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Acid-stress-induced changes in enterohaemorrhagic *Escherichia coli* O157 : H7 virulence

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Enterohaemorrhagic *Escherichia coli* (EHEC) O157 : H7 is naturally exposed to a wide variety of stresses including gastric acid shock, and yet little is known about how this stress influences virulence. This study investigated the impact of acid stress on several critical virulence properties including survival, host adhesion, Shiga toxin production, motility and induction of host-cell apoptosis. Several acid-stress protocols with relevance for gastric passage as well as external environmental exposure were included. Acute acid stress at pH 3 preceded by acid adaptation at pH 5 significantly enhanced the adhesion of surviving organisms to epithelial cells and bacterial induction of host-cell apoptosis. Motility was also significantly increased after acute acid stress. Interestingly, neither secreted nor periplasmic levels of Shiga toxin were affected by acid shock. Pretreatment of bacteria with erythromycin eliminated the acid-induced adhesion enhancement, suggesting that *de novo* protein synthesis was required for the enhanced adhesion of acid-shocked organisms. DNA microarray was used to analyse the transcriptome of an EHEC O157 : H7 strain exposed to three different acid-stress treatments. Expression profiles of acid-stressed EHEC revealed significant changes in virulence factors associated with adhesion, motility and type III secretion. These results document profound changes in the virulence properties of EHEC O157 : H7 after acid stress, provide a comprehensive genetic analysis to substantiate these changes and suggest strategies that this pathogen may use during gastric passage and colonization in the human gastrointestinal tract.

INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) infection is a leading cause of bloody diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome (HUS), the latter representing the major cause of acute renal failure in children (Kaper & Karmali, 2008). Of the EHEC serotypes, O157 : H7 has the highest correlation worldwide with HUS, and in North America it is the predominant EHEC serotype, contributing to over 75 000 human infections annually (Kaper & Karmali, 2008; Mead *et al.*, 1999; Rangel *et al.*, 2005).

EHEC O157 : H7 virulence factors include many fimbrial and non-fimbrial adhesins, flagella, toxins including Shiga toxins, and a type III secretion system (TTSS). Non-fimbrial adhesins include intimin, considered to play a major role in bacterial–host adhesion, as well as the plasmid-encoded *toxB*, the chromosomal genetic locus *efa1* (EHEC factor for adherence), and the chromosomally encoded adhesins Iha (*Vibrio cholerae* IrgA homologue), Cah (calcium-binding antigen 43 homologue) and OmpA (outer-membrane protein A) (Johnson *et al.*, 2005; McKee *et al.*, 1995; Tatsuno *et al.*, 2001, 2001; Torres *et al.*, 2005). Fimbrial structures that have been identified include two long polar fimbriae, F9 (a type 1 pilus homologue), two type IV pili (HCP in EHEC O157 and TFP in a non-O157 EHEC), the sorbitol-fermenting EHEC O157 : H- plasmid-encoded fimbriae, SFP and ECP (*E. coli* common pilus) produced by both pathogenic and non-pathogenic *E. coli* (Blackburn *et al.*, 2009; Brunder *et al.*, 2001; Low *et al.*, 2006; Xicohtencatl-Cortes *et al.*, 2009). Flagella are also critical virulence factors, primarily through permitting bacterial swimming motility. Flagella have also been reported to function as EHEC adhesins, promoting adherence to mucins, the major component of the mucus that lines the gastrointestinal tract (Erdem *et al.*, 2007; Mahajan *et al.*, 2009).

Abbreviations: EHEC, enterohaemorrhagic *Escherichia coli*; EPEC, enteropathogenic *E. coli*; RT, room temperature; TTSS, type III secretion system.

The GEO accession number for the microarray data associated with this paper is GSE14069.

A supplementary table of primers is available with the online version of this paper.

In addition to adhesive virulence factors, EHEC O157 : H7 produce Shiga toxins (Stx1 and/or Stx2), which inhibit the synthesis of host-cell proteins and contribute to EHEC-induced renal disease (Tarr *et al.*, 2005). Other EHEC virulence factors include the locus of enterocyte effacement (LEE)-encoded TTSS translocation and effector proteins, as well as numerous non-LEE-encoded effector proteins (Deng *et al.*, 2004; Tobe *et al.*, 2006), all of which enable the pathogen to modulate host-cell signalling to favour bacterial replication, survival and host colonization.

These virulence factors are important in the establishment of infection and the progression of disease. Virulence gene expression in EHEC has been shown to be regulated by a number of environmental factors including temperature, culture medium and host-cell factors (Abe *et al.*, 2002; Nakanishi *et al.*, 2006; Sperandio *et al.*, 2002). However, the impact of acid stress, particularly that encountered during gastric passage, on EHEC virulence gene expression is not well understood. EHEC O157 : H7 is remarkable in its ability to tolerate acidity; it possesses four different acid-resistance systems that provide protection against exposure to pH as low as 2–2.5 (Foster, 2004; Lin *et al.*, 1996). Interestingly, it uses these acid-resistance systems differentially for survival in foods versus the bovine intestinal tract (Foster, 2004; Price *et al.*, 2004), thus reflecting the fact that these systems are differentially dependent on culture conditions and growth phase. Since the infectious dose of EHEC is typically low (50–100 organisms) (Tuttle *et al.*, 1999), acid tolerance and resistance are critical virulence traits.

In the related pathogen, enteropathogenic *E. coli* (EPEC), the plasmid-encoded regulator, Per, which regulates expression of many EPEC virulence factors, is negatively regulated at pH 5.5 and positively regulated at pH 8.0, suggesting that virulence gene expression is repressed during mild acid stress and enhanced at neutral to alkaline pH, typical of the small intestine, which it colonizes (Shin *et al.*, 2001). In another study, a *gadE* (encoding an acid-resistance regulator) mutation resulted in increased adhesion of *E. coli* O157 : H7 to colonic epithelial cells, again suggesting negative regulation of one or more adhesins during acid stress (Tatsuno *et al.*, 2003). Other studies have reported that production of Shiga toxin is sensitive to culture conditions including pH (Duffy *et al.*, 2000; Yuk & Marshall, 2004). However, to our knowledge there have been no studies of EHEC virulence changes after more severe acid stress, typical of that encountered during gastric passage, nor studies linking an acid-stressed EHEC virulence phenotype with transcriptional changes.

The goal of this study was to determine how acid stress influences EHEC virulence properties and to define the genetic basis for these changes. Understanding how acid stress modulates the virulence potential of this pathogen is essential for delineating the pathogenesis of disease and may offer novel approaches to prevent and treat EHEC infection.

