

# Proteome Analysis of a Food Borne Pathogen Enteroaggregative *Escherichia coli* under Acid Stress

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## Abstract

Enteroaggregative *Escherichia coli* (EAEC) is emerging as a significant diarrheal pathogen in multiple population groups. Like any other enteropathogen, EAEC may encounter life-threatening levels of inorganic acids (H<sup>+</sup>) during their natural route of infection. In this study, we showed that EAEC orchestrates acid tolerance by modulating the levels of acid-induced outer membrane proteins (OMP). EAEC (T8) was grown *in vitro* by mimicking *in vivo* pH conditions of both in stomach and intestine. The lowest pH where EAEC showed growth was 4.0 in Luria Bertani (LB) media, and surprisingly during log phase two *de novo* OMP of sizes 41 kDa and 48 kDa were exclusively observed at this pH. Further, acid-induced proteins (ASP) at pH 4.0 were identified by 2D gel electrophoresis and Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF). The role of the most notable acid/pH response regulator *rpoS* in ASP expression was determined by gene knockout in the clinical strain EAEC (T8). Mutation of *rpoS* gene suppressed growth and down-regulated the expression of ASP in EAEC (T8) at pH 4.0 confirming probable role of *rpoS* in EAEC.

**Keywords:** Acid shock proteins; Acid stress; Diarrhea; Enteroaggregative *Escherichia coli*; *rpoS*

**Abbreviations:** 2D PAGE: Two Dimensional Polyacrylamide Gel Electrophoresis; AA: Aggregative Adherence; ACN: Acetonitrile; Ambic: Ammonium bicarbonate; AR: Acid Resistance; ASP: Acid Shock Protein; ATR: Acid Tolerance Response; EAEC: Enteroaggregative *Escherichia coli*; EAEC (T8): Clinical isolate of EAEC; CFU: Colony Forming Units; DA: Diffusely Adherent; HEp-2: Human Epithelial pharyngeal cell line; EHEC: Enterohemorrhagic *Escherichia coli*; IEF: Isoelectric focusing; IPG: Immobilized pH Gradient; IPTG: Isopropyl  $\beta$ -D-thiogalactopyranoside; LA: Luria Agar; LB: Luria Bertani; MALDI-TOF: Matrix Assisted Laser Desorption Ionization-Time of Flight; MS: Mass Spectrometry; MT8: *rpoS* gene mutant in EAEC (T8); OMP: Outer Membrane Protein; PMF: Peptide Mass Fingerprint; PMSF: Phenyl Methyl Sulphonyl Fluoride

## Introduction

Enteroaggregative *Escherichia coli* (EAEC) are increasingly recognized as an emerging pathotype responsible for acute and persistent diarrhea in infants both in developing and developed countries (Huang and Dupont, 2004), in human immunodeficiency virus/acquired immunodeficiency syndrome patients (HIV) (Mayer and Wanke, 1995) and in travelers (Adachi et al.,

2002). It has been shown that EAEC can induce growth impairment and malnutrition among children even without diarrhea (Nataro et al., 1998). The defining feature of EAEC is its ability to elicit characteristic “stacked brick” like aggregative adherence (AA) to HEp-2 cells. EAEC infections are mediated by 60MDa plasmid (pAA) encoding aggregative adherence fimbriae (AAF) for AA phenotype to human erythrocytes, and clump and biofilm formation (Bhardwaj et al., 2006). EAEC isolates also express chromosome-encoded virulence markers such as the 116 kDa secreted mucinase, Pic (a protein involved in intestinal colonization) (Henderson et al., 1999). EAEC pathogenesis involves production of mucus-containing biofilm, which leads to mucoid stools and persistent colonization. Finally, EAEC leads to an inflammatory response with cytokine release, mucosal toxicity and intestinal fluid secretion by enterotoxins (Fagundes-Neto et al., 2000).

