was observed (Fig. S2), suggesting a delayed physiological adaptation process.

To investigate whether EA utilization as a nitrogen source by EDL933 requires the eut gene cluster, we constructed isogenic mutants by replacement of each eut gene with the gene conferring resistance to kanamycin. Five mutants defective for EutH (active transport of EA across the bacterial membrane), EutB (subunit of EAL), EutE (acetaldehyde dehydrogenase), EutG (alcohol dehydrogenase) or EutD (phosphotransacetylase) were constructed. The EDL933::eutB::kanR isogenic mutant was unable to grow in M9 minimal medium with EA as the sole nitrogen source (Fig. S2), while complementation in...
trans with the eutB gene [ΔeutB(pEutB) strain] restored a bacterial growth (Fig. S2). These results clearly demonstrated that a functional EAL including EutB was required for the release of ammonia from EA and its utilization by the bacteria as a nitrogen source. In contrast, the mutant strains defective for EutE, EutG, EutH and EutD, respectively, showed growth phenotypes similar to those of the wild-type strain (Fig. S3), confirming that acetaldehyde dehydrogenase, alcohol dehydrogenase and phosphotransacetylase are not involved in the utilization of EA as a nitrogen source by EDL933.

The fact that the wild-type strain and the mutant EDL933ΔeutH::kanR showed similar growth patterns (Fig. S3) suggests that EA can diffuse passively across the bacterial membrane and that an active transport of EA was not required under the growth conditions used in the experiments. In a previous study, Penrod and colleagues showed that EA is found under two forms (unprotonated [EA0] and charged [EA+] depending on the external pH and EA concentration (Penrod et al., 2004). The charged form does not enter cells while the uncharged form diffuses freely across the membrane (Penrod et al., 2004). Accordingly, the EA concentration in the M9 minimal medium used in this study (30 mM) seemed to provide enough EA0 for an efflux rate that supported cell growth with or without EutH.

**EA present in BIC is catabolized by EDL933**

The presence of EA in the bovine intestinal fluid and its consumption by EDL933 were investigated. To this end, we developed an LC-MS/MS method to quantify EA in sBIC and in the culture supernatant of bacterial strains incubated in sBIC. A concentration of 2.18 mM of free EA was detected in sBIC before incubation, and a decrease in EA concentration was observed after incubation of the EHEC strain EDL933 (Fig. 3). EA concentration also decreased after growth of the ΔeutD::kanR, ΔeutE::kanR and ΔeutG::kanR mutants (Fig. 3). In contrast, only a slight decrease in EA concentration was detected after growth of the ΔeutB::kanR and ΔeutH::kanR mutants, while complementation in trans with the eutB and eutH gene [ΔeutB(pEutB) and ΔeutH(pEutH) strains], respectively, restored a decrease of the EA concentration (Fig. 3). Taken together, these results indicate that EA present in sBIC was consumed by EHEC and was probably only used as a nitrogen source. Indeed, EutE, EutD and EutG are required to utilize EA as a carbon source but not as a nitrogen source (Fig. 1). Only EutB involved in the release of ammonia from EA and EutH involved in active transport of unprotonated EA (EA0) across the bacterial membrane are required to utilize EA as a nitrogen source. The concentration of EA0 in sBIC was calculated using the Henderson–Hasselbach equation (the pH of sBIC was 7.3 and the pK for EA protonation is 9.5). The EA0 concentration in sBIC (13.8 μM) was in the range over which the EutH transporter of S. enterica enhances the ability to utilize EA (between 3 and 25 μM) (Penrod et al., 2004). The presence of eutH in the eut gene cluster of enterobacteriaceae suggests that the bacteria probably encounter EA at low concentrations in biological fluids.

A kinetic analysis of EDL933 growth in sBIC showed that maximal eutB expression occurred between 3 and 5 h of incubation and correlated with the consumption of EA by EDL933 (Fig. 4). These results further confirm the involvement of eutB in the utilization of EA by the bacteria and suggest that eut gene expression was not only induced by the presence of EA but could also be regulated by the growth phase or by depletion of nutrient sources.