METHODS

Bacterial strains and growth conditions. Bacteria were maintained as glycerol stocks at −80 °C. Prior to use, bacteria (Table 1) were cultured on Luria–Bertani (LB) agar supplemented with the appropriate antibiotics. Since EHEC virulence factors have been demonstrated to be induced during exponential growth in high-glucose DMEM (Rosenshine *et al.*, 1996), prior to infection and binding assays, overnight LB broth cultures were diluted 1 : 5 in DMEM and grown to mid-exponential phase (2–4 h, 37 °C, 5 % CO_2). Bacterial viability was assessed by serial dilution and plating on tryptose phosphate agar with 0.1 % sodium pyruvate.

Cell culture. HEp-2 cells (human laryngeal cell line, ATCC) were grown in MEM (with Earle's salts and L-glutamine; GibcoBRL) with 10 % fetal calf serum (FCS), 0.1 % gentamicin at 37 °C in 5 % CO_2. The human colonic cell line CaCo-2 (ATCC) was grown similarly, but without L-glutamine and with 1 % gentamicin (GibcoBRL). Vero cells (ATCC) were grown in α-minimal essential medium with 5 % FCS and 40 μg gentamicin ml^{-1} at 37 °C in 5 % CO_2.

Bacterial–host-cell adhesion assays. Adhesion of bacteria before and after acid stress to human epithelial cells was assessed by plate count, flow cytometry and fluorescent microscopy as described previously (Barnett Foster *et al.*, 2000, 1999; Dytoc *et al.*, 1993). For the plate count method, approximately 10^6 viable cells were infected with 10^8 bacteria in MEM without supplementary antibiotics for 3–6 h at 37 °C, 5 % CO_2. Colony counts of adherent bacteria were determined using MacConkey agar plates without crystal violet. To assess the involvement of newly synthesized protein adhesins after stress, bacteria were pretreated with a subinhibitory concentration of

Table 1. Bacterial strains used in this study

Strain	Description	Reference
86-24	Wild-type EHEC (O157 : H7), $Stx2^+$	Griffin *et al.* (1988)
85-170	EHEC (O157 : H7), Stx^-	Tzipori *et al.* (1987)
CL56	EHEC (O157 : H7), $Stx1^+$	Karmali *et al.* (1985b)
86-24Δeae	*eae* isogenic 86-24 mutant	McKee & O'Brien (1996)
86-24Δiha	*iha* isogenic 86-24 mutant	Tarr *et al.* (2000)
HS	Non-pathogenic commensal *E. coli*	Lloyd *et al.* (2007)
ORN 172	Non-adherent lab *E. coli*	Tarr *et al.* (2000)
ORN172 pSK	Complemented with pSK vector	Tarr *et al.* (2000)
ORN 172 pIha	Complemented with pSK containing cloned *iha*	Tarr *et al.* (2000)

erythromycin (0.06 mg ml^{-1}) for 30 min at room temperature (RT), prior to cell infection, as described previously (de Jesus *et al.*, 2005; Huesca *et al.*, 1996).

In the flow-cytometric analysis of bacteria–host-cell binding, approximately 10^6 cells (70–80 % confluent monolayer) were infected with 10^8 bacteria for 2 h at 37 °C. After incubation, both detached and adherent (trypsinized) cells were harvested and separated from non-adherent bacteria by centrifugation through an isotonic 15 % sucrose solution. Bacterial binding was detected using a bacteria-specific antibody and a goat anti-rabbit fluorescein isothiocyanate (FITC-GAR) conjugate (Sigma) and quantified by FACS analysis using a FACS Scan Becton Dickson flow cytometer. All samples were analysed with Cell Quest software. Histogram plots showing cell count versus fluorescence intensity were generated.

To assess bacterial–host-cell binding via fluorescent microscopy, a modification of a previously published protocol was used (Dytoc *et al.*, 1993). Approximately 10^8 bacteria (EHEC O157 : H7 GFP86-24) were incubated for 4–6 h at 37 °C, 5 % CO_2 with 10^6 cells grown on glass coverslips (VWR). For the 6 h adhesion assays, medium was removed after 3 h, cells were washed with PBS, fresh MEM (without additives) was added and incubation continued for an additional 3 h. After infection times, coverslips were washed with PBS, and cells were fixed with 4 % paraformaldehyde (Sigma-Aldrich) in PBS and examined using a Carl Zeiss LSM 510 scanning confocal microscope. Images were taken with an argon ion laser 488 514 nm using two channels, channel D, 543, 488 and channel 1, 488. Image analysis was completed with the LSM 510 software package.

Acid-stress protocols. The effect of both brief acute acid stress and acid-adapted acid stress on EHEC virulence properties was investigated. For brief acute acid stress, overnight LB cultures of bacteria (10^9 c.f.u. ml^{-1}) were subcultured into high-glucose DMEM for virulence induction (as above), and aliquots (10^7 c.f.u. ml^{-1}) were centrifuged, resuspended and incubated in DMEM adjusted to pH 3 (RT, 15/30 min), washed and resuspended in pH 7.0 DMEM (designated UA15, UA30 respectively). Unstressed bacteria were incubated in pH 7.0 DMEM, RT, for the same time periods (UU15, UU30).

Acid adaptation may provide partial protection against more acute acid stress and may be physiologically relevant; to address this we investigated the effect of 1 h acid adaptation at pH 5.0 prior to low pH stress. Briefly, overnight LB cultures of bacteria (10^9 c.f.u. ml^{-1}) were induced in high-glucose DMEM (as above), and aliquots containing 10^8 c.f.u. were centrifuged and resuspended in DMEM adjusted to pH 5.0 for 1 h; the bacteria were then centrifuged and resuspended in DMEM adjusted to 3.0 for 15 or 30 min (designated AA15, AA30 respectively). Since the pH drops during the adaptation period, we also assessed the effect of buffering during adaptation. In the unbuffered adaptation step, bacteria were adapted at RT for 1 h in either pH 5.0 (adapted) or pH 7.0 (unadapted) DMEM without any buffering agents. In the buffered adaptation step, DMEM was supplemented with 25 mM MES (pH 5.0) and the adaptation step was carried out at 37 °C and 5 % CO_2 while the control was incubated with DMEM buffered with MOPS (pH 7.0). Acid shocking was conducted at pH 3.0 (unbuffered) at RT for all treatments.

Motility assays. Motility assays were conducted on motility soft agar plates containing 0.3 % (w/v) agar as previously described (Li *et al.*, 2007). Final measurements were taken at 20–24 h post-inoculation.