EAEC is a food-borne pathogen and it is well known that *rpoS*, a regulatory gene, is a key element in the survival of several food-borne human pathogens (Ibanez-Ruiz et al., 2000; Price et al., 2000). *rpoS*, an alternate sigma factor ( $\sigma$ ) of RNA polymerase, is involved in stress resistance and protection under adverse environmental conditions such as nutrient limitation or osmotic shock and acid stress (Rosche et al., 2005). Enteric bacteria like *E. coli*, *E. faecalis*, *S. typhimurium* and *H. pylori* prefer to live and grow at neutral pH, however, acid resistance (AR) contributes to the pathogenesis of Enterohemorrhagic *Escherichia coli* (EHEC) (Lin et al., 1996) and the induction of virulence factors such as ToxR in *V. cholerae* (Miller et al., 1987). *rpoS* has been reported to be an acid shock gene important for the induction of the acid tolerance response (ATR) (Lee et al., 1995). In *S. flexneri* and *E. coli* strains, the induction of two of three AR pathways under aerobic growth conditions is positively regulated by *rpoS* (Bhagwat, 2003). AR 1 is referred as the glucose-repressible oxidative pathway and it protects EHEC from acid stress above pH 3.0. However, the structural components of the AR system by which it protects the cells, are still unknown (Audia et al.,

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2001). In this study, we have identified acid-induced OMP of EAEC by simulating the natural route of infection (pH 1.5-5.5). Also, we examined the effect of stress regulator *rpoS* on the expression of these acid-induced OMP in order to correlate with EAEC-induced pathogenesis.

## Materials and Methods

### Bacterial strain and growth conditions

EAEC (T8) strain was procured from the National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India as a gift. The EAEC (T8) strain was preserved for short-term on 1.5% Luria Agar (LA, Sigma-Aldrich, USA) after biochemical and phenotypic characterization. The growth pattern under acid stress (inorganic) conditions in EAEC (T8) was studied by adjusting media pH with 1N HCl to variable pH (3.0-5.5) and control pH 7.4. A single colony of EAEC (T8) from LA plate was allowed to grow overnight at 37°C with shaking (215 rpm) in 10 ml LB media with control pH 7.4 and was subcultured (1:500) in the pre-warmed LB media (variable pH) and grown under similar conditions. A bacterial sample (1 ml) was taken and absorbance (OD<sub>600nm</sub>) was measured every hour in a spectrophotometer (Kontron 860 Spectrophotometer, Netherlands) until OD<sub>600nm</sub> reached approximately 2.0. To confirm the growth pattern at pH 4.0 further, the growth curve was also checked in LB media by CFU Plot. For this, culture inoculum (100 µl) was plated on a LA plate by diluting (when appropriate) in sterile saline solution at different time intervals. The plates were incubated at 37°C overnight and the next morning, the colonies were counted. SigmaPlot (version 2000) was used for the CFU plot analysis.

### Preparation of outer membrane proteins (OMP)

OMP were prepared as previously described (Leyh and Griffith, 1992). Briefly, the culture was grown overnight in LB at control pH 7.4 and subcultured (1:500) in fresh LB media with variable pH (3.0-5.5) and control pH 7.4. It was then grown at 37°C with shaking (215 rpm) up to stationary phase and cells were harvested (6,000 rpm for 10 min), washed twice with 10 mM Tris buffer (pH 7.4) to remove excess medium, and resuspended in the same buffer containing 2 mM protease inhibitor Phenyl Methyl Sulphonyl Fluoride (PMSF; Sigma-Aldrich). Cells were then disrupted by ultrasonication (10 cycles of 30 sec with 30 sec interval in between) at 8 W for 10 min on ice. The cell debris was removed by centrifugation (6,000 rpm, 4°C for 20 min). The supernatant obtained was ultracentrifuged (1,00,000g for 1 h) and the pellet was resuspended in 2% lauryl sarcosine (ICN, USA). After incubation for 1 h at 37 °C, the supernatant was ultracentrifuged at the same speed. The pellet (detergent insoluble fraction) was obtained and washed twice with the same buffer with PMSF and stored as OMP fraction at -20°C.

### Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The crude protein preparation was separated on SDS-PAGE in a midi gel apparatus (Bangalore Genei, India) as described previously. The 12.5% resolving and 5% stacking gels were made using a discontinuous buffer system. The protein samples were solubilized at 100°C for 5 min in a sample buffer. The protein sample (80 µg) in each lane was loaded with a Molecular Weight (MW) protein marker and electrophoresis was performed at con-

stant current (15 mA). The gels were stained in 0.2% Coomassie Brilliant Blue R-250 (prepared in 50% methanol and 7% acetic acid) and destained (50% methanol and 7% acetic acid). The MWs for acid-induced OMP were determined by densitometric analysis (Bio-Rad, USA).