**The eut operon is absent from the genome of bacteria constituting the normal bovine digestive microbiota**

We next evaluated the spreading of the eut genes among bacterial genomes. Comparison of DNA sequences was performed among nucleotide sequences from bovine gut metagenomes and among the 592 completed bacterial genomes available on databases. The eut gene sequences were absent from bovine metagenome libraries. In contrast, the complete eut gene cluster (14 769 bp) was found in the genomes of all the E. coli strains present in the ‘microbes genomic’ BLAST database. These E. coli strains were isolated from humans or animals and are associated with enteric infections (EHEC, enteropatho-
genetic *E. coli*, enterotoxigenic *E. coli*, enteroaggregative *E. coli* or extraintestinal infections (uropathogenic *E. coli*). The *eut* gene cluster was also found in the genome of the human *E. coli* commensal intestinal strain HS and the non-pathogenic K12-derivative *E. coli* strain MG1655. All the *eut* *E. coli* sequences are closely related to the *eut* gene cluster of EDL933 (96–97% identity) and show a similar genetic organization. However, a 6782 bp DNA fragment (including integrases and cryptic prophages encoding genes) is inserted between the *eutA* and *eutB* genes of the K12-derivative *E. coli* MG1655 genome. Among the remaining *Enterobacteriaceae*, the *eut* gene cluster of EDL933 was also closely related to the *eut* sequences of *Shigella* (*S. sonnei*, *S. dysenteria*, *S. boydii*) (96–97% identity), *S. enterica* (85% identity), *Citrobacter koseri* (84% identity) and *Klebsiella pneumoniae* (82% identity), but was not present in the genome of *Yersinia pestis*.

No similarity was found at the nucleotide level, when the entire *eut* gene cluster was compared with the genomic sequences of bacteria included in the three major phyla (*Bacteroidetes*, *Firmicutes* and *Actinobacteria*) constituting 99% of the normal bovine digestive microbiota. However, a distant relationship could be detected in translated sequences from the genome of *Enterococcus faecalis*, *L. monocytogenes* and *Clostridium* spp., all included in the *Firmicutes* phylum and involved in food-borne gastrointestinal infections or nosocomial diseases. For example, identity scores of 37–63% were found for *EutB*, *EutC*, *EutH* and *EutL* when the amino acid sequences of EDL933 and the *E. faecalis* V583 strain were compared. The absence of *eut* sequences in the genome of bacteria constituting the normal bovine microbiota, except in commensal *E. coli*, suggests that EA cannot be used as a nutrient or is used with less efficiency by microorganisms naturally present in the bovine gut.

**EA is poorly consumed by the endogenous microbiota of the bovine gut**

To analyse the capacity of the normal bovine microbiota to catabolize EA, BIC samples containing live endogenous microbiota (BIC-LEM) were collected from three cows at slaughter. Bacterial counts from the three BIC-LEM samples freshly collected revealed the presence of $4.5 \times 10^5$ to $7 \times 10^6$ ml$^{-1}$ of strict anaerobes and $3 \times 10^5$ to $2 \times 10^6$ ml$^{-1}$ of facultative anaerobes. After 9 h of incubation at 39°C without aeration, the bacterial population reached $8 \times 10^7$ to $3 \times 10^8$ ml$^{-1}$ and $7.5 \times 10^7$ to $1 \times 10^8$ ml$^{-1}$ of strict and facultative anaerobes, respectively, demonstrating that the endogenous bacterial population present in BIC was able to grow under our culture conditions. EA present in BIC-LEM samples was poorly consumed by the endogenous microbiota as a low decrease in EA concentration was observed after 9 h of incubation (2.18 and 1.78 mM before and after incubation respectively).

**Utilization of EA confers a competitive advantage to EHEC strains**

Because EA present in BIC was poorly catabolized by the endogenous microbiota, its consumption could constitute a nutritional advantage for EHEC. To test this hypothesis, competitive assays were performed between EHEC strains and their isogenic *eut* mutants incubated in BIC-LEM samples. The bacterial enumeration was performed with and without kanamycin as described in the *Experimental procedure* section. A competitive index (CI) was calculated as described in Table 1.

EDL933 was first co-incubated with the EDL933*eutB*::kanR or the EDL933*eutH*::kanR mutant. Identical growth phenotypes were observed during the first 5 h of co-incubation (Fig. 5), indicating that similar nutrients present in BIC were used with equal efficiencies or that the two strains were not competing for similar limiting nutrients. However, from 6 h of co-incubation (corresponding to the entry of bacteria in the stationary growth phase) the CI values were below 1 (Table 1), indicating that EDL933 outcompeted the *eutB*::kanR and *eutH*::kanR mutants. Statistical analysis confirmed the growth defect of each mutant from 7 to 9 h of co-incubation with the wild-type strain (Fig. 5). To broaden these results, two additional O157:H7 EHEC strains, Sakai and 86-24, and their respective *eutB*::kanR isogenic mutants, were included in the competition experiments (Table S1). Similarly to EDL933, growth patterns and CI values indicated that from 7 h of...
incubation these two EHEC strains outcompeted their respective mutants defective in their capacity to catabolize EA (Fig. S4 and Table 1). In addition, a large decrease in EA concentration was observed when Sakai and 86-24 were incubated in sBIC samples (Fig. 6A).