Shiga toxin production. Shiga toxin production was assayed as previously described (Karmali *et al.*, 1985a; Tam & Lingwood, 2007). Briefly, 1.5×10^4 Vero cells were seeded into 96-well tissue culture plates and grown for 24 h at 37 °C, 5 % CO_2 prior to the experiment. Serial dilutions of bacterial supernatants, periplasmic extracts or a Shiga toxin 1 (Stx1) standard were added to Vero cells and plates were incubated for another 48–72 h at 37 °C, 5 % CO_2, after which Vero cell survival was assessed by crystal violet staining based on absorbance at 570 nm.

Secreted Shiga toxin was prepared from bacterial supernatants as previously described (Yoh *et al.*, 1997). Briefly, an LB overnight culture of strain 86-24 was grown, then induced in DMEM, and subjected to buffered acid-adapted acid stress (or no stress) as described above. Bacteria were pelleted by centrifugation and supernatants were filter-sterilized and stored at −20 °C. To obtain periplasmic extracts, bacterial pellets were resuspended in Colymycin (0.1 mg ml^{-1}, Parker Davis) and incubated statically at 37 °C for 30 min. Samples were then centrifuged and extracts were filter-sterilized and stored at −20 °C. Results were standardized to discount any differences in initial and final c.f.u. ml^{-1} for each treatment prior to and after overnight growth.

As a positive control, a Stx1 stock standard of 1.1 μg ml^{-1} was used (kindly provided by Dr Lingwood, Hospital for Sick Children, Toronto). Yoh *et al.* (1997) have shown that the cytotoxicity of Stx1 and Stx2 on Vero cells was similar, permitting the use of Stx1 as a standard. Final Stx1 standard concentrations in the wells ranged from 2.2×10^1 ng ml^{-1} to 2.2×10^{-7} ng ml^{-1}. The control wells included 50 μl MEM (no additives).

Induction of apoptosis. To establish whether acid stress influences the levels of EHEC-induced apoptosis in infected epithelial cells, the extent of apoptosis was determined by fluorescent dye staining as described previously (Wu *et al.*, 2004). Briefly, subconfluent epithelial cells were infected with EHEC (either unstressed or stressed) at an m.o.i. of 50 : 1 for 1 h, after which non-adherent bacteria were removed and infection allowed to continue for 8 h. Negative controls included addition of DMEM alone. Cells were then stained with acridine orange-ethidium bromide (100 μg ml^{-1}) and cell death was determined by fluorescent microscropy. The percentage of apoptotic cells was determined by counting at least 200 cells in multiple randomly selected fields using blind scoring.

Statistical analysis. Results are expressed as mean ± SD. Analysis of variance was used for statistical analysis of intergroup comparisons. $P \leqslant 0.05$ was considered significant.

Microarray analysis. For the microarray studies, bacteria were grown in LB culture overnight with shaking, then subcultured into DMEM the following day, and grown at 37 °C, 5 % CO_2 statically to mid-exponential phase (OD_{600} 0.4–0.6). Bacteria were exposed to either unadapted acute acid stress for 15 or 30 min (UA15; UA30) or buffered acid-adapted acute acid stress (AA30) and then neutralized to pH 7.0 as described above. Parallel control samples (unstressed) were pre-induced in MOPS-buffered DMEM and incubated in MOPS-buffered DMEM for the same time periods. Each treatment included three independent biological replicates. Following incubation, two volumes of RNA Protect Bacterial Reagent (Qiagen) was added to each culture. Bacteria were pelleted by centrifugation, supernatant removed and samples stored at −80 °C for subsequent purification of RNA. Total RNA was extracted using a modification of a method previously described (Hanna *et al.*, 2001). The quality and integrity of total RNA in each sample was verified using an Agilent Technologies Bioanalyser 2100.

An MWG *E. coli* O157 array (GenBank accession no. GPL 533) was used for gene expression analysis. The array was composed of duplicate spots of 6176 50-mer oligonucleotides (Ocimum Biosolutions oligonucleotide set). Detailed protocols for the cDNA generation and hybridization of the microarrays can be found at the UHN Microarray Centre website (http://www.microarrays.ca). Microarrays were scanned using the Agilent G2565BA scanner,

yielding two 16-bit TIFF images per array, one corresponding to the Cy3 channel and one corresponding to the Cy5 channel. The images were quantified using GenePix Pro 3.0 (Molecular Devices). Before normalization, each individual array spot was trimmed to exclude bad or empty spots identified by GenePix Pro 3.0. Normalization was performed using the LOWESS algorithm. For each point, three biological and two technical replicates were measured, resulting in a maximum of six gene expression values per gene per treatment. Signal intensities were averaged among the technical replicates. Two types of data analysis were performed: to identify density-dependent changes in gene expression, single averaged normalized signal intensities for each treatment point were compared to the mean of corresponding unstressed control signal intensities. Only genes with a relative signal $\log_2$ ratio (SLR) value above 1.0 or below −1.0 were selected for further analysis. Significant genes within each treatment were identified using a one sample *t*-test ($P<0.05$).

Real-time PCR. Real-time PCR was performed using a Roche LightCycler and a Quantitect SYBR-Green PCR kit (Qiagen). Primers were designed using MAC Vector software to generate amplicons ranging from 100 to 170 bp in size (for primer sequences see Supplementary Table S1, available with the online version of this paper). Real-time PCR was performed in triplicate in 20 µl reaction mixture containing 10 µl 2× QuantiTec SYBR Green PCR Master Mix, 1 µl primers (0.5 µM) and 5 ng cDNA. Reaction mixture without template was run as a control. Cycling conditions were as follows: 95 °C for 15 min followed by 40 cycles of three steps consisting of 94 °C for 15 s, primer annealing at optimal temperature for 30 s, and 72 °C for 30 s. For each primer set, the cycle threshold values (CP, crossing point), defined as the first cycle showing an amount of PCR product above background, was determined. Relative expression was based on the expression ratio between target gene versus reference gene. As a reference gene, glyceraldehyde-3-phosphate dehydrogenase, *gapA*, was selected as it showed a constant low expression throughout the treatment protocols and has also been chosen by other authors (Fitzmaurice *et al.*, 2004; Noller *et al.*, 2003). The mean CP of the genes, the CP variation *n* and the coefficient of the variation (CV) were calculated to determine reproducibility and variation. Linearity and amplification efficiency were determined for each primer pair, and only values corresponding to high amplification efficiency in the exponential range were used. Every real-time PCR was performed to high amplification efficiency in the exponential range used, and was performed in triplicate.