### Two dimensional polyacrylamide gel electrophoresis

The 2D PAGE analysis of acid-induced OMP was performed as previously published (O'Farrell, 1975). The sequential extraction kit was used for protein analysis performed in a PROTEAN IEF system as described by the manufacturer (Bio-Rad). Approximately 100 µg of sample was mixed with 70 µl ReadyPrep rehydration buffer (Bio-Rad) and centrifuged (6,000 rpm for 2 min) and 125 µl was loaded in the rehydration tray with immobilized pH gradient (IPG) range 5-8 (7 cm, Bio-Rad). The IPG strips were left for rehydration overnight at room temperature (RT). Nanopure water (8 µl) was added onto paper wick placed on both ends of the channels in the focusing tray. The IPG strip from the rehydration tray was transferred to the corresponding channel in an isoelectric focusing (IEF) tray. The IPG strip was again covered with mineral oil. Separation in the first dimension by IEF was achieved at 20,000V-h. For the second dimension, the strips were run in gel with 1 X Tris glycine SDS running buffer and silver stained. The experiments were performed in triplicate and representative gels were shown.

### Protein identification by mass spectrometry (MS)

The excised spots from 2D PAGE were processed for MS analysis by standard published in-gel trypsin digestion. Briefly, the spots were washed with 50 mM ammonium bicarbonate (ambic) and acetonitrile (ACN) (1:1) for 10 min, then dehydrated with 100% ACN and finally 10 mM dithiothreitol (DTT) in 50 mM ambic was added and incubated for 45 min at 56°C for reducing the peptide linkages. Then, 50 mM iodoacetamide (IAA) in 50 mM ambic was added in dark for alkylation of disulfide linkages. The gel pieces were washed again as before. Trypsin digestion was carried out overnight at RT and peptides were extracted in 0.1% trifluoro acetic acid (TFA) and ACN (2:1). The sample was spotted on a matrix-assisted laser desorption ionization mass spectrometry (MALDI) target plate mixed with 5 mg/ml matrix (4x  $\alpha$ -cyano-4-hydroxycinnamic acid solution in 1 ml, 1:1 ACN: ethanol), then analyzed on an Applied Biosystems 4700 MALDI-TOF/TOF (Bruker Daltonix, Ultraflux, version, Germany). MALDI-TOF spectra were acquired on a Reflex IV in positive reflectron mode, in the m/z 500-3000 range. Peptide mass fingerprints (PMF) spectra thus obtained were submitted against the prerelease translated databases using Mascot MS/MS search engine (Matrix Science Ltd, London, UK).

### *rpoS* gene mutant construction in EAEC (T8)

The *rpoS* gene mutant was prepared by TargeTron™ Gene Knockout mutation kit (Sigma-Aldrich), which provided rapid and specific disruption by re-targeting group II introns efficiently into desired DNA target by site-specific insertion as described by the manufacturer. Briefly, DNA of EAEC (T8) cells was prepared by standard published protocol. Lysozyme (200 µg/ml), SDS (1%) and Proteinase K (100 g/ml) was added and incubated at 56°C for 3 h. Then, DNase free RNase 100 µl/ml was added and incubated at 37°C for 1 h. This mixture was extracted with Tris-saturated phenol (pH 8.0) followed by extraction with

phenol:chloroform (1:1), and aqueous phase was ethanol precipitated. After washing the precipitate in 70% ethanol, the DNA was resuspended in TE buffer and stored at -20°C.