EA is not consumed by commensal E. coli strains

Three commensal E. coli strains of our laboratory collection isolated from the ovine (4C) or bovine (BG1) digestive tract and from the faeces of a healthy human (5A) were tested for their capacity to use EA as a nutrient. The eutB gene was detected by PCR amplification in the genome of the three isolates. In addition, all the

The competitive index (CI) was calculated as follows:

A. (eut mutant CFU recovered/wild-type CFU recovered)/(eut mutant CFU inoculated/wild-type CFU inoculated); CI < 1 indicated that the wild-type strain outcompeted the mutant.

B. (EHEC CFU recovered/E. coli commensal CFU recovered)/(EHEC CFU inoculated/E. coli commensal CFU inoculated). CI = 1 indicated that none of the two strains have a competitive advantage over the other.

C. (EHEC ΔeutB::kanR CFU recovered/E. coli commensal CFU recovered)/(EHEC ΔeutB::kanR CFU inoculated/E. coli commensal inoculated); CI < 1 indicated that the E. coli commensal outcompeted the EHEC ΔeutB::kanR.

The CI values calculated at each of the four first hours were not included in Table 1. CI values < 0.80 are in bold.

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Table 1. Competitive index.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Incubation time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>A</td>
<td></td>
</tr>
<tr>
<td>EDL933 / EDL933ΔeutB::kanR</td>
<td>0.85</td>
</tr>
<tr>
<td>Sakai / SakaiΔeutB::kanR</td>
<td>1.14</td>
</tr>
<tr>
<td>86-24 / 86-24ΔeutB::kanR</td>
<td>0.86</td>
</tr>
<tr>
<td>EDL933 / EDL933ΔeutH::kanR</td>
<td>0.89</td>
</tr>
<tr>
<td>BG1 / BG1ΔeutB::kanR</td>
<td>1.11</td>
</tr>
<tr>
<td>4C / 4CΔeutB::kanR</td>
<td>1.16</td>
</tr>
<tr>
<td>5A / 5AΔeutB::kanR</td>
<td>0.92</td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>EDL933 / BG1</td>
<td>0.87</td>
</tr>
<tr>
<td>Sakai / BG1</td>
<td>0.91</td>
</tr>
<tr>
<td>86-24 / BG1</td>
<td>0.99</td>
</tr>
<tr>
<td>C</td>
<td></td>
</tr>
<tr>
<td>BG1 / EDL933ΔeutB::kanR</td>
<td>0.84</td>
</tr>
<tr>
<td>BG1 / SakaiΔeutB::kanR</td>
<td>0.83</td>
</tr>
<tr>
<td>BG1 / 86-24ΔeutB::kanR</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Fig. 6. Quantification of EA and eutB mRNA after incubation of EHEC and commensal E. coli strains in sBIC. A. EA quantification. The bacterial strains were incubated in BIC-LEM for 7 h. B. Absolute eutB quantification was performed with 4 h of incubation of the E. coli strains in sBIC. Values are the mean ± 1 SEM of three independent experiments.

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commensal E. coli strains were able to grow in M9 medium with EA as the sole nitrogen source (data not shown), demonstrating the presence of functional Eut proteins. However, levels of eutB mRNA were ~25-fold lower in the commensal isolates than in the EHEC strains (Fig. 6B), suggesting that pathogenic O157:H7 EHEC strains degrade EA more efficiently than commensal E. coli. Accordingly, a slight decrease in EA concentration was observed after growth of the three commensal E. coli in BIC-LEM (Fig. 6A), indicating that EA present in the bovine intestine was poorly consumed by commensal E. coli.

In addition, mutants defective for EutB were constructed from BG1, 4C and 5A commensal E. coli and competition assays were performed in BIC-LEM samples. Each of the wild-type strains co-incubated with its respective ΔeutB::kanR mutant showed indistinguishable growth curves (Fig. S5) and CI values close to 1 (Table 1). These results confirm that EA present in BIC was not used as nutrient by commensal E. coli strains.

