Differential effects of short-chain fatty acids and iron on expression of *iha* in Shiga-toxigenic *Escherichia coli*

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Shiga-toxigenic *Escherichia coli* (STEC) colonizing the bowel are exposed to a variety of short-chain fatty acids (SCFAs), including acetate, propionate and butyrate, produced by gut microflora. However, the total concentrations and relative amounts of SCFAs in the lumen vary with intestinal niche. Here we report that conditions simulating SCFA concentrations present in the human gut trigger expression of the *iha* gene, which encodes an adherence-conferring outer-membrane protein of pathogenic *E. coli*. We show that growth under conditions simulating colonic, but not ileal, SCFA concentrations increases *iha* expression in three tested STEC strains, with the strongest expression detected in LEE-negative STEC O113:H21 strain 98NK2. Expression of *iha* is known to be subject to Fur-mediated iron repression in O157:H7 STEC, and the same occurs in 98NK2. However, exogenous iron did not repress *iha* expression in the presence of colonic SCFAs in either 98NK2 or the O157:H7 strain EDL933. Moreover, exposure to the iron chelator 2,2'-dipyridyl caused no further enhancement of *iha* expression over that induced by colonic SCFAs. These findings indicate that SCFAs regulate *iha* expression in STEC independently of iron. Increased expression of *iha* under colonic but not ileal SCFA conditions possibly may contribute to preferential colonization of the human colon by STEC.

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INTRODUCTION

Shiga-toxigenic *Escherichia coli* (STEC) cause food-borne infections in humans, commencing with watery or bloody diarrhoea that can progress to haemorrhagic colitis and the life-threatening haemolytic uraemic syndrome (HUS) (Karmali, 1989; Nataro & Kaper, 1998; Paton & Paton, 1998). STEC are classified into two major groups in accordance with the presence of the locus of enterocyte effacement (LEE). LEE-positive strains have the ability to produce attaching and effacing lesions on the intestinal epithelium, characterized by intimate attachment of the bacteria to the epithelial cell surface, rearrangement of the cell cytoskeleton and effacement of the epithelial microvilli, as reviewed by Frankel *et al.* (1998). However, the presence of LEE is not essential for pathogenesis, as both sporadic cases and even outbreaks of HUS can be caused by LEE-negative STEC (Karmali *et al.*, 1985; Paton *et al.*, 1999). Thus, alternative accessory virulence factors, in particular adherence-mediating molecules, may also play a role in pathogenesis. Among STEC a range of novel adhesins have been identified, including the chromosomally encoded adherence-conferring protein Iha, a homologue of *Vibrio*

cholera IrgA (Tarr *et al.*, 2000). Iha was first characterized in *E. coli* O157:H7, but it is distributed widely among LEE-negative and LEE-positive STEC, as well as in uropathogenic *E. coli* (UPEC) (Johnson *et al.*, 2000; Tarr *et al.*, 2000). Tarr *et al.* (2000) reported that an *iha* deletion mutant of O157:H7 STEC was impaired in adherence to HeLa cells, although the difference did not reach statistical significance. Nevertheless, there was a highly significant increase in adherence to both HeLa and MDBK cells when *iha* was expressed from a plasmid in a non-piliated recombinant *E. coli* host. The high prevalence (91 %) of *iha* in STEC belonging to different seropathotypes and the presence of multiple *iha* copies in some strains point to the potential importance of this novel adhesin (Tarr *et al.*, 2000; Toma *et al.*, 2004). In the UPEC strain CFT073, *iha* functions as a urovirulence factor, by contributing to colonization in a mouse urinary tract infection model (Johnson *et al.*, 2005). Furthermore, Iha from UPEC strain UCB34 functions as a catecholate siderophore receptor in *E. coli* K-12. The capacity of Iha to transport siderophores is TonB-dependent, whereby the protein complex TonB/ExbB/ExbD provides the energy required for active transport (Leveille *et al.*, 2006; Postle & Kadner, 2003). Additionally, *iha* expression is regulated by the ferric-uptake regulator protein Fur (Rashid *et al.*, 2006b). In the presence of iron, dimerized Fur binds via a

Abbreviations: LEE, locus of enterocyte effacement; HUS, haemolytic uraemic syndrome; SCFA, short-chain fatty acid; STEC, Shiga-toxigenic *Escherichia coli*; UPEC, uropathogenic *E. coli*.

sequence-specific protein–DNA interaction to *iha* promoter regions and represses *iha* transcription. Under iron-limiting conditions, Fur is unable to interact with the DNA and as a consequence, *iha* transcription is de-repressed (Leveille *et al.*, 2006; Rashid *et al.*, 2006b). Free iron levels are typically low at mucosal surfaces, due to binding by host lactoferrin, and so induction of iron-scavenging mechanisms is an important bacterial *in vivo* survival strategy. However, Fe levels need to be carefully regulated to avoid oxidative stress induced by the Fenton reaction (Touati, 2000). Iron is an important environmental cue, and iron-limiting conditions induce virulence-related genes in a number of pathogens (Litwin & Calderwood, 1993; Touati, 2000).