RESULTS

Bacterial survival after acid stress

The viabilities of three well-characterized EHEC O157 : H7 strains (two prototypic strains, 86-24 and 85-170, and one clinical isolate, CL56) were examined after exposure to acid stress. In all cases, acid-stressed EHEC O157 : H7 had significantly lower viabilities relative to time zero (prior to the acid-stress treatment) ($P<0.05$). However, there was never more than a log-fold difference in viability after acid stress, indicating that the EHEC O157 : H7 strains tested were able to withstand both brief acute acid stress and acid-adapted acute acid stress. Viabilities (% survival) after exposure to brief acute acid stress (pH 3) were 51.0 ± 7.0 % and 45.7 ± 17.4 % for 15 and 30 min of stress respectively. After unbuffered adapted-acid stress (unbuffered pH 5.0 adaptation followed by pH 3.0 acid stress), viabilities were 88.0 ± 8.3 % and 78.4 ± 5.8 % for 15 and 30 min of acute acid stress respectively, indicating that adaptation had a protective effect. Viabilities after buffered adapted-acid stress (86.0 ± 10.0 % and 85.7 ± 10.1 % for 15 and 30 min of acute acid stress respectively) were similar to the unbuffered adapted-acid stress, suggesting that the buffering during the adaptation stage did not significantly enhance survival during acute acid stress.

Adhesion of EHEC O157 : H7 to epithelial cells is increased after acid stress

Adhesion of several EHEC O157 : H7 strains was tested before and after acid stress to two different epithelial cell lines (HEp-2 and CaCo-2) using three different adhesion assays (plate count, fluorescent microscopy and flow cytometry). Since Shiga toxin has been reported to enhance adhesion of EHEC to human epithelial cells (Barnett Foster *et al.*, 2000; Robinson *et al.*, 2006), strain 85-170, which is Stx-negative, was included in this study to rule out any contribution of Stx-mediated adherence. Adhesion of acid-adapted acid-stressed EHEC O157 : H7 to both cell lines was significantly increased relative to the unstressed controls (Fig. 1). Adhesion enhancement ranged from 160 to 486 % relative to the controls. Adhesion of acid-adapted acid-shocked EHEC O157 : H7 strain CL56 to the colonic epithelial cell line CaCo-2, as measured by plate count assay, was significantly enhanced, showing up to 486 % increase in a 6 h adhesion assay after acid-adapted 30 min acute acid stress (Fig. 1a). Similar results were achieved with two other EHEC O157 : H7 strains, 86-24 and 85-170, with significant adhesion increases after acid-adapted 30 min acid stress ranging from 257 % to 322 % in a 3 h HEp-2 cell-adhesion assay. Fluorescent microscopy also showed dramatic increases in the adhesion of GFP-labelled 86-24 after acid-adapted acid shock (Fig. 1b). Flow cytometric analysis similarly showed a significant increase in adhesion of strain CL56 to HEp-2 cells after just 5 min of acute acid shock preceded by acid adaptation (not shown). In contrast, the adhesion of the commensal strain HS was not increased after the same acid treatment (not shown). When the DMEM acid-adaptation step was buffered using MES, the adhesion enhancement changes were more modest, indicating that the adhesion enhancement was sensitive to even subtle pH changes during the stress protocol (Fig. 1c).

Adhesion of acid-shocked EHEC O157 : H7 is reduced by pretreatment with erythromycin

Since the adhesion enhancement may result from increased expression of known and/or new adhesins or modification of existing adhesins, it was important to determine the impact of *de novo* protein synthesis on acid-induced adhesion. Erythromycin used at subinhibitory concentrations has been shown to inhibit bacterial protein synthesis and has been used to inhibit protein synthesis after bacterial stress (de Jesus *et al.*, 2005; Huesca *et al.*, 1996). Pretreatment with erythromycin, at a subinhibitory

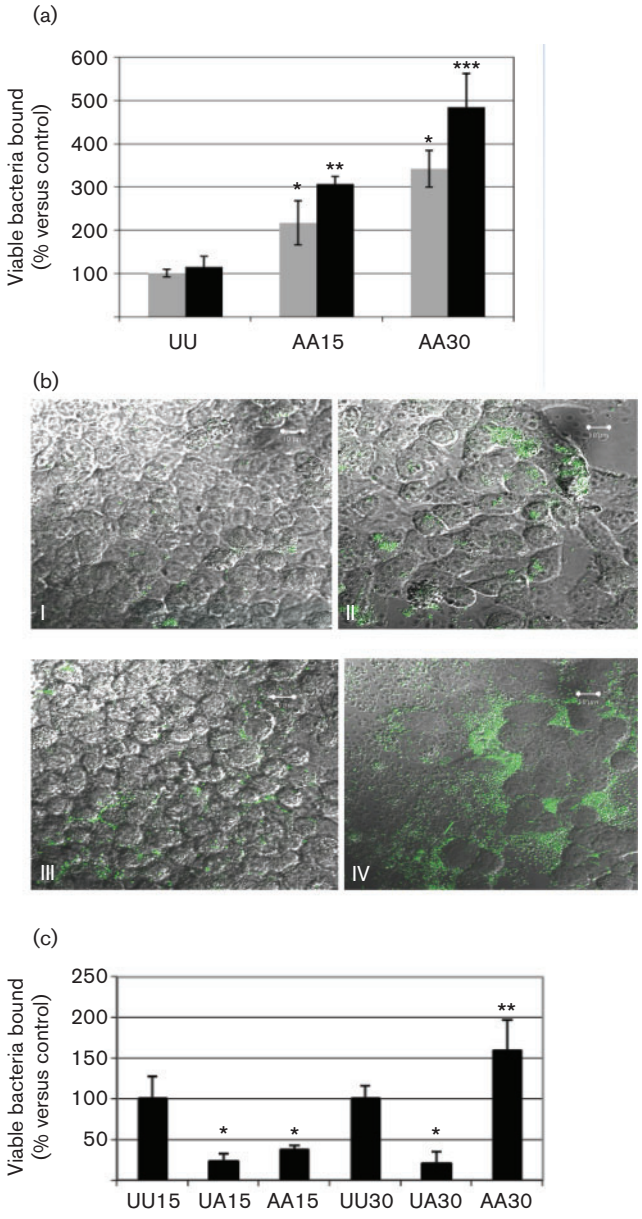

Fig. 1. Epithelial cell adhesion of acid-shocked EHEC O157 : H7. (a) Adhesion of acid-adapted acid-shocked CL56 to CaCo-2 cells in a 3 h (grey) or 6 h (black) adhesion assay. *Significantly different from 3 h unadapted unshocked (UU) control. **Significantly different from 6 h UU control. ***Significantly different from 6 h UU control and 6 h AA15. (b) Confocal microscopic analysis of adhesion of GFP-labelled 86-24 to HEp-2 cells. I, UU control (4 h incubation); II, AA30 (4 h incubation); III, UU control (6 h incubation); IV, AA30 (6 h incubation). Scale bars, 10 μm. (c) Adhesion of buffered adapted acid-shocked 85-170 to HEp-2 cells in a 3 h adhesion assay. *Significantly different from corresponding control (UU15 or UU30). **Significantly different from corresponding control (UU30), UA30 and AA15.

concentration of 0.06 mg ml^{-1}, completely eliminated the adhesion enhancement of acid-adapted acid-shocked EHEC O157 : H7 but had no effect on the adhesion of unstressed EHEC O157 : H7 (Fig. 2).