Primers were designed from published *E. coli rpoS* gene specific primers (Mulvey and Loewen, 1989). The PCR conditions for the *rpoS* gene amplification were 95°C for 1 min; 57°C for 1 min; 72°C for 1 min for 35 cycles. The PCR products were resolved with marker [ $\phi$ X174 DNA *Hae*III and  $\lambda$  DNA *Hind*III double digest (Finnzymes, Finland); 100 bp Ladder (Bangalore Genei)] on 1-2% agarose gels and visualized on Chemilmager™ 4400 (Applied Biosystems, USA). For the target site selection for mutation, *rpoS* gene fragment (1.4 Kb) of EAEC (T8) was used to put into the algorithm at kit manufacturer's website (USA-aldrich.com/targetronaccess; Sigma-Aldrich) for designing specific primers capable of inducing mutation. The sequence of three unique primers IBS (AAAAAAGCTTATAATTATCCTTAGCGGCCAGAGGCGTGCGCCAGATAGGGTG), EBS2 (TGAACGCAAGTTCTAATTTCCGATGCGCTCGATAGAGGAAAGTGTCT), and EBS1d (CAGATTGTACAAATGTGGTGATAACAGATAAGTCAGAGGCGATAACTTACCTTTCTTTGT) with the lowest E-value (<0.5) and EBSU (CGAAATTAGAACTTGC GTTCAGTAAAC) were required to re-target the intron to insert at a specific site. The optimal PCR specificity and yield was obtained with primers of HPLC purity. PCR reaction was again performed in a thermal cycler (described above), which re-targeted the intron by primer-mediated mutation. A reaction containing four-primer master mix was made to mutate the intron at several positions spanning a 350 bp region with 25  $\mu$ l of JumpStart REDTaq ReadyMix (provided in the kit) in a 50  $\mu$ l reaction mixture. The PCR conditions for the re-targeting reaction were 95°C for 1 min 30 sec; 53°C for 2 min and 72°C for 1 min 30 sec. Following purification of the PCR product (mutated 350 bp PCR fragment) by PCR Clean-up Kit (Qiagen, Germany), cohesive ends in the PCR product were generated by restriction enzymes *Hind*III and *Bsr*GI by double digestion reaction first at 37°C and successively at 60°C for 30 min each. Finally, the reaction was stopped at 80°C for 10 min for ligation into a linear vector pACD4K-C (provided in the kit) and transformed in DH5 $\alpha$  cells by following procedure.

For transformation in DH5 $\alpha$  *E. coli*, the reaction mixture was heated (60°C for 30 sec) and cooled on ice for 1 min. One  $\mu$ l of T4 Ligase (NEB, USA) was added to the reaction mixture and incubated for 30 min. Transformation of the ligated product was performed in DH5 $\alpha$  *E. coli* cells prepared by CaCl<sub>2</sub> method. The ligation reaction was assessed by plating the reaction on LB-chloramphenicol (25  $\mu$ g/ml)-X-gal-IPTG (20 mg/ml X-gal and 100 mM IPTG spread on plates) and observing the ratio of white (successfully re-targeted) to blue (non-mutated/parental) colonies in DH5 $\alpha$  *E. coli* cells. The plasmid DNA was prepared from the white colonies (recombinant) by standard alkaline lysis method. The culture (3 ml) was centrifuged (6,000 rpm for 1 min) and the pellet was resuspended in TE buffer, followed by the addition of solution (1% SDS in 0.2 N NaOH). The contents were incubated on ice (5 min). To this lysate, 3 mM potassium acetate (pH 5.5) was added for 10 min and centrifuged (6,000 rpm for 10 min). The supernatant containing plasmid DNA was precipitated with two volumes of ethanol. After subsequent washing in 70% ethanol, the precipitate was suspended in TE and was run on the agarose gel to check the increase in size. The

plasmid from the blue colonies was taken as a control.

For knockout of *rpoS* in EAEC (T8), the recombinant plasmid (8  $\mu$ l) was added to chemically prepared EAEC (T8) competent cells (50  $\mu$ l). Heat shock was given to cells (30 sec for at 42°C) and LB media (450  $\mu$ l) was added and were allowed to grow (37°C for 1 h). Further, 100  $\mu$ l of this transformation reaction was added to LB (3 ml) containing 25  $\mu$ g/ml chloramphenicol, 1% glucose and incubated overnight and subcultured in the same media to allow growing to an OD<sub>600</sub> nm of approximately 0.2. Then 100 mM stock IPTG was added and incubated (30°C for 30 min), the cells were centrifuged (6,000 rpm for 1 min), resuspended in LB (1 ml) containing 1% glucose without chloramphenicol at 30°C for 1 h. The culture (100  $\mu$ l) was plated on a LA kan (50  $\mu$ g/ml) plate. The recombinant white (Cam<sup>s</sup> Kan<sup>r</sup> Glu<sup>r</sup>) colonies were picked and DNA was prepared for confirming intron insertion by PCR and was run in 2% agarose along with the DNA marker.