EA catabolism is required to maintain the growth rate of EHEC at the same level as that of commensal E. coli in BIC

In competition assays between EHEC and commensal E. coli, indistinguishable growth curves and CI values close to 1 were obtained when EDL933 and BG1 NaI® were co-incubated (Fig. 7A and Table 1). These data suggest that the two strains were not competing for similar limiting nutrients in the bovine intestine. In contrast, competition experiments between BG1 NaI® and EDL933ΔeutB::kanR indicated that the commensal strain significantly outcompeted the EHEC strain defective for EutB from 7 to 9 h of co-incubation (Fig. 7B and Table 1). Similar results were obtained when the EHEC strains Sakai and 86-24 or their ΔeutB::kanR isogenic mutants were co-incubated with BG1 NaI® (Fig. S6 and Table 1). Taken together, these results indicate that EHEC strains needed to consume EA to maintain their growth rate at the same level as that of commensal E. coli strains.

Discussion

We speculated that EA may be present in the gastrointestinal tract of cattle and used as a source of nutrients by EHEC. In this study, we detected for the first time free EA in the contents of bovine small intestine and we demonstrated that the capacity to metabolize it confers a nutritional advantage to EHEC O157:H7.

In the bovine intestine, PE can be provided by the turnover of epithelial cells, cell debris from the intestinal and ruminal microbiota and, by plant cell membranes of the forage included in the feed. It is well documented that EHEC, but not commensal E. coli strains, bind specifically to PE (Barnett Foster et al., 1999; 2000). This specific binding induces apoptosis of epithelial cells, triggers elevated host PE levels (Barnett Foster et al., 1999; 2000; Wu et al., 2004) and consequently increases the amount of PE released into the luminal content. Furthermore, mammalian intestinal cells have phospholipase D activity resulting in hydrolysis of endogenous PE (Kiss and Tomono, 1995; Martin et al., 2007) and consequently in the release of free EA into the intestinal luminal contents. Therefore, in contrast to commensal E. coli, binding of EHEC strains to PE in the mammalian intestine could constitute a strategy for EA acquisition by EHEC.

Prokaryotic and eukaryotic cells (including enterocytes) do not synthesize PE de novo (Bakovic et al., 2007). To
synthesize PE, eukaryotic cells use the EA metabolically produced or provided from exogenous lipids supplied through the diet. This could explain the low rate of free EA that we have quantified in BIC. In contrast, the bacteria are unable to utilize EA for PE biosynthesis (all of the PE present in bacterial cell membranes derives from phosphatidylserine decarboxylation) (Bakovic et al., 2007). Therefore, the EA consumed by EHEC cannot be used for the synthesis of PE in bacterial membranes, but is probably used exclusively by bacteria as a nutrient.

Interestingly, 2.2 mM of EA was detected in BIC samples whereas a lower rate was present in other compartments of the bovine digestive tract [rumen (7.7 nM), caecum (12.3 nM) and colon (17.3 nM)] (Results not shown). Although the recto-anal junction is considered as the primary E. coli O157:H7 colonization site for persistent shedding in cattle (Naylor et al., 2005b; Lim et al., 2007), the rumen, the small intestine and the proximal colon are also minor sites of EHEC carriage (Baines et al., 2008; Nart et al., 2008). Moreover, E. coli O157:H7 experimental infection involves both the small and large intestine in cattle, but because of the location of the small intestine in the digestive tract, this site is more critical to maintaining infections and persistent shedding (Baines et al., 2008).

EA, which is constantly renewed, seems to be an important nitrogen source allowing EHEC to survive and multiply in the bovine small intestine.

There is no obvious explanation for the inability of EDL933 to use EA as a carbon source. The presence of a functional EAL was demonstrated by the growth of EDL933 in EA as a nitrogen source. In addition to EAL, other enzymes such as aldehyde dehydrogenase (EutE), isocitrate lyase (Icd1), malate synthase (AceB) and isocitrate lyase (AceA) activities are required for the utilization of EA as a carbon source (Starai et al., 2005). The inability of EHEC to grow on minimal medium with EA as sole carbon source suggests a deficiency in one or more of these enzymes. The growth of E. coli on EA as a carbon source requires a high concentration of EA (82 mM), whereas only 30 mM EA is required for S. enterica growth (Jones and Turner, 1984; Stojiljkovic et al., 1995; Brinsmade and Escalante-Semerena, 2004; Penrod et al., 2004; Sheppard et al., 2004; Starai et al., 2005). Furthermore, not all E. coli strains are able to grow with EA as a carbon source and considerable differences were observed in EAL enzyme activity among different E. coli strains (Jones and Turner, 1984). We also tested the growth conditions described by Jones and colleagues and did not observe growth of EDL933 with EA (82 mM) as the sole of carbon source (data not shown). A relatively low amount of EA was quantified in BIC samples and an identical growth phenotype was observed during the first hours of co-incubation of EHEC and their eutB mutant (Figs S4 and 5). These results suggested that EA is one of the nitrogen sources present in the bovine small intestine and that other nitrogen sources are probably first used by EHEC strains.