Shiga toxin production remains unaltered after acid stress

The effect of acid stress on Shiga toxin production (both secreted and periplasmic extracts) of EHEC O157 : H7 86-24 was examined using the Vero cell cytotoxicity assay. None of the experimental acid-stress treatments (UA15, UA30, AA15, AA30) resulted in a significant change in Shiga toxin production, either in the periplasmic extracts or in the supernatant filtrates, as compared to the unadapted, unshocked controls. All filtrates and extracts were analysed over a broad range of dilutions with three replicates for each dilution. The CD_{50} (dose to cause death of 50 % of cells) for the Stx1 standard was 0.5 pg ml^{-1}, which is consistent with that reported in the literature (Hoey *et al.*, 2003). Culture supernatants from both unshocked and acid-shocked *E. coli* 86-24 typically contained 20 ng ml^{-1}. Results were representative of three independent experiments.

Swimming motility of EHEC O157 : H7 is increased after acid stress

EHEC O157 : H7 motility was modestly, but significantly, increased, by 128–137 %, after acute acid stress as compared with unstressed EHEC O157 : H7 (Fig. 3).

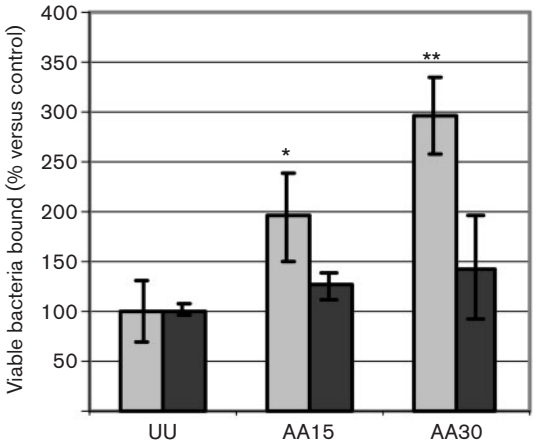

Fig. 2. Effect of pre-incubation with erythromycin (0.06 mg ml^{-1}) on adhesion of acid-adapted acid-stressed EHEC O157 : H7 85-170 to HEp-2 cells as assessed by plate count assay (n=4–6). UU, control (unadapted, unshocked); AA15 and AA30, acid adapted and acid shocked for 15 and 30 min respectively without erythromycin (grey), with erythromycin (black). Values are means ± SD. *Significantly different from AA15 with erythromycin; **significantly different from AA30 with erythromycin.

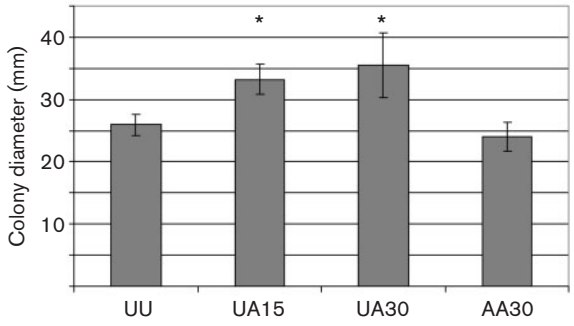

Fig. 3. Motility of EHEC O157 : H7 86-24 before and after acid stress. Motility was measured in a 24 h soft agar swimming assay; results are expressed as motility diameter means ± SD ($n=4$) and are representative of three experiments. *Significantly different from control UU.

Apoptosis of epithelial cells infected with acid-stressed EHEC O157 : H7 is increased

Epithelial cells infected with acid-stressed EHEC O157 : H7 showed significantly increased levels of apoptosis relative to cells infected with unstressed EHEC O157 : H7 as determined by acridine orange-ethidium bromide staining (Fig. 4). Levels of apoptosis increased 200–400 % with increased time of acid shock, correlating with epithelial cell-adhesion increases (Fig. 1). This finding is consistent with our previous study showing a strong correlation between extent of host-cell apoptosis and host-cell adhesion of EHEC O157 : H7 (Barnett Foster *et al.*, 2000).

Microarray and real-time PCR analysis of changes in EHEC O157 : H7 virulence gene expression after acid stress

To investigate the genetic basis for the changes in EHEC adhesion and virulence observed after acid stress, DNA microarray analysis was conducted. The transcriptome of EHEC O157 : H7 (strain 85-170) was analysed under three different acid-treatment protocols; the full dataset is available through the GEO database (GEO accession no. GSE14069). For each treatment, the expression of each gene was normalized to its expression in unstressed conditions. With a cut-off value of 2 for the fold change in expression ($P \leqslant 0.05$), we found 2.8–4.7 % of the genome upregulated and 0.45–2.1 % of the genome downregulated after acid stress (dependent on the acid-stress protocol) (Table 2). Brief acute acid stress downregulated 2.1 % of the genome but when the acid stress was prolonged, with or without prior adaptation, there was a recovery of expression (UA30, 0.45 %; AA30, 0.9 % downregulated) and upregulation, particularly after unadapted acute acid stress (UA30, 4.7 %; AA30, 2.8 %). There were significant changes in the expression of genes encoding known virulence factors, including flagella, TTSS proteins and fimbrial proteins (Table 3). By contrast, there was no significant change in the expression of the housekeeping gene *gapA* (encoding glyceraldehyde-3-phosphate dehydrogenase), either by array or by real-time PCR (not shown). Acute acid stress (UA30), as well as acid-adapted acid stress (AA30), upregulated many flagellar biosynthesis and regulatory genes (Table 3). Numerous genes encoding TTSS factors (including EspA, EspB, EspD, EscF, CesT and Tir) were significantly downregulated after brief acute acid stress (UA15), but showed recovery after either prolonged acute acid stress or acid-adapted acid stress (Table 3). Real-time PCR demonstrated that the expression of genes encoding EspA and intimin was decreased under brief acid stress (UA15, AA15) but showed recovery, and increased expression, after prolonged acute acid stress (UA30) (Fig. 5a).