### Characterization of *rpoS* gene mutant in EAEC (T8)

For characterization of MT8 mutant, various assays were performed. Briefly, for MT8 colony morphology, the growth pattern of MT8 was also checked in LB broth at pH 4.0. The OMP profile of MT8 at pH (4.0) was compared with the wild type EAEC (T8) by SDS-PAGE. Also, the clump formation was checked for MT8 as described previously (Albert et al., 1993). Briefly, EAEC (T8) strain was subcultured on LA and subcultured (1:500) in Mueller Hinton Broth (MHB) (Difco Laboratories, Detroit, USA) in duplicate tubes and incubated in an orbital shaker (215 rpm) at 37°C. The formation of a clump or a pellicle as a ring at the side of the test tube at the end of incubation (20 h) was regarded as a positive result.

HEp-2 adherence assay for EAEC (T8) characterization was performed by the method described previously with minor modifications (Cravioto et al., 1991). Cells were grown to 50-70% confluency as monolayers in a 6-well flat bottom tissue culture plate and washed with phosphate buffered saline (PBS). Fresh DMEM media pH was set with 1 N HCl to the desired pH (filter sterilized) and was added along with 2% fetal calf serum (FCS) and 0.5% D-mannose without antibiotics to the 6-well plates (Greiner, USA). EAEC (T8) was grown in LB media with control pH 7.4 overnight at 37 °C and was inoculated (1:8) in the media with pH 4.0 and control pH 7.4 and incubated at 37°C overnight with 5% CO<sub>2</sub>. After incubation, the cells were washed and fixed with 100% methanol and stained with 2.5% Giemsa for 15 min. The adherence patterns were examined under 40 X magnification and photographed at 100 X magnification with digital camera (Carl Zeiss, Sony, Japan) with oil immersion in a light microscope (Leica MPS32, USA).