Approximately, 500 endogenous bacterial species are present in the mammalian gut (Moore and Holdeman, 1974) and to coexist, each member of the microbiota must be able to utilize a few limiting nutrients better than the others. Except in E. faecalis and C. perfringens, the eut genes were rarely detected in the genome of bacteria included in the three major phyla constituting the mammalian gut microbiota (Bacteroidetes, Firmicutes and Actinobacteria). The genetic information required to utilize EA as nutrient is mainly carried by the genome of enterobacteriaceae, frequently involved in intestinal or extra-intestinal infections in mammals (Klebsiella, Salmonella, Shigella and Citrobacter). In this report, we showed that in contrast to EHEC, the resident microbiota of the bovine intestine consumed EA weakly. EA confers a nutritional advantage to pathogenic E. coli strains in the mammalian gut and represents an alternative nitrogen source for EHEC in the digestive environment. In agreement with our results, previous reports indicate that activation of the eut genes can help pathogenic bacteria to survive in the mammalian environment. Indeed, the eutABC genes of L. monocytogenes are induced in the cytosol of Caco-2 cells and the use of EA as a nutrient might be critical for growth of the bacteria in cultured cells (Joseph et al., 2006). In addition, it has been shown that S. enterica eut mutant strains are attenuated in a mouse model of infection, indicating that EA may be an important source of nutrients for the bacteria in vivo (Stojiljkovic et al., 1995). Microarray analyses also reveal that the eutMNPQT genes are overexpressed during growth of a non-pathogenic K12-derivative E. coli strain (MG1655) on mouse caecal mucus (Chang et al., 2004). However, MG1655 eut mutants do not present any colonization defect in the streptomycin-treated mouse model (Chang et al., 2004). This could be due to the much lower expression of eut genes in non-pathogenic E. coli than in EHEC observed in the present study. In addition, the nutritional compositions of BIC and of mouse intestine content probably differ considerably because of differences in resident microbiota and diet. Altogether the data suggest that EA degradation is more critical for maintenance of EHEC O157:H7 in BIC than for colonization of the mouse intestine by a non-pathogenic E. coli.

Freter’s niche theory postulates that the growth rate of a particular bacterium in the intestine is defined by its ability to occupy different ecological niches (Freter et al., 1983a,b). Our data indicate that EHEC could avoid competing with the species of the ruminant digestive microbiota by using EA as a nitrogen source. Therefore, a strategy to reduce EHEC carriage by ruminants may
involve competition for nutrients using exogenous bacteria. Carbon and energy sources used by EHEC in BIC are currently under investigation. In-depth knowledge of the physiology of EHEC in the digestive tract of the ruminant could help to select 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**

Characteristics of *E. coli* strains are summarized in Table S1. The O157:H7 EHEC strains EDL933, Sakai and 86-24 were used in this study. *E. coli* commensal strains were isolated from ovine (4C) and bovine (BG1) digestive tract at the slaughterhouse and, from faeces of healthy humans (5A). Commensal *E. coli* isolates were identified by plating undiluted digestive samples on sorbitol MacConkey agar plates incubated overnight at 37°C. All the sorbitol fermenting colonies were then observed after Gram staining and the *E. coli* species were finally validated using the API® 20E gallery (bioMérieux). PCR amplifications were performed to confirm that the strains did not possess any of the virulence genes *stx*, *espA* and *eae* using specific primers previously described (Pradel *et al.*, 2000; Bertin *et al.*, 2004). Spontaneous nalidixic acid-resistant mutants of BG1, 4C and 5A commensal *E. coli* were also used in this study (Table S1). Each wild-type *E. coli* strain and its corresponding spontaneous *Nal* mutant showed an identical growth curve pattern when cultured in LB broth at 39°C without aeration.

**Preparation of BICs containing live endogenous microbiota**

Bovine intestinal content containing LEM was collected to perform bacterial competition experiments. Three Holstein steers were slaughtered in the facilities located at the INRA research centre (Theix), in accordance with the guidelines of the local Ethics Committee. The jejunum and ileum were removed as a single piece and the intestinal content was collected from the last two meters of the jejunum to a few centimetres before the end of the ileum in O₂-free N₂ saturated sterile flasks. The BIC was immediately transferred to the laboratory, rapidly filtered through four layers of cheese-cloth and stored at –80°C.