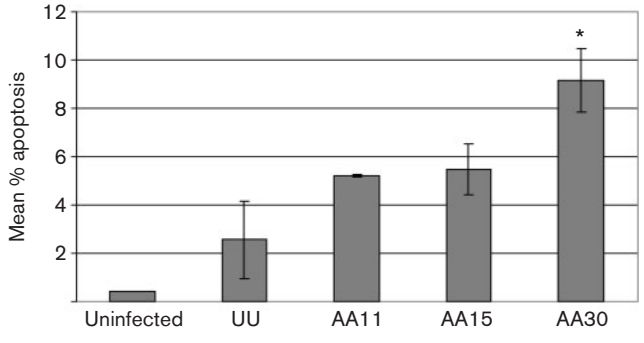

Fig. 4. Acid stress of EHEC O157 : H7 85-170 augments induction of host-cell apoptosis. The ordinate shows percentage of apoptotic cells in a population of >200 cells averaged over duplicates. Results are representative of two independent experiments. *Significantly different from control UU.

The dataset was also scanned for changes in fimbrial and adhesin-related genes (Table 3). Consistent with the flagella and TTSS gene changes, gene expression of several fimbrial and adhesin-related genes decreased after brief acute acid stress (UA15), and then recovered and showed upregulation after either prolonged acute acid stress or acid-adapted acid stress, suggesting that these gene products may play a role in the enhanced adhesion of acid-stressed EHEC. Several genes within the *ecp* (*yag*) operon were upregulated across acid treatments, with *yagZ* significantly upregulated for UA30 treatment, suggesting that this adhesin may participate in host adhesion under all the acid-stress treatments tested. The data also showed upregulation of some *lpf* genes at UA30, suggesting that long polar fimbriae may play a role in host adhesion under this acid treatment.

Since our adhesion data showed a significant increase in host adhesion under acid-adapted acid stress (AA30) relative to the other two acid treatments, we were also interested in identifying genes that showed upregulation under this treatment. Some potential candidates emerge including *yadK*, curli and *lpf2*. The involvement of these adhesins in acid-adapted acid stress is currently being investigated.

Table 2. Summary of total gene changes for each acid-stress treatment as determined by DNA microarray

Number (%) of genes significantly ($P \leqslant 0.05$) up- or downregulated twofold or more relative to the unstressed control for each of three treatments: UA15 and UA30, unadapted, acid shocked for 15 and 30 min; AA30, acid adapted, acid shocked for 30 min.

Treatment	No. (%) of total genes up- or downregulated (6261 total oligos)	
	Twofold up	Twofold down
UA15	205 (3.3 %)	131 (2.1 %)
UA30	292 (4.7 %)	28 (0.45 %)
AA30	177 (2.8 %)	54 (0.9 %)

The microarray results also showed upregulation of numerous decarboxylase genes, two being statistically significant (ornithine decarboxylase, fivefold UA30; glycine decarboxylase, fourfold UA15). Real-time PCR demonstrated that after acid-adapted acid stress (AA15 and AA30), genes *adiA* (arginine decarboxylase, acid resistance 3), *gadA* (glutamate decarboxylase, acid resistance 2) and *sucA* (succinate dehydrogenase) were upregulated twofold or more ($P \leqslant 0.05$) (Fig. 5b). These genes have been reported to be induced under acid conditions (Foster, 2004; Kannan *et al.*, 2008; Maurer *et al.*, 2005).

Intimin and Iha are not required for acid-induced adhesion enhancement of EHEC O157 : H7

Intimin and Iha are known EHEC adhesins (McKee *et al.*, 1995; Tarr *et al.*, 2000); however, their role in the acid-induced adhesion to epithelial cells has not been established. Real-time PCR results indicated that intimin was significantly upregulated after acid-adapted acid stress and could therefore be involved in the adhesion of acid-adapted acid-stressed EHEC O157 : H7 (Fig. 5a). However, when the adhesion of wild-type and intimin-negative (*eae*) 86-24 strains were compared, similar increases in adhesion of both strains after acid stress relative to their unstressed correlates were observed, ruling out an essential role for intimin in the adhesion enhancement of acid-stressed EHEC O157 : H7.

The microarray data indicated a large though not a statistically significant increase in *iha* expression after acid-adapted 30 min acid stress (AA30; upregulated by 3.10 ± 1.8-fold, $P=0.08$). However, when the adhesion of wild-type 86-24 and the *iha* isogenic mutant before and after stress were compared, similar increases in adhesion of both strains after acid stress relative to the unstressed correlates were observed, suggesting that Iha was not essential to the adhesion enhancement after acid stress. To confirm this, we also compared the adhesion of a non-pathogenic ORN 172 strain to its isogenic *iha*-complemented mutant (ORN 172 pIha) before and after stress. In a finding similar to that for the non-pathogenic commensal strain, HS, no increase in adhesion of the non-pathogenic ORN 172 strain after acid stress was observed. Similarly, no increase in adhesion of acid-stressed ORN 172 pIha was seen, reinforcing the previous finding that Iha was not essential to the adhesion enhancement following acid stress.

DISCUSSION

Many opportunities for acid-stress exposure exist for EHEC, including the anorectum of cattle, animal feed, food-processing conditions, acidic foods and the human gastrointestinal tract. To establish infection, this pathogen must breach the acidic environment of the stomach, which, under normal fasting conditions, can be as low as pH 2.5–3.0. Although the molecular strategies utilized by this pathogen to survive acid stress are well understood (Foster, 2004), there is little information on the influence of acid stress on EHEC O157 : H7 virulence. Several other gastrointestinal pathogens (*Helicobacter pylori*, *Legionella pneumophila*, *Clostridium difficile*, *Salmonella typhimurium* and *Legionella monocytogenes*) show enhanced virulence after exposure to acid stress, including increased host-cell adhesion and invasion, macrophage apoptosis, and lethality in mouse models (Conte *et al.*, 2000; de Jesus *et al.*, 2005; Fernandez *et al.*, 1996; Hennequin *et al.*, 2001; Hoffman & Garduno, 1999; Huesca *et al.*, 1996; O'Driscoll *et al.*, 1996; Waligora *et al.*, 1999). The present study now demonstrates that there are also significant changes in EHEC O157 : H7 virulence properties after acid stress.