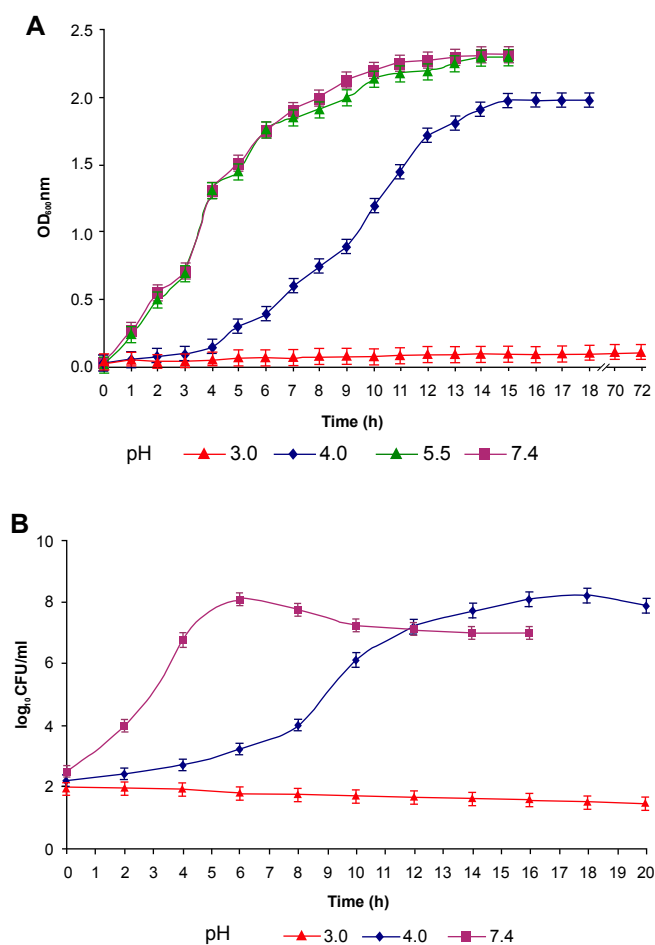
## Results

### Growth analysis of EAEC (T8) under acid stress conditions

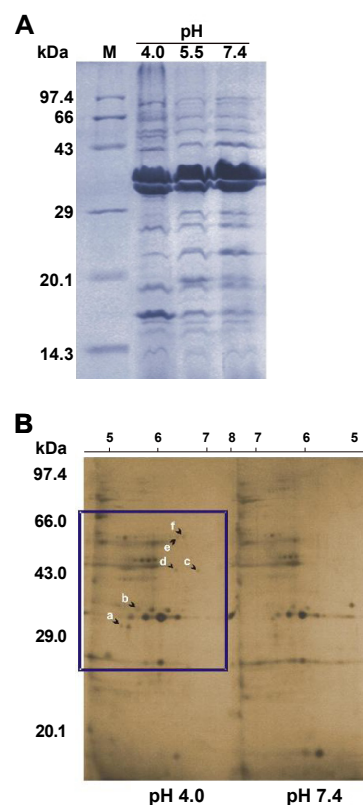
The growth pattern of EAEC (T8) was studied *in vitro* in LB media at pH 3.0, 4.0, 5.5 and control pH 7.4. Initially, a single colony of EAEC (T8) was inoculated in LB media with control pH 7.4 and incubated at 37°C for overnight in a shaker (215 rpm). This overnight culture was further subcultured (1:500 dilution) in LB media (pre-warmed at 37°C) at different pH. The growth pattern was recorded every hour on a spectrophotometer until OD<sub>600</sub> nm reached 2.0. The lowest pH at which EAEC (T8)

in LB broth showed linear growth was 4.0. In contrast, at pH 3.0 even after 3 days in the shaker (215 rpm) no signs of growth was revealed by EAEC (T8) (Figure 1A). Lesser growth of EAEC (T8) at pH 4.0 could be correlated with the time taken by the cells to reach log phase, which is approx. 7 h. However, pH 5.5 and control pH 7.4 in EAEC (T8) showed a similar growth rate with a doubling time of 3 h (Figure 1A). No appreciable lag phase was observed for growth either at pH 5.5 or control pH 7.4, whereas the doubling time of growth was prolonged at pH 4.0.

The lowest pH (4.0) at which EAEC (T8) showed growth in LB was further checked for the viability of cells by CFU count (Figure 1B). The viability of EAEC (T8) cells was also checked at pH 3.0 and control pH 7.4. The inoculum was taken aseptically every hour and was diluted in PBS ( $10^{-3}$ - $10^{-5}$ ) when required and plated on LA plates and incubated at 37°C overnight. The numbers of colonies was counted and mean value was taken at different dilutions. EAEC (T8) again showed a slow growth rate and was log phase reached in approximately 9 h ( $10^5$  cells/ml) whereas at control pH 7.4, the same count was achieved in 2.5 h (Figure 1B). During stationary phase that is after 10 h, cessation of growth was observed. The difference between the growth rate at pH 4.0 and control pH 7.4 in EAEC (T8) by CFU count was initially different during the lag phase but thereafter,



**Figure 1:** Growth analysis of EAEC (T8) under acid stress. EAEC (T8) grown overnight at control pH 7.4 in LB was subcultured (1:500) in LB with different pH as described in Materials and Methods section. After every 1 h, cell count was measured at 600 nm in spectrophotometer at different pH (A) and the cells were plated on LA plate for 16 h to count the viable cell number by taking CFU/ml (B). All values represent the mean S.D. of three experiments.



**Figure 2:** Isolation and identification of acid-induced OMP. EAEC (T8) was grown at different pH (4.0 and 5.5) and control pH 7.4 in LB and the cells were harvested during log phase for OMP preparation as described in Materials and Methods section. Equal loading (80 µg) of the OMP in each lane was run in a SDS-PAGE after protein assay by Lowry and Coomassie blue staining. The samples were run three times and the representative gel was analyzed for MW by densitometry. Lane M: Protein MW marker. Numbers at left are MW of marker protein. The MW of the acid-induced OMP showing major changes in the expression after being confirmed by densitometry analysis (A). OMP (100 µg) from EAEC (T8) were separated on pH 5-8 range IPG strip (7cm) and electrophoresed on a 12.5% gel. Separated proteins were silver stained. Numbers at left are MW of marker protein. The box with arrow marks indicates the protein spots expressed at specific pH 4.0 (B). The gels were run three times and the representative gel was analyzed.

with increased time, the difference became negligible towards the stationary phase. However, at pH 3.0, no colony was observed after incubation and subsequently, all other experiments in the our study were carried out at pH 4.0 (pH for acid shock).