We first determined whether we could perform the competition experiments under micro anaerobic conditions and, second, whether we could keep the samples frozen at –80°C until use without altering the viability of the endogenous microbiota. To this aim, BIC samples were incubated under both strictly anaerobic conditions (100% N₂ atmosphere) and normal atmosphere at 39°C without aeration. Strict and facultative anaerobes were enumerated before and after freezing, and before and after incubation.

To enumerate total facultative anaerobes, 10-fold serial dilutions of samples (collected from each of the three cows) were performed in the mineral solution described by Bryant and Burkely (1953). The bacterial dilutions were plated on Petri dishes containing G20 solid medium (Chassard *et al.*, 2008) and incubated in aerobic conditions at 39°C for 48 h. To enumerate the total viable count of strictly anaerobic bacteria, 10-fold serial dilutions of BIC samples were performed in mineral solution under N₂ flow. Each dilution was inoculated into O₂-free CO₂ saturated roll tubes (Hungate *et al.*, 1966) using the complete CC medium described by Leedle and Hespell (1980). Three roll tubes inoculated per dilution were then incubated for 4 days at 39°C, after which the colony counts were determined.

After 9 h of incubation, comparable levels of viable strict anaerobes and facultative anaerobes were found whatever the incubation conditions (Table S2). Therefore, we decided to perform BIC incubations under normal atmosphere at 39°C without shaking.

After two months of storage at –80°C, BIC samples still contained more than 90% of viable strict and facultative anaerobes, indicating that the conditions of sample preparation and storage were appropriate to keep most of the micro-biota viable.

**Preparation of sBIC samples**

To obtain sBIC, the intestinal contents were diluted with an equal volume of sterile PBS and then centrifuged (2000 g for 20 min). The supernatant was filtered through a 0.22 μm nylon filter and the resulting pooled aliquots were stored at –80°C until use. Samples from other compartments of the bovine gastro-intestinal tract (rumen, caecum and colon) were also collected at slaughter. As with the BIC preparation, all the samples were diluted (1:1) in PBS buffer, centrifuged at 2000 g for 20 min and filtered. The samples were then frozen at –80°C before use.

**Culture media and growth conditions**

*Escherichia coli* EDL933 was cultured on BIC or M9 minimal medium supplemented with glucose (4 g l⁻¹), MgSO₄ (1 mM), CaCl₂ (0.1 mM), vitamin B12 (cyanocobalamin) (150 nM), vitamin B1 (5 mg l⁻¹) and trace metals (0.1 mM ZnSO₄, 0.045 μM FeSO₄, 0.2 μM Na₂SeO₃, 0.2 μM Na₂MoO₄, 2 μM MnSO₄, 0.1 μM CuSO₄, 3 μM CoCl₂ and 0.1 μM NiSO₄) (2 ml l⁻¹) (M9-gluc). EDL933 precultures in Luria–Bertani (LB) broth inoculated from single colonies were performed for 8 h at 37°C with aeration. The precultures were then diluted 50-fold in M9-gluc and BIC and grown overnight without aeration at 39°C. The next day, 18 mm test tubes containing 7 ml of BIC or M9-gluc medium were inoculated by overnight BIC or M9-gluc broth cultures, respectively, and incubated at 39°C without shaking.

The ability of *E. coli* EDL933 to grow with EA as sole carbon or nitrogen source was tested on minimal media. A modified M9 minimal salt medium containing KH₂PO₄ (22 mM), Na₂HPO₄ (48 mM) and NaCl (8.5 mM) was supplemented with MgSO₄ (1 mM), CaCl₂ (0.1 mM), vitamin B12 (cyanocobalamin) (150 nM), vitamin B1 (5 mg l⁻¹) and trace metals (2 ml l⁻¹). Utilization of EA as a nitrogen source was investigated by addition of EA hydrochloride (30 mM) and...
glycerol (20 mM) to the modified M9 minimal medium. To test the capacity of EDL933 to use EA as the sole carbon source, the modified M9 minimal medium was supplemented with NH₄Cl (19 mM) and EA hydrochloride (30 mM). All chemicals were from Sigma. For each condition, three LB broth cultures were each started from a single colony and grown overnight at 37°C with aeration. Cells were then pelleted by centrifugation (4000 g for 10 min), resuspended in M9 minimal medium with EA as the sole nitrogen or carbon source and diluted 50-fold in the corresponding minimal media. Cultures were then incubated with shaking (200 r.p.m.) at 37°C and the growth was monitored in three parallel cultures by following optical density (λ = 600 nm).