STEC efficiently colonize the human gut, and, although direct evidence is limited, the colon is believed to be the primary site of such colonization. The gastrointestinal tract is a complex microenvironment containing, among other substances, a variety of short-chain fatty acids (SCFAs), which are a major metabolic product of probiotic bacteria. The three principal organic acids present in the intestine are acetate, propionate and butyrate, but the composition and concentration varies between the ileum and colon (Cummings *et al.*, 1987; Cummings & Macfarlane, 1991; Macfarlane *et al.*, 1992; Salminen *et al.*, 1998). Diet also affects relative SCFA composition; for example, the main product of starch fermentation is butyrate, whereas pectin fermentation yields more acetate (Cummings & Macfarlane, 1991; Gilbert *et al.*, 2005). The total concentration of SCFAs in the gut is also affected by time after ingestion, as SCFAs are absorbed rapidly (Salminen *et al.*, 1998). Nevertheless, Cummings *et al.* (1987) have estimated concentrations of SCFAs at 13 ± 6 mmol kg⁻¹ in the terminal ileum, 80 ± 11 mmol kg⁻¹ in the caecum and 131 ± 9 mmol kg⁻¹ in the descending colon. Another study estimated concentrations of SCFAs at approximately 97 mmol kg⁻¹ in the descending colon and 198 mmol kg⁻¹ in the caecum (Macfarlane *et al.*, 1992). Furthermore, in the food industry fatty acids are widely used as preservatives at concentrations ranging from 1 to 4% (reviewed by Erickson & Doyle, 2007). STEC infections result from consumption of contaminated food products and so pathogens may be exposed to SCFAs during food processing, as well as during colonization of the human gut. A number of studies have shown that SCFAs can act as environmental cues, triggering alterations in gene expression, as well as directly inhibiting the growth of pathogenic bacteria (Arnold *et al.*, 2001; Gantois *et al.*, 2006; Kwon & Ricke, 1998; Lawhon *et al.*, 2002; Shin *et al.*, 2002). Variations in SCFA composition in different regions of the gastrointestinal tract (Cummings *et al.*, 1987, 2001; Cummings & Macfarlane, 1991; Lawhon *et al.*, 2002; Macfarlane *et al.*, 1992) may also enable differential expression of virulence factors in specific host microenvironments. Here we investigate the effects of SCFAs, simulating concentrations in the colon and ileum, on expression of *iha* by the hyper-virulent LEE-negative STEC

O113:H21 strain 98NK2 and LEE-positive STEC O157:H7 strains EDL933 and 86-24.

METHODS

Bacterial strains and plasmids. The *E. coli* strains and plasmids used in this study are listed in Table 1. Cells were routinely grown in Luria–Bertani (LB) medium with or without 1.5% Bacto agar. As recovery medium for transformants, SOC medium (20 g tryptone l⁻¹, 5 g yeast extract l⁻¹, 20 mM glucose, 8.6 mM NaCl, 2.5 mM KCl, 20 mM MgSO₄) was used. All bacteria were grown at 37 °C, except the temperature-sensitive strain 98NK2(pkD46). Antibiotics were used at 50 µg ml⁻¹ for kanamycin and 100 µg ml⁻¹ for ampicillin.

Incubation with SCFAs. To simulate gastrointestinal levels of SCFAs, overnight cultures of respective strains were diluted 1 : 120 in LB buffered with 100 mM MOPS (pH 6.7) and supplemented with either 95 mM acetate, 60 mM propionate and 17 mM butyrate (colonic SCFA medium) or with 25 mM acetate, 2.5 mM propionate and 2.5 mM butyrate (ileal SCFA medium). The NaCl concentration of the control medium (MOPS-buffered LB, pH 6.7) was adjusted to ensure equivalent osmolality to the respective (ileal or colonic) SCFA medium. Cells were grown at 37 °C with agitation for 3 and/or 4 h. All experiments were performed at least twice on different cultures, except when otherwise indicated. To examine the growth of 98NK2 following treatment with SCFAs, 100 µl aliquots of cell culture, and serial dilutions thereof, were plated in duplicate on LB agar to determine c.f.u. ml⁻¹ hourly for 5 h.

Growth in iron-rich and iron-limited media. *E. coli* strains were grown at 37 °C with agitation in LB medium supplemented with 100 mM MOPS (pH 6.7) and SCFAs, in standard LB medium (pH 7.5) or in DMEM (Gibco) (without agitation), supplemented with either 0.2 mM 2,2'-dipyridyl (iron-limited media) or 10 µM FeSO₄ (iron-rich media).

Mutagenesis of 98NK2. Deletion of the *fur* and *tonB* genes and insertion of a kanamycin-resistance cassette were performed using the lambda Red recombinase system as described by Datsenko & Wanner (2000). In order to do this, a kanamycin-resistance gene was amplified from plasmid pkD4 using high-fidelity PCR and the primer pairs d-tonB-f/d-tonB-r and d-fur-f/d-fur-r, for *tonB* and *fur*, respectively (Table 2). The resultant PCR products were purified and electroporated into *E. coli* strain 98NK2 harbouring the temperature-sensitive plasmid pkD46. Kanamycin-resistant, ampicillin-sensitive recombinants were checked by PCR using respective flanking region primers and the kanamycin-specific primers K1, K2 and Kt described by Datsenko & Wanner (2000). Sequencing reactions were performed in order to confirm the deletion of the major portion of the respective gene, and confirmed mutants were designated 98NK2Δ*fur*::*kan* and 98NK2Δ*tonB*::*kan*.