Acid stress was shown to significantly enhance the adhesion of EHEC O157 : H7 to human epithelial cells. The adhesion increases were consistently observed for several different EHEC O157 : H7 strains, with two different epithelial cell lines including a colonic cell line. This phenotype was not observed with the non-pathogenic strains tested. When EHEC were acid adapted and then acid stressed, adhesion increases were larger (up to 486 % of the unstressed control). Research has shown that after a large meal, human stomach pH can rise to as high as pH 6 before dropping to pH 2 (Foster, 2004), suggesting that the acid-adaptation response may be physiologically relevant to colonization of the human intestines. EHEC may also experience mild acid stress in acidic foods followed by more acute acid stress upon ingestion (Erickson & Doyle, 2007). The relevance of the adaptation response is also reinforced by the finding that EHEC O157 : H7 that are already expressing an acid-resistance system remain acid-resistant for at least a month under refrigeration (Lin *et al.*, 1996). We found that pretreatment with subinhibitory concentrations of erythromycin eliminated the adhesion enhancement, indicating that the acid-induced adhesion increase requires protein synthesis, presumably of existing or new adhesins, or both. We also detected increased host cell apoptosis, which is consistent with the increases seen in bacterial adhesion and with earlier findings that host-cell apoptosis is induced by EHEC O157 : H7 binding (Barnett

Table 3. Changes in expression of selected categories of genes: microarray results showing fold changes in expression of flagella, fimbrial and adhesin-related and TTSS genes

Boldface values represent significant changes ($P \leqslant 0.05$).

Gene ID	Hypothetical function	UA15	UA30	AA30
Flagellar genes				
ECS1453	Flagellar biosynthesis, initiation of hook assembly; FlgD	−2.31	**4.38**	2.41
ECS2602	Regulator, flagellar biosynthesis, acts on class 2 operons; FlhD	**2.29**	2.16	1.64
Z1711	Cell-proximal portion of basal-body rod; FlgB	1.62	**4.29**	**3.20**
Z1716	Cell-distal portion of basal-body rod; FlgG	−1.48	1.76	**3.14**
Z1717	Basal-body outer-membrane L (LPS) ring protein; FlgH	−1.67	1.02	**1.95**
Z1719	Flagellar biosynthesis; FlgJ	−1.93	**4.59**	−1.19
Z1948	Reoxidizes DsbA after S–S bond formation in flagellar P-ring; DsbB	**2.03**	1.44	1.26
Z3014	Filament capping protein; enables filament assembly; FliD	−1.07	**2.68**	1.42
Z3015	Flagellar biosynthesis; repressor of class 3a, 3b operons; FliS	3.61	**4.43**	**1.97**
Z3031	Flagellum-specific ATP synthase; FliI	−2.76	−1.79	**−2.49**
Z3034	Flagellar biosynthesis; FliL	−4.40	1.19	**2.18**
Z3040	FliR	16.15*	**8.48**	**3.42**
Fimbrial and adhesin-related genes				
B0107	Putative membrane protein – biogenesis of fimbriae; HofB	1.64	**7.46**	5.45
B1202	Putative adhesion and penetration protein; b1202	1.10	4.48*	1.03
B0136	Putative fimbrial protein; YadK	1.00	5.87	**4.94**
B0293	ORF, hypothetical protein; YagZ	2.89	5.63*	4.98
B2000	Outer-membrane fluffing protein, similar to adhesin; Flu	−1.52	−1.46	**−4.20**
B2335	Putative fimbrial protein; b2335	−2.97	−1.07	**−2.85**
B2339	Putative fimbrial-like protein; b2339	−1.14	0.81	6.85
B1042	Curlin major subunit, coiled-surface structures; cryptic; CsgA	**−5.54**	1.35	2.01
ECS0320	Putative receptor; YagW	3.29	28.84*	7.94
ECS0321	Putative enzyme; YagX	3.37	5.98*	6.62
ECS0322	ORF, hypothetical protein; YagY	2.57	5.28*	3.99
ECS0597	Fimbrial Z protein; probable signal transducer; FimZ	2.58	**3.36**	2.38
ECS1396	AidA-I adhesin-like protein	−2.69	1.32	1.67
ECS1417	Putative 2-component regulator 2nd curli operon; CsgD	1.14	2.15	**3.02**
ECS3217	Putative fimbrial-like protein	−2.85	**3.36**	1.73
ECS3218	Putative fimbrial protein	−2.20	**2.94**	1.71
ECS4665	Putative fimbrial protein	1.14	**2.26**	1.19
ECS4668	Hypothetical protein	−2.64	3.77	10.10*
ECS4669	Putative fimbrial chaperone	−7.60	−1.14	**1.95**
Z0152	Putative fimbrial-like protein	−2.76	**6.09**	2.66
Z0356	ORF, hypothetical protein; YagV	9.02*	**4.06**	3.31*
Z0639	Adhesin/invasin-like protein	1.24	**14.29**	3.47
Z2897	ORF, hypothetical protein; YebE	−1.27	−1.39	**−5.54**
Z2905	ORF, hypothetical protein; YebK	**2.18**	1.64	1.24
Z2908	ORF, hypothetical protein; YebA	−1.09	−1.18	**−2.58**
Z2909	Putative adhesin; YebL	**2.00**	1.97	−1.27
Z4504	Putative fimbrial protein; YraK	−2.69	−1.50	−3.45
Z4969	Putative fimbrial chaperone protein precursor	2.17	**3.58**	2.75
Z4971	Putative fimbrial major protein precursor; LpfA	26.72*	2.60	1.85
Z5112	NLE=Tir, translocated intimin receptor Tir	**−16.07**	−2.23	−1.13
TTSS genes				
ECS3721	TTSS protein EprS	−1.51	**14.09**	**6.90**
ECS3723	TTSS protein EpaR1	10.13	4.94	**4.01**
Z4199	Hypothetical protein	**59.16**	9.51*	11.79*
Z4201	ORF, hypothetical protein; b2863	11.82	**2.38**	1.69
Z5103	EscF protein	−7.21	−1.84	1.87
Z5104	Hypothetical protein	**−20.82**	−1.93	1.01
Z5105	NLE=EspB, EspB protein	**−28.51**	−5.07	1.20
Z5106	EspD protein	**−16.34**	−3.30	1.79
Z5107	EspA protein	**−115.63**	−6.56	1.82

Table 3. cont.

Gene ID	Hypothetical function	UA15	UA30	AA30
Z5111	CesT protein	**−3.47**	−1.81	1.26
Z5112	NLE=Tir, translocated intimin receptor Tir	**−16.07**	−2.23	−1.13
Z5120	TTSS protein EscV	**−3.75**	−1.89	2.24
Z5140	Ler protein	1.49	−1.77	**−3.00**

*All replicates twofold or more upregulated.

Foster *et al.*, 2000). Finally, there was a significant but modest increase in swimming motility after acute acid stress but not after acid-adapted acid stress, which may be part of the pathogen's survival strategy in the face of acid stress.