RNA extraction and real-time PCR (q-PCR)

Bacterial suspensions were centrifuged at 10 000 g for 15 min. The supernatants were stored at −20°C for further investigations and bacterial pellets were rapidly resuspended in two volumes of RNAProtect Bacteria reagent (Qiagen) to stabilize the RNA. The suspension was then centrifuged (10 000 g for 15 min) and total RNA purification was performed on the bacterial pellet using the NucleoSpin RNA II kit (Macherey-Nagel). DNA contaminations were digested using an RNase-free DNase I column during the RNA preparation as described by the manufacturer (Macherey-Nagel). 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 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 for both relative and absolute mRNA quantification.

Absolute mRNA quantification was performed as previously described (Vareille et al., 2007; de Sablet et al., 2008). The standard curves for both eutB and tufA were obtained by amplification of EDL933 genomic DNA purified with the DNeasy Blood and Tissue Kit (Qiagen). Amplifications were performed with AccuTaq LA DNA Polymerase (Sigma-Aldrich) and the eutB-F (GACGACGTGCAAAGTATCG)/eutB-R (GAGCGTTGCCATATGCC) and tufA-F (CAGGTAGGGTCCGTACAT)/tufA-R (CCTGACCAGTTCATTTCT) primer pairs respectively. The level of eutB and tufA mRNA was quantified and converted to molecule number as previously described (Fronhofs et al., 2002). The results are presented as the ratios between the copy number of eutB mRNA and the copy number of tufA mRNA.

Relative mRNA quantification was performed using primers designed to specifically amplify fragments of 90–200 bp (Table S3). 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.

**Competitive experiments**

Precultures of *E. coli* strains (inoculated from a single colony) were incubated in LB broth with the appropriate antibiotic. The bacterial cultures were diluted 50-fold in LB broth and grown overnight at 39°C without shaking. A fresh sample of BIC with live endogenous microbiota (BIC-LEM) was inoculated with approximately 5 × 10⁷ bacteria per millilitre of each of the two strains tested in competition assays and then incubated at 39°C without shaking. At each time point, the co-culture was serially diluted 10-fold in PBS pH 7.2 and spotted on SMAC agar plates. Co-cultures of wild-type/eutB::kanR mutants were spotted on SMAC plates without antibiotic and on SMAC plates containing kanamycin (50 μg ml⁻¹). Co-cultures of EHEC/commensal *E. coli* strains were spotted on SMAC plates without antibiotic and on SMAC plates containing nalidixic acid (50 μg ml⁻¹). The plates were then incubated overnight at 37°C before counting colony-forming units (CFU). When the wild-type EHEC strains (sensitive to the antibiotics used in this study) were included in competition assays, the CFU count was calculated by subtracting the number of CFU resistant to kanamycin or nalidixic acid from the number of CFU counted on agar plate without antibiotic. Each experiment was replicated three times and a CI was calculated as described in Table 1.

**Mutant construction and molecular cloning**

Replacements of eutB, eutD, eutE, eutH and eutG genes by the gene conferring resistance to kanamycin in EDL933 were obtained by using the one-step PCR-based method of Datsenko and Wanner (2000). Similarly, eutB::kanR isogenic mutants of two additional EHEC strains (Sakai and 86-24) and three commensal strains (4C, BG1 and 5A) were also constructed. Primers used to construct the mutants (Table S4) were designed according to the EDL933 genome sequence. Gene knockouts were confirmed by PCR analysis using primers specific for the kanamycin gene and the ORFs flanking the mutated genes.

For cloning of the eut genes, primers consisting of the first or last nucleotides of the corresponding ORF and of the restriction sequence of the enzymes EcoRI and BamHI (eutB, eutD, eutE eutG) or KpnI and PstI (eutH) were used to amplify the eut genes from EDL933 genomic DNA (Table S4). The PCR products were then purified by the Qiaquick Purification PCR kit (Qiagen), digested with the relevant enzymes and ligated into the expression vector pTrc99A (conferring resistance to ampicillin, under the control of the isopropyl β-D-thiogalactoside-inducible trc promoter) using T4 DNA ligase (Invitrogen). Each of the resulting recombinant plasmids was then electroporated into the corresponding EDL933::eut::kanR mutant to complement the gene defect. Gene complementation was checked by PCR amplification and restriction enzyme digestion.