Isolation of total RNA. RNA was isolated from bacterial pellets using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions, and treated with RNase-free DNase (Roche) following the recommendations of the manufacturer. The purity of each RNA preparation was confirmed by one-step reverse transcription (RT)-PCR (Access RT-PCR Core Reagents, Promega), with or without reverse transcriptase, using the 16S rRNA-specific primers rrsH-f and rrsH-r (Table 2). The products were visualized after electrophoresis on a 2% TAE-agarose gel. In DNA-free RNA samples, PCR products were only seen in the presence of reverse transcriptase. RNA was further purified using an RNeasy Mini kit and the concentration and quality of RNA was determined by A_{260/280} measurement. First-strand cDNA synthesis from equal amounts of total RNA was performed

Table 1. Bacteria and plasmids used in this study

Strain or plasmid	Description*	Reference/source
<i>E. coli</i> strains		
98NK2	STEC O113:H21	Paton <i>et al.</i> (1999)
98NK2-Cu	STEC O113:H21, cured of megaplasmid	Paton <i>et al.</i> (2001)
EDL933	STEC O157:H7	Riley <i>et al.</i> (1983)
86-24	STEC O157:H7	Tarr <i>et al.</i> (1989)
JM109	<i>E. coli</i> K-12 derivative	Yanisch-Perron <i>et al.</i> (1985)
98NK2 Δ <i>iha</i> _{pO113} :: <i>kan</i>	STEC O113:H21 Δ <i>iha</i> _{pO113} :: <i>kan</i> , Kn ^R	Srimanote (2003)
98NK2 Δ <i>fur</i> :: <i>kan</i>	STEC O113:H21 Δ <i>fur</i> :: <i>kan</i> , Kn ^R	This study
98NK2 Δ <i>tonB</i> :: <i>kan</i>	STEC O113:H21 Δ <i>tonB</i> :: <i>kan</i> , Kn ^R	This study
M15(pREP4)	<i>E. coli</i> K-12 derivative, Kn ^R	Qiagen
M15(pREP4)(pQ _{iha})	Produces His ₆ -Iha _{pO113} fusion protein, Ap ^R Kn ^R	This study
Plasmids		
pQE-31	Expression vector, Ap ^R	Qiagen
pQ _{iha}	Expression vector, contains <i>iha</i> _{pO113} , Ap ^R	This study
pKD4	Vector template for mutagenesis, Ap ^R Kn ^R	Datsenko & Wanner (2000)
pKD46	Red recombinase expression vector, Ap ^R	Datsenko & Wanner (2000)

*Kn^R, kanamycin resistance; Ap^R, ampicillin resistance.

using random primers (Invitrogen) and SuperScript III Reverse Transcriptase (Invitrogen), as described by the manufacturer. cDNA was purified using a MiniElute PCR Purification kit (Qiagen) according to the recommendations of the Genisphere RNA amplification kit (http://www.genisphere.com/pdf/SenseAMP_Plus_12-15-06.pdf) and eluted with 10 μ l elution buffer.

Two-step real-time PCR. The amount of mRNA extracted from bacteria and reverse transcribed into cDNA was measured by relative real-time PCR. Real-time PCR was performed in triplicate on a Rotorgene RG-2000 (Corbett Research) according to the manufacturer's instructions. Primers for *iha*, *tonB* and *fur* (Table 2) were designed using Oligo Explorer 1.2 (<http://www.genelink.com/tools/glo-oe.asp>). PCR amplifications were performed with 1 μ l 1:60-diluted

cDNA in a 25 μ l reaction mix containing 200 nM each primer and 12.5 μ l Platinum SYBR Green qPCR Super Mix-UDG (Invitrogen). The amplification conditions were as follows: 50 °C for 2 min, 95 °C for 2 min, and then 40 cycles of 95 °C for 30 s and 60 °C for 30 s. The specificity of each PCR product was confirmed by melting curve analysis according to the manufacturer's instructions. For each gene the fold-differences were determined using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001). Levels of expression of *iha*, *tonB* and *fur* were related to the constitutively expressed housekeeping gene *rrsH* (16S rRNA). Expression data for *iha*, *fur* and *tonB* were analysed relative to the level of the respective gene in the untreated sample (control), and presented as a relative fold increase or decrease between treated and untreated samples, normalized to the level of *rrsH* RNA. Statistical significance of differences between normalized C_t values were

Table 2. Oligonucleotide primers used in this study

Primer sequences derived from *E. coli* K-12, *E. coli* O157:H7 strain EDL933 and strain 86-24 or *E. coli* O113:H21 strain 98NK2 and strain E41 sequences, as deposited in the NCBI database.

Primer	Sequence 5'-3'
d-tonB-f	CGTTACTTTTCGGTCTGCATTTCATGGTGCTGTTGTGGCGGGTCTGTGTAGGCTGGAGCTGCT
d-tonB-r	ATTCACCACAATCCCACTGCCTGGCTTACCCGGCTCATAACGCCATATGAATATCCTCCTTAG
d-fur-f	ACCGCCCTAAAGAAAGCTGGCCTGAAAGTAACGCTTCCTCGTTGTGTAGGCTGGAGCTGCTTC
d-fur-r	AATCGCCTTCGGCACAGTGACCGTAAAGATAGAGACTGTGGTTCATATGAATATCCTCCTTAG
fur-f	TGGTTAGTCAGGCGAATGCC
fur-r	GACACAGCAACATCACCACG
iha-f	CTGACTAATGCAGCCGCCAG
iha-r	CCTCCGGTTTTACCCGTACC
iha _{pO113} -f	TGAGAAGGATCCGACTAATGCAGCCGCC
iha _{pO113} -r	TTTTGCAAGCTTCATGCCTGCTAACGGATA
rrsH-f	GAACGGTAACAGGAAGAAGC
rrsH-r	CGACGTTATGCGGTATTAGC
tonB-f	TTCATGGTGCTGTTGTGGCG
tonB-r	TTGTGGCGGTTTCAGATCAG

analysed by Student's unpaired *t*-test (two-tailed) using Graphpad Prism version 5.0.