Neither acute acid stress nor acid-adapted acid stress resulted in any change in Shiga toxin production. Others have reported either no change or reduced Stx production after acid adaptation alone (Duffy *et al.*, 2000; Leenanon *et al.*, 2003; Yuk & Marshall, 2004). To our knowledge this is the first study to report no effect of either acute acid stress or acid-adapted acid stress on Stx production. Since Stx is likely transcytosed across the intestinal epithelium at the site of bacterial attachment in the large intestine (Hurley *et al.*, 2001), it is possible that Stx production may be more responsive to cues from the microenvironment of the large intestine rather than the acid stress of gastric passage.

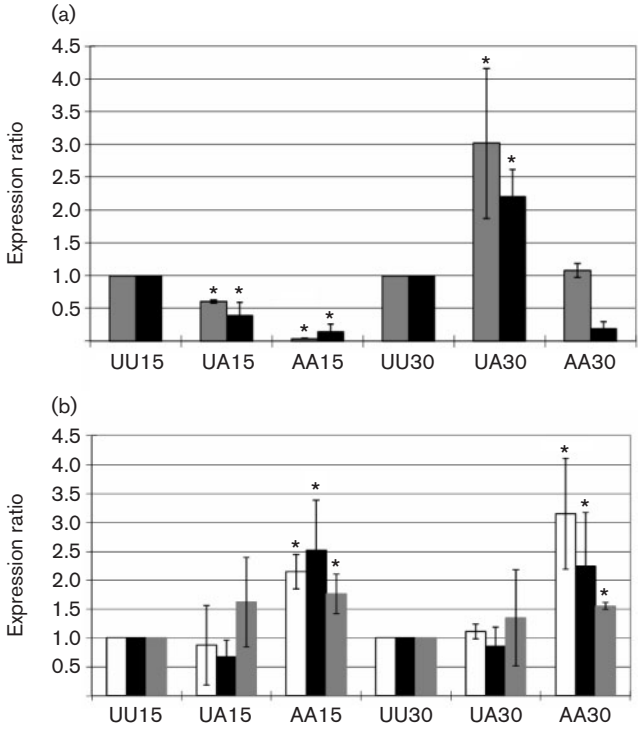

Fig. 5. Relative expression ratios of acid-stressed EHEC O157 : H7 85-170 genes normalized to *gapA* and to the relative unadapted unshocked controls. Results for: (a) intimin (grey) and *espA* (black) (n=3); (b) *adiA* (white), *gadA* (black), *sucA* (grey) (n=3). Results were obtained using the Pfaffl mathematical model for gene normalization and relative quantification. Statistical analysis was done using parametric *t*-tests. *Significantly different from matched control.

The microarray results confirm the acid-triggered changes in virulence properties. The data show significant changes in gene expression of virulence and adhesin-associated genes for each of the three acid-treatment protocols. There was significant upregulation of many flagella-related genes after UA30 treatment, which is consistent with the increased motility associated with this treatment. This finding is in contrast to our previous work on acid-induced changes in the related pathogen, EPEC, where no increase in flagella expression after acid shock was observed (de Jesus *et al.*, 2005). This may reflect functional differences between these two flagella types, since H6 flagella of EPEC have been shown to promote adhesion to human epithelial cells whereas H7 flagella of EHEC O157 : H7 do not (Giron *et al.*, 2002). Another study indicated that flagellar synthesis genes are strongly induced at pH 5.0 while *che* and *mot* genes are weakly repressed at the same pH (Maurer *et al.*, 2005). We found that acute acid stress corresponded to a modest increase in motility, a significant upregulation of numerous flagellar synthesis genes but no significant change in the majority of *che* and *mot* genes. It has been established that low external pH combined with unaltered cytoplasmic pH contributes to the proton-motive force which drives flagellar rotation (Khan & Macnab, 1980; Polen *et al.*, 2003). However, weak organic acids that depress the cytoplasmic pH can decrease the proton-motive force and impair the mechanochemical reaction cycle of the flagellar motor but not the actual torque-generation step (Minamino *et al.*, 2003; Nakamura *et al.*, 2009). Taken together, these findings suggest a complex regulation of flagella and flagella-related genes related to different aspects of flagellar structure and function. It should also be noted that other studies have focused on changes in extracellular pH over a range from 7 to 5 while our study has examined more dramatic changes in extracellular pH.

Expression of genes encoding type III secretion factors was significantly decreased after brief acute acid shock but

showed recovery and/or increase after prolonged acute acid stress and acid-adapted acid stress. Expression of LEE genes has been shown to be regulated by environmental factors including culture conditions, temperature, biocarbonate ion and nutrient levels (Abe *et al.*, 2002; Kenny *et al.*, 1997; Nakanishi *et al.*, 2006; Puente *et al.*, 1996; Rosenshine *et al.*, 1996; Sperandio *et al.*, 2002) and in EPEC it is also regulated by acid-tolerance response mechanisms (Shin *et al.*, 2001).

A similar trend was observed for genes encoding fimbrial genes and other adhesins. Interestingly, different patterns of expression for the three treatment groups were noted, suggesting a complex regulation of adhesins under acid stress and possibly coordination among selected adhesins under different acid-stress conditions. Our adhesion data combined with the microarray data suggest that adhesion under different acid-stress treatments involves different sets of adhesins, with some adhesins downregulated and others upregulated for a particular acid-stress treatment. It is likely that the acid-induced adhesion enhancement involves several adhesins that work synergistically depending on the nature of the acid-stress treatment. Adhesion assays using isogenic intimin and *iha* mutants indicate that that these adhesins are unlikely to be involved in the acid-induced adhesion enhancement. The microarray data identified a number of known and putative adhesins including some that are expressed in non-pathogenic *E. coli* as well. There was no acid-induced adhesion increase for the non-pathogenic strains tested, suggesting that regulation of these adhesin genes is different in O157 : H7 and/or that post-transcriptional events minimize the involvement of these particular adhesins. Since several fimbrial and non-fimbrial adhesins were upregulated in our microarray study, the roles of these adhesins individually and in concert in acid-induced EHEC host-cell adhesion are being investigated.

This study highlights the importance of acid stress in altering virulence properties of EHEC O157 : H7. Although acid stress reduces survival of this pathogen as a population, the adhesive capacity of the surviving organisms is dramatically enhanced, a trait that may be critical for the colonization of the large intestine and for disease progression. Acid-triggered increases in flagella expression and motility may also favour enhanced virulence of this pathogen. Given that the infectious dose of this pathogen is very low, the importance of these adhesion and motility increases should not be trivialized. Understanding how this pathogen adapts to and uses host-environmental cues to influence its virulence phenotype is critical to the development of prevention and treatment strategies.

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