**EA quantification by mass spectrometry**

Prior to mass spectrometry quantification, each sample was deproteinized by sulfosalicylic acid precipitation. Briefly, samples (1 ml) were incubated for 15 min with 40% sulfosalicy-
EHEC uses ethanolamine as a nitrogen source in the bovine intestine

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cyclic acid (100 μl) and then centrifuged at 11 000 g for 15 min. The pellets were removed and EA contained in the supernatant was derivatized with dansyl chloride to be quantified by mass spectrometry. Dansylated derivatives of the amines were formed by adding 300 μl of saturated NaHCO₃ solution, 2 ml of dansyl chloride solution (10 mg ml⁻¹ in acetone) and 1 ml of EA solution in 200 μl of NaOH. Fresh dansyl chloride solutions were prepared each time just before use. After shaking, samples were left in the dark at room temperature for 20 min, and residual dansyl chloride was removed by addition of 100 μl of NH₄OH 25% (V/V). The volume was then adjusted to 5 ml with acetonitrile. After filtration, 20 μl of the solution was injected for the analysis. The liquid chromatography mass spectrometry system consisted of an 1100 LC system (Agilent technology) with a triple quadrupole mass spectrometer API 2000 equipped with an APCI source in the positive mode (Applied Biosystems). Sciex software (version 1.4.1) from Applied Biosystems was used for instrument control, data acquisition and data processing. The mass spectrometer was operated at 420°C. Acquisition was set with MRM mode at m/z 295/157. The column used was a symmetry shield, 150 ¥ 2.1 mm, 5 μm (Waters). The mobile phase consisted of acetonitrile/water (65/35, V, V) in isocratic mode. The flow-rate was 300 μl min⁻¹ with a run time of 8 min. EA, dansyl chloride and HPLC grade solvents were from Sigma. Water for HPLC analysis was obtained using a MilliQ Plus water purification system (Millipore). The extraction solvent (HCLO₄) was from Carlo Erba. Sodium hydroxide and other reagents were from VWR international.

Sequence analysis

In silico analyses were performed to detected eut genes among bacterial genomes. The xBASE (http://xbase.bham.ac.uk/) and the BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) servers were used. The nucleotide sequence of the eut operon was compared with the nucleotide sequences of the 592 bacterial complete genomes present in the ‘Microbes genomic’ BLAST database. Amino acid sequences were aligned and compared using the Blastp algorithms in the Genomic BLAST database. Amino acid sequences were aligned and compared using the Blastp algorithms in the Genomic BLAST database.

Statistical analyses

ANOVA with the Games–Howell post hoc test was used to identify significant differences among multiple test groups. Statistical analysis of competitive assays was conducted using the Mann–Whitney non-parametric U-test.

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Fig. S1. Bacterial growth curves of EDL933. The EHEC strain EDL933 was incubated at 39°C without shaking in sBIC (open circle) and M9 minimal medium (M9-gluc) (filled circle). The arrows indicate the times at which the RNA samples were collected. Each time point is the mean of three independent experiments.

Fig. S2. Bacterial growth curves of EDL933 and EDL933*ΔeutB-kanR* in M9-gluc. The wild-type strain EDL933 and its isogenic *eutB* mutant were incubated at 37°C with shaking in M9 minimal medium. EDL933 was incubated in M9-gluc (filled squares), M9 with EA as the sole source of carbon (open circles) and M9 with EA as the sole source of nitrogen (open squares); EDL933*ΔeutB-kanR* (open triangles) and EDL933*ΔeutB* (filled triangles) were incubated in EA as the sole source of nitrogen.

Fig. S3. Growth curves of EDL933 and its isogenic *eut* mutants in M9-gluc. The wild-type strain EDL933 and its isogenic *eut* mutants were incubated at 37°C with shaking in M9 minimal medium with EA as the sole source of nitrogen. Each time point is the mean of three independent experiments.

Fig. S4. Competition assays between the EHEC strains and their corresponding *eut* mutants. The BiC-LEM samples were inoculated with a 1:1 mixture of the two *E. coli* strains tested. Bars represent the SEM of three independent experiments. **P < 0.0001** (Mann–Whitney test). The corresponding CI values are shown in Table 1.

Fig. S5. Competition assays between the commensal *E. coli* and their corresponding *eut* mutants. The BiC-LEM samples were inoculated with a 1:1 mixture of the two *E. coli* strains tested. Bars represent the SEM of three independent experiments. The corresponding CI values are shown in Table 1.

Fig. S6. Competition assays between the EHEC or EHEC *Δeut:kanR B* strains and the commensal *E. coli* strain BG1. The BiC-LEM samples were inoculated with a 1:1 mixture of the two *E. coli* strains tested. Bars represent the SEM of three independent experiments. **P < 0.0001** (Mann–Whitney test). The corresponding CI values are shown in Table 1.

Table S1. Bacterial strains.

Table S2. Bacterial population levels in BiC-LEM samples.

Table S3. Sequences of primers used in relative mRNA quantification.

Table S4. Sequences of primers used in construction of mutants and in gene cloning.

Supporting information

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