Southern hybridization. DIG-labelled *iha* probe was generated by PCR using the PCR DIG Labelling Mix (Roche) with primers *iha-f* and *iha-r* and 98NK2 DNA as template. Three micrograms of *Eco*RI- and *Bst*XI-digested DNA was separated by electrophoresis on a 0.8 % agarose gel. Southern transfer of separated DNA onto positively charged nylon membrane (Biodyne, Pall Corporation) was carried out as described by Maniatis *et al.* (1982). After transfer, DNA was fixed to the membrane by UV cross-linking. Membranes were prehybridized at 42 °C for 30 min in prehybridization buffer [5 × SSC, 0.1 % (w/v) *N*-lauroylsarcosine, 0.2 % (w/v) SDS, 1 % (w/v) Blocking reagent; Roche] and incubated overnight with hybridization solution [5 × SSC, 0.1 % (w/v) *N*-lauroylsarcosine, 0.2 % (w/v) SDS, 1 % (w/v) Blocking reagent] containing the appropriate DIG-labelled *iha*-probe. Membranes were washed twice with 2 × SSC/0.1 % SDS followed by two wash steps for 15 min with 0.5 × SSC/0.1 % SDS at 65 °C under constant agitation. After blocking with 1 % (w/v) Blocking reagent in 0.1 M maleic acid/0.15 M NaCl, pH 7.5, the membrane was probed with anti-DIG-AP Fab fragment (Roche) and bands were detected using chemiluminescent substrate CDP Star (Invitrogen).

Preparation of anti-Iha_{pO113} serum. In order to generate an Iha_{pO113}-specific serum, *iha*_{pO113} was expressed using Qiaexpressonist (Qiagen), resulting in production of a His₆-Iha_{pO113} fusion protein, whereby the His₆-tag was fused to the N terminus of mature Iha_{pO113}, removing the 38 aa signal sequence. In order to do this, *iha*_{pO113} was PCR-amplified using primers *iha*_{pO113F} and *iha*_{pO113R}, which incorporated *Bam*HI and *Hind*III restriction sites, respectively (Table 2). The resultant PCR product was digested and ligated into pQE31 (Qiagen). Correct in-frame insertion of the *iha*_{pO113} fragment was confirmed by sequence analysis and the construct (pQiha) was transformed into the expression host *E. coli* M15(pREP4). For large-scale purification, an exponential-phase broth culture of *E. coli* M15(pREP4)(pQiha) containing 100 µg ampicillin ml⁻¹ and 25 µg kanamycin ml⁻¹ was induced by the addition of 2 mM IPTG (Inalco Pharmaceutical) and incubated for 3 h with shaking at 37 °C. The cells were harvested by centrifugation and resuspended in 12 ml 6 M guanidine.HCl, 0.1 M disodium hydrogen orthophosphate, 0.01 M Tris/HCl, pH 8.0, and stirred for 1 h at room temperature. The cell lysate was centrifuged (10 000 g, 25 min, 4 °C) and the supernatant was loaded onto a Ni-NTA (Probond, Invitrogen) column, pre-equilibrated with 10 column volumes of buffer A (0.5 M NaCl, 15 mM imidazole), at a rate of 15 ml per hour. After washing the column with 10 column volumes of buffer A, 20 column volumes of buffer B (8 M urea, 0.1 M disodium hydrogen orthophosphate, 0.01 M Tris/HCl, pH 8.0) and 20 column volumes of buffer C (8 M urea, 0.1 M disodium hydrogen orthophosphate, 0.01 M Tris/HCl, pH 6.3, 0.25 M NaCl, 5 mM imidazole), the protein was eluted using 30 ml of a 0–500 mM imidazole gradient. Fractions (3 ml) were collected and analysed by SDS-PAGE. Peak fractions were pooled and dialysed overnight at 4 °C against a decreasing concentration of urea (8 M–2 M), with dialysis continuing for a further 4 h in PBS pH 7.5 containing 2 M urea in order to refold the protein. Six BALB/c mice were immunized by intraperitoneal injection of 10 µg purified Iha_{pO113} in complete Freund's adjuvant, followed by two doses of 10 µg purified Iha_{pO113} in incomplete Freund's adjuvant at 10 day intervals. Blood was collected by cardiac puncture 2 weeks after the third immunization, and serum was stored at 4 °C.

Western blot analysis. After incubation under various conditions, the equivalent of 1 × 10⁸ bacteria were pelleted by centrifugation (4000 g, 5 min, 4 °C) and resuspended in 100 µl SDS sample buffer [62.5 mM Tris pH 6.8, 2 % (w/v) SDS, 10 % (v/v) glycerol, 5 %

mercaptoethanol, 0.05 % (w/v) bromophenol blue]. Proteins were separated by SDS-PAGE using a 4–20 % Bistris gradient gel (Invitrogen) according to the manufacturer's instructions and electrophoretically transferred onto PVDF membranes (Hybond, Amersham). Membranes were blocked for 1 h with 1 × TTBS (Tris-buffered saline, 0.05 % Tween 20) containing 5 % skim milk and probed with mouse anti-His₆-Iha_{pO113} serum, followed by incubation with goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase (Bio-Rad). Bands were detected using a chromogenic nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate.