### Protein profiling of EAEC (T8) under acid stress conditions

Expression of acid-induced OMP in EAEC (T8) was studied by loading approximately 80 µg of protein at different pH (4.0 and 5.5) and control pH 7.4 by 12.5% SDS-PAGE. The MW of the proteins was analyzed by densitometry analysis. Two OMP of sizes 41 kDa and 48 kDa were observed at pH 4.0 only and were not detectable at control pH 7.4 (Figure 2A). The expression of these two OMP was also not detectable at mild acidic pH 5.5 and therefore these might be acid-induced OMP, which were synthesized *de novo* under acid stress conditions (pH 4.0). Besides these two *de novo* OMP, the expression of only one OMP of size 17 kDa was 3-fold high at pH 4.0 than at pH 5.5 and control pH 7.4. The 23 kDa OMP showed approximately 2-fold

Spot	Accession number <sup>2</sup>	Identified protein (Function and source) <sup>3</sup>	Top score <sup>4</sup>	Matched peptides <sup>5</sup>	Theoretical MW (in kDa) <sup>6</sup>	Measured MW (in kDa)/pI <sup>7</sup>
a	G84107	Lipopolysaccharide (biosynthesis gene, <i>B. holodurans</i> )	53	10	32.38	31/5.2
b	AAL15430	AY055229 NID ( <i>Moraxella nonliquefaciens</i> )	68	12	39.7	34/5.5
c	A89980	Hypothetical protein	52	11	41.91	45/7.0
d	A97335	Probable membrane protein	48	9	42.2	45/6.5
e	Q9L2K2	Putative lipoprotein ( <i>Streptomyces</i> )	42	7	43.6	54/6.2
f	A70320	Nitrate transporter ( <i>Aquifex</i> )	45	9	51.5	55/6.2

<sup>1</sup>The data obtained after processing the acid-induced protein spots by MALDI-TOF/TOF was analyzed using the MASCOT database-searching algorithm.

<sup>2</sup>NCBI accession numbers for the identified proteins in the known protein database.

<sup>3</sup>Identified protein in the known protein database with function and source.

<sup>4</sup>Top score of the identified protein indicates the protein matching on the basis of the number of peptides.

<sup>5</sup>Number of peptides identified by MS/MS for each protein is listed.

<sup>6</sup>Theoretical MW (in kDa) of the identified protein in the protein database.

<sup>7</sup>Calculated MW (in kDa)/isoelectric points (pI) of the identified protein from the densitometry analysis.

**Table 1:** Identification of six-induced OMP<sup>1</sup>

lower protein expression at pH 4.0 than the control pH 7.4. However, two OMP of sizes 27 kDa, 34 kDa and other high kDa OMP showed almost similar expression at pH 4.0 and control pH 7.4.

The expression of two *de novo* OMP 41 kDa and 48 kDa was observed only during the logarithmic phase of growth (OD<sub>600</sub> nm was 0.6) only at pH 4.0 (Figure 2A).

Changes in the OMP expression in EAEC (T8) observed by SDS-PAGE were further identified by 2D gel electrophoresis. OMP (100 µg) prepared from EAEC (T8) (log phase) was run on 2D PAGE on IPG with pI 5-8. Following separation, the gel was visualized by silver staining. For each protein sample, the reproducibility of the 2D PAGE was assessed by repeated analysis (at least three times) under similar conditions. Six protein spots were detected in the pI range 5-8 (Figure 2B) at pH 4.0. Approximately 45 kDa protein showed two spots (c and d), which might be the isoforms characterized by differences in the observed MW and/or pI values (horizontal spot pattern). Some spots showed additional changes in their MW, which probably reflect differential posttranslational modification.