RESULTS

Homologues of *iha* in 98NK2

iha homologues are found in several STEC and UPEC strains as single or multiple copies (Tarr *et al.*, 2000). Therefore, we initially investigated the number of *iha* homologues in the hyper-virulent LEE-negative O113:H21 STEC 98NK2. Southern blot analysis of *Eco*RI- and *Bst*XI-digested DNA of 98NK2 and its megaplasmid-cured derivative (98NK2-Cu) revealed two *iha* probe-reactive bands in each of the 98NK2 digests, but only one in either of the 98NK2-Cu digests (result not presented). Thus, 98NK2 contains two copies of *iha*, one located on megaplasmid pO113 (as already deposited in GenBank; accession nos NC_007365 and AF399919) and one residing on the chromosome.

Regulation of *iha* expression in 98NK2 by iron

As a further preliminary experiment, we examined whether *iha* expression in the LEE-negative STEC O113:H21 strain 98NK2 is regulated by iron and the Fur protein, as previously reported for UPEC and the LEE-positive STEC O157:H7 strain 86-24 (Leveille *et al.*, 2006; Rashid *et al.*, 2006b). Since Iha functions as a siderophore receptor and the capacity of Iha to transport siderophores is TonB-dependent, we also investigated *tonB* expression under the same conditions. Given the fact that Fur functions as a repressor of a broad range of genes, including *iha*, we also examined *fur* gene expression. In order to do this, 98NK2 was grown in LB and DMEM media with or without 0.2 mM 2,2'-dipyridyl to simulate iron-limiting conditions. RNA was isolated from exponentially growing cultures after 3 h incubation in the case of LB and after 6 h in the case of DMEM, since the growth rate of *E. coli* in tissue culture media is retarded. Two-step real-time RT-PCR was then used to measure levels of *iha*, *tonB* and *fur* mRNA, normalized against 16S rRNA (*rrsH*), in treated versus untreated cultures and results are presented as a relative fold increase/decrease (Fig. 1a). After growth in LB supplemented with 2,2'-dipyridyl we observed a 20.3-fold increase in *iha* expression. Coincidentally, a 42.4-fold increase in *tonB* expression was detected, whereas *fur* expression in 98NK2 was relatively unaffected (1.8-fold increase). In DMEM, an enhancement in expression of *iha* (9.3-fold) and *tonB* (3.1-fold) was detectable following growth in DMEM supplemented with 2,2'-dipyridyl

Fig. 1. Effect of 2,2'-dipyridyl (DPY) on expression of *iha*, *tonB* and *fur* in *E. coli* O113:H21 strain 98NK2, determined by real-time RT-PCR. Cells were grown for 3 h in LB and for 6 h in DMEM, supplemented with 0.2 mM 2,2'-dipyridyl. (a) Data are presented as the increase (*n*-fold) in mRNA expression compared to the control grown in LB or DMEM without 2,2'-dipyridyl, normalized to *rrsH* expression. Data are the mean $\pm$ SD of two independent experiments, each assayed in triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; Student's unpaired two-tailed *t*-test. (b) Relative *iha* mRNA levels in 98NK2 determined by real-time RT-PCR as described above. Real-time RT-PCR data for each gene were normalized against those obtained for the 16S rRNA (*rrsH*) mRNA control using the formula $1/(C_{t(iha)} - C_{t(rrsH)})$. Data are the mean $\pm$ SD of two independent experiments, each assayed in triplicate.

relative to DMEM alone. Furthermore, a 13.2-fold increase in expression of *fur* was observed.

We also investigated effects on Iha production by SDS-PAGE and Western immunoblotting using anti-Iha_{pO113}. Whole-cell lysates were analysed after 4 h incubation in LB supplemented with 2,2'-dipyridyl or FeSO₄, or after 7 h in the case of DMEM (Fig. 2). Immunoreactive bands of approximately 70 kDa, corresponding in size to the deduced molecular mass of mature Iha_{pO113}, were evident in all samples. In cells grown in LB, basal production of Iha was low, presumably due to the presence of sufficient amounts of iron in the medium to repress *iha* (Fig. 2). In

Fig. 2. Immunoblot analysis of whole-cell lysates of 98NK2 using anti-Iha_{pO113}. Cells were grown in LB for 4 h or in DMEM for 7 h without supplements (lane 1), or with 0.2 mM 2,2'-dipyridyl (lane 2) or 10 μ M FeSO₄ (lane 3). Lysates were separated by SDS-PAGE, blotted and probed with anti-Iha. The bands represent the 70 kDa Iha antigen. The blot shown is representative of three independent experiments.

DMEM, basal Iha production was higher than in LB, because the level of iron is sufficiently low to partially de-repress *iha*. Increased *iha* expression in DMEM is also apparent when the absolute level of *iha* mRNA relative to the internal standard *rrsH* ($C_{t(iha)} - C_{t(rrsH)}$; see Methods) is compared for DMEM- and LB-grown cells (Fig. 1b). The observation that Iha is better expressed in DMEM than LB medium has been reported previously for O157:H7 STEC, non-O157:H7 STEC and UPEC strains (Johnson *et al.*, 2005; Rashid *et al.*, 2006b; Schmidt *et al.*, 2001).