#### MALDI-TOF MS/MS analysis

The expression of unknown OMP under acid stress (at pH 4.0) was observed by 2D PAGE and the expression of differentially expressed proteins was confirmed by PDQuest software. Automated gel scanning and computer-assisted analysis was used for the overlapping of 2D PAGE (control pH 7.4 vs. 4.0). The protein spots were marked with different colors and it was found that at pH 4.0, six protein spots were differentially expressed by PDQuest (data not shown). In a relevant set of data not hampered by one of the usual drawbacks of 2D PAGE, inter gel variability was generated, which distinguished the different isotopes and translated the signals to a two color display (green and red) where all proteins common to both samples ("protein noise", black or brownish) could easily be rejected.

After confirmation of differential protein expression at pH 4.0 by PDQuest, peptide mass fingerprints (PMF) were performed by MALDI-TOF MS. The six protein spots at pH 4.0 were cut, digested with trypsin and demonstrated by MALDI-TOF mass spectrometry. The spectra thus obtained were analyzed by a

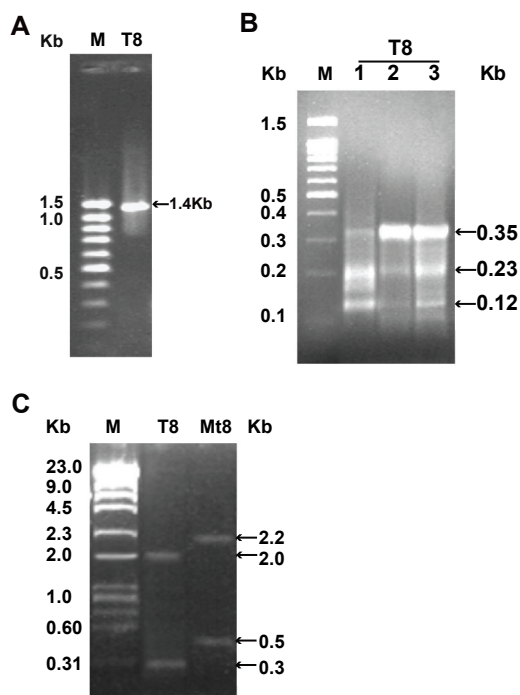
MASCOT Search engine where mass fingerprints were matched with the already reported sequences in NCBI-translated databases. In this study, the six proteins were identified from EAEC (T8) under acid stress by their amino acid composition, pI and MW, using MS. Maximum homolog of spots with their theoretical and measured MW (a-f) matched with the known protein are listed in Table 1. The probable membrane protein, lipoprotein biosynthesis gene, hypothetical proteins, putative lipoprotein and nitrate transporter were identified in EAEC (T8) under acid stress.

#### *rpoS* gene mutant construction of EAEC (T8)

Genomic DNA isolated from EAEC (T8) showed amplicon of 1.4 Kb (Figure 3A) with *rpoS* gene specific primers. For site specific mutation in *rpoS* gene, the amplicon (1.4 Kb) was re-targeted with intron specific primers by PCR. For this, *rpoS* PCR product was diluted 1:100 in TE buffer and used as a template. The re-targeted PCR showed three amplicons on agarose gel (4%) and out of which the 350 bp amplicon was the desired product (Figure 3B). The plasmid vector pACD4K-C (7678 bp; TargeTron® Vector pACD4K-C-loxP Vector Map; Sigma-Aldrich) was used for re-targeting of *rpoS* gene by intron specific primers. The 350 bp amplicon was gel purified and digested for ligation in the vector as described in Materials and Methods section. The blue-white colony selection was performed after transformation in DH5α cells. The white (recombinant) colonies were selected and plasmid DNA was prepared. This recombinant plasmid with re-targeted *rpoS* gene was further sub-cloned in the wild type strain EAEC (T8). For this, the re-targeted plasmid was sub-cloned in competent cells of wild type EAEC (T8) and white (recombinant) colony was selected. Genomic DNA was prepared and the mutant (MT8) strain was confirmed by PCR, which showed increase in size of amplicon by 200 bp (Figure 3C).

#### *rpoS* gene analysis of the mutant MT8

After confirmation of *rpoS* gene disruption in EAEC (T8), MT8 (mutant) strain was characterized in different ways. *rpoS* in MT8 strain cultured on LA kan media plate showed small and round colonies (<0.5 mm) after 16 h of incubation at 30°C (Figure 4A; bottom panel) as compared to medium sized 0.5-1 mm (Figure 4A; top panel) size colonies in control EAEC (T8) strain