In LB supplemented with 2,2'-dipyridyl, Iha production was strongly induced (Fig. 2). In spite of the higher baseline expression of Iha in DMEM, addition of 2,2'-dipyridyl resulted in a further increase in Iha expression, presumably due to full de-repression (Fig. 2). Under de-repressed conditions, overall Iha expression was similar in both media, at both the protein (Fig. 2) and mRNA level (Fig. 1b). As expected, addition of FeSO₄ to either DMEM or LB fully repressed Iha production, with levels similarly low in both media (Fig. 2). Collectively, the above RT-PCR and immunoblot findings are consistent with Fur-mediated repression of *iha* in 98NK2 by iron in both media.

Effect of SCFAs on *iha* expression

SCFAs are the dominant anions in the mammalian intestinal tract, with acetate, propionate and butyrate accounting for 85–95 % of total SCFAs in all regions of the colon (Cummings & Macfarlane, 1991). To simulate colonic SCFA concentrations we used a mixture of 95 mM acetate, 60 mM propionate and 17 mM butyrate (55 % acetate, 35 % propionate and 10 % butyrate) in MOPS-buffered LB (see Methods); for ileal SCFA concentrations, we used a mixture of 25 mM acetate, 2.5 mM propionate and 2.5 mM butyrate (85 % acetate, 7.5 % propionate and 7.5 % butyrate) (Cummings *et al.*, 1987; Cummings & Macfarlane, 1991; Lawhon *et al.*, 2002; Macfarlane *et al.*, 2006). We then examined *iha* gene expression in response to exposure to SCFAs.

Initially, effects of SCFAs on growth of 98NK2 were investigated. Growth in ileal SCFA medium had no effect on growth rate (Fig. 3a). However, colonic SCFA medium retarded growth, increasing generation time to about 60 min, compared with 30 min for control medium (MOPS-buffered LB of equivalent osmolality) (Fig. 3b).

To examine the expression profile of *iha* and other relevant STEC genes, real-time RT-PCR analysis was carried out on RNA isolated from 98NK2 after 3 and/or 4 h incubation in ileal or colonic SCFA media, or control medium (see Methods). Time points were chosen on the basis of the growth curves in Fig. 3. No significant increase in expression of either *iha*, *fur* or *tonB* was observed after 3 h growth in ileal SCFA medium (result not shown). Western blot analysis confirmed these results at the protein level for Iha (data not shown). However, 98NK2 exhibited marked *iha* upregulation following growth in colonic SCFA medium (20.1- and 11.9-fold after 3 and 4 h, respectively) (Fig. 4). A similarly marked increase in *tonB* expression was also observed (60.9- and 43.2-fold after 3 and 4 h, respectively). Upregulation of *fur* was less dramatic, but nevertheless significant (4.2-fold at both time points).

Fig. 4. Gene expression levels of *iha*, *tonB* and *fur* as determined by real-time RT-PCR after growth of 98NK2 for 3 h or 4 h in colonic SCFA medium. Data are presented as the increase (*n*-fold) in gene expression levels compared to 98NK2 grown in control medium, normalized to *rrsH* expression. Data are the mean $\pm$ SD of three independent experiments, each assayed in triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; Student's unpaired two-tailed *t*-test.

Fig. 3. Effect of ileal (a) and colonic (b) concentrations of SCFAs on growth of *E. coli* 98NK2. Growth was measured by viable count (c.f.u. ml⁻¹; data are the mean $\pm$ SD, $n=2$), as described in Methods. The dotted lines indicate time points selected for RNA isolation.

Next, we investigated whether growth in colonic SCFA medium had the same effect on *iha* expression in the LEE-positive STEC strains EDL933 and 86-24. As indicated in Fig. 5, both strains exhibited statistically significant upregulation of *iha* expression, albeit to a lesser extent

Fig. 5. Gene expression levels of *iha*, *tonB* and *fur* in 98NK2-Cu, 86-24 and EDL933 after growth in colonic SCFA medium for 3 h. Data are presented as the increase/decrease (*n*-fold) in expression levels compared to cells grown in control medium, normalized to *rrsH* expression. Data are the mean $\pm$ SD of two independent experiments, each assayed in triplicate. *, $P < 0.05$; **, $P < 0.01$; Student's unpaired two-tailed *t*-test.

than in 98NK2. EDL933 contains two identical copies of *iha* (located on O islands OI-41 and OI-48) (Taylor *et al.*, 2002) and exhibited a 3.7-fold increase after 3 h, whereas 86-24, with only one *iha* copy (Tarr *et al.*, 2000), showed a stronger induction (8.2-fold after 3 h). Induction of *tonB* was only modest compared to that seen in 98NK2 (2.8-fold for 86-24 and 2.4-fold for EDL933). Colonic SCFA medium had no significant effect on *fur* expression in either strain (Fig. 5).

Production of Iha was also investigated by Western immunoblotting with Iha_{pO113} antibody (Fig. 6). All STEC strains exhibited marked upregulation of Iha production when grown in colonic SCFA medium relative to the control medium. However, total Iha production in colonic SCFA medium was greater for 98NK2 than for 86-24, which in turn was greater than for EDL933.

To investigate whether regulatory elements present on the megaplasmid pO113 affect *iha* expression, we also investigated the response of the megaplasmid-cured derivative of 98NK2 (98NK2-Cu) to growth in colonic SCFA medium for 3 h. As previously shown for the wild-type, 98NK2-Cu exhibited a marked upregulation of *iha* expression at the mRNA level (285.6-fold) (Fig. 5) and protein level (Fig. 6). Also, *tonB* and *fur* showed similar induction to that observed in 98NK2 (58.7- and 2.3-fold, respectively) (Fig. 5). We also examined the degree of colonic SCFA-induced *iha* upregulation in 98NK2Δ*iha*_{pO113}::*kan*, in which the megaplasmid-encoded copy of *iha* has been inactivated by insertion of a non-polar kanamycin resistance cassette. We observed a 17.2-fold increase in expression of the remaining chromosomal copy of *iha* in this mutant (data not presented), which is similar to the observed *iha* expression in 98NK2 after the same period of induction. Likewise, colonic SCFA-induced increases in expression of *tonB* and *fur* were similar to those for 98NK2 (24.6-fold and 2.6-fold, respectively) (data not presented).

Examination of the absolute *iha* mRNA levels for the strains 98NK2, 98NK2-Cu, 98NK2Δ*iha*_{pO113}::*kan*, EDL933 and 86-24 relative to the internal standard *rrsH* ($C_{t(iha)} - C_{t(rrsH)}$) shows that the *iha* mRNA level under

both basal and colonic SCFA conditions was greater for LEE-negative STEC 98NK2 than for either LEE-positive STEC EDL933 or 86-24 (Fig. 7). This is not simply a gene dosage effect; the *iha* mRNA concentration under both induced and non-induced conditions in 86-24 (which has a single chromosomal copy of *iha*) is not dissimilar to that in EDL933 (which has two chromosomal *iha* copies) under the respective conditions. 98NK2 has a single chromosomal copy of *iha*, as well as one on the (low copy number) megaplasmid pO113. However, 98NK2Δ*iha*_{pO113}::*kan*, which retains only the single chromosomal copy of *iha*, has a similar *iha* mRNA concentration to that observed in 98NK2, in either base or colonic SCFA medium (Fig. 7). On the other hand, the megaplasmid-cured derivative of 98NK2 exhibited a decreased *iha* mRNA level compared to 98NK2 under non-induced conditions, but the *iha* mRNA level under induced conditions was similar to 98NK2.

Regulatory interactions between colonic SCFAs and iron

We have shown above that supplementation of LB with either 2,2'-dipyridyl (Fig. 1) or colonic SCFAs (Fig. 4) causes an almost identical upregulation of *iha*, *tonB* and *fur* in 98NK2 (approx. 20-, 40- and 2–4-fold, respectively, in both conditions). To assess whether Fe availability and SCFA composition interact in terms of *iha* regulation, 98NK2 was grown in colonic SCFA medium supplemented with either 0.2 mM 2,2'-dipyridyl or 10 μM FeSO₄. Addition of 2,2'-dipyridyl to colonic SCFA medium caused no further increase in expression of any of the genes (*iha*, *tonB* and *fur* were upregulated 15.4-, 39.4- and 2.1-fold, respectively) (Fig. 8a). Addition of FeSO₄ to colonic SCFA medium also resulted in a similar expression pattern to

Fig. 6. Immunoblot analysis of whole-cell lysates of 98NK2, 98NK2-Cu, 86-24 and EDL933 with anti-Iha_{pO113}. Cells were grown in colonic SCFA medium (+) or control medium (-) for 4 h. Lysates were then separated by SDS-PAGE, blotted and probed with anti-Iha. The bands represent the 70 kDa Iha antigen. The blot shown is representative of two independent experiments.

Fig. 7. Relative *iha* mRNA levels in 98NK2, 98NK2-Cu, 98NK2Δ*iha*_{pO113}::*kan*, 86-24 and EDL933 after growth in colonic SCFA medium or control medium for 3 h. Real-time RT-PCR data for each gene were normalized against those obtained for the 16S rRNA (*rrsH*) mRNA control using the formula $1/(C_{t(iha)} - C_{t(rrsH)})$. Data are the mean ± SD of two independent experiments, each assayed in triplicate.

Fig. 8. Effect of iron and SCFAs on *iha* expression. (a) Expression levels of *iha*, *tonB* and *fur* in 98NK2 determined by real-time RT-PCR. Cells were grown for 3 h in colonic SCFA medium supplemented with either 10 μ M FeSO₄ (Fe) or 0.2 mM 2,2'-dipyridyl (DPY), as indicated. Data are presented as the increase/decrease (*n*-fold) in expression levels compared to cells grown in control medium, normalized to *rrsH* expression. Data are the mean $\pm$ SD of two independent experiments, each assayed in triplicate. *, $P < 0.05$; **, $P < 0.01$; Student's unpaired two-tailed *t*-test. (b) Western immunoblot. 98NK2 or EDL933 was grown in colonic SCFA medium or control medium, with or without 10 μ M FeSO₄, for 4 h. Lysates were then separated by SDS-PAGE, blotted and probed with anti-Iha. The bands represent the 70 kDa Iha antigen. The blot shown is representative of two independent experiments.

that in colonic SCFA alone (21.5-, 37.8- and 2.4-fold upregulation for *iha*, *tonB* and *fur*, respectively) (Fig. 8a). The effect of Fe on Iha expression in the presence of

colonic SCFAs was also examined by Western blotting in both 98NK2 and EDL933 (Fig. 8b). In both cases, addition of 10 μ M FeSO₄ had no effect on the strong induction of Iha by colonic SCFAs. Thus, colonic SCFAs are capable of overriding Fe-mediated repression of *iha* expression in STEC.

iha expression in 98NK2 Δ *fur*::*kan* and 98NK2 Δ *tonB*::*kan*

To examine whether Fur has any role in the regulation of *iha* transcription in 98NK2 under colonic conditions, a *fur* deletion mutant was constructed as described in Methods. However, colonic SCFA conditions strongly inhibited growth of 98NK2 Δ *fur*::*kan*, preventing transcriptional analysis. We also examined the effect of deletion of *tonB*, in view of the fact that its expression in response to iron-chelation or colonic SCFA conditions paralleled that of *iha* in all STEC strains studied. After 3 h growth of 98NK2 Δ *tonB*::*kan* in colonic SCFA medium, *iha* expression was upregulated approximately 20-fold (data not shown). Thus, deletion of *tonB* has no effect on *iha* expression.

DISCUSSION

Iha is a potentially important accessory virulence factor for several pathotypes of *E. coli*, including STEC, UPEC and avian-pathogenic strains (Ewers *et al.*, 2007; Johnson *et al.*, 2000; Ons *et al.*, 2007; Rodriguez-Siek *et al.*, 2005; Tarr *et al.*, 2000; Touati, 2000), functioning as an adhesin and, at least for UPEC, as a catecholate siderophore receptor (Leveille *et al.*, 2006). This latter function is consistent with the fact that *iha* is induced under iron-limiting conditions and is subject to Fur-mediated repression in iron-replete environments in both UPEC and O157:H7 STEC strains (Leveille *et al.*, 2006; Rashid *et al.*, 2006b), as well as in the hyper-virulent LEE-negative STEC strain 98NK2, as shown in this study. However, the mammalian gut is a complex environment and many other compounds may impact on virulence gene expression. For example, the gut, particularly the colon, is rich in SCFAs such as acetate, propionate and butyrate, which are produced by commensal fermentative flora e.g. *Lactobacillus* sp. and *Bifidobacteria* sp. (Cummings *et al.*, 1987, 2001; Cummings & Macfarlane, 1991; Macfarlane *et al.*, 1992, 2006; Salminen *et al.*, 1998). In this study we have shown that growth in media supplemented with SCFAs simulating colonic conditions strongly induced *iha* transcription and expression of Iha protein in two well-characterized O157:H7 STEC strains (EDL933 and 86-24) and in the O113:H21 strain 98NK2. No such induction was observed under ileal SCFA conditions. These findings are consistent with the belief that the colon, rather than the ileum, is the preferred site of STEC colonization in humans (Paton & Paton, 1998).

Whilst all three STEC strains exhibited *iha* induction in colonic SCFA medium, the strength of the induction (i.e.

fold-increase) at the mRNA level (judged by real-time RT-PCR), and the total amount of Iha produced (judged by Western blotting) appeared to be greater in 98NK2 than in either of the O157:H7 strains. This is also evident from comparison of the absolute *iha* mRNA levels for the various strains relative to the internal standard *rrsH*. Under both basal and colonic SCFA conditions, *iha* mRNA concentration was greater for 98NK2 than for either EDL933 or 86-24 (Fig. 7). One possible explanation may be strain-specific DNA sequence polymorphisms upstream of the *iha* promoter. Comparison of GenBank entries indicates that there are 14 nucleotide differences in the 100 nt region upstream of the *iha* transcription start site, including polymorphisms at positions -85, -10 and -1, and within the putative Fur-binding site, between 98NK2 and both 86-24 and EDL933 (data not presented). Rashid *et al.* (2006b) have not only shown that a nucleotide exchange at position -85 caused higher *iha* expression in CFT073 than in 86-24, but also suggested that additional strain-specific factors, such as repressors, activators and additional nucleotide polymorphisms, may play a role in differential *iha* expression. These polymorphisms may well account for the observed differences in overall *iha* expression both under basal conditions, and in the presence of colonic SCFAs.

An important finding of the present study is that for both the LEE-negative O113:H21 STEC 98NK2 and the LEE-positive O157:H7 STEC EDL933, colonic SCFA-mediated induction of *iha* in 98NK2 could not be repressed by supplementation of the medium with FeSO₄. The inability of exogenous iron to repress *iha* in colonic SCFA medium is not due to poor expression of *fur* in either strain. Indeed, the total *fur* mRNA concentration was greater after growth in colonic SCFAs than in control medium (result not shown). Although the precise mechanism whereby colonic SCFAs induce *iha* expression in STEC is not understood, one can imagine a model whereby a SCFA-induced transcriptional activator interacts with the *iha* promoter region, activating transcription by competitive displacement of Fur.

The findings of this study indicate the potential for SCFA composition to modulate expression of virulence-related STEC genes in different parts of the human gastrointestinal tract. Inter-species variations in gut microflora affecting intestinal SCFA composition may also explain the previously observed differences in expression of several STEC virulence genes (including *iha*) in extracts of bovine versus human faeces (Rashid *et al.*, 2006a). At least for *iha*, upregulation in the human colon (but not in the ileum) may enhance colonization capacity in what is believed to be the preferred host niche. Moreover, given the fact that Iha may function as both an adhesin and a siderophore receptor (Leveille *et al.*, 2006; Rashid *et al.*, 2006b), upregulation of Iha by colonic SCFAs, along with TonB, which is also required for siderophore uptake, may increase the iron-scavenging capacity in the more anaerobic colonic environment. This may be important for *E. coli* energy-metabolism, since deployment of anaerobic energy-gen-

erating enzymes containing multiple Fe-S clusters, rather than iron-free flavoproteins in the respiratory chain, would dramatically increase the demand for iron.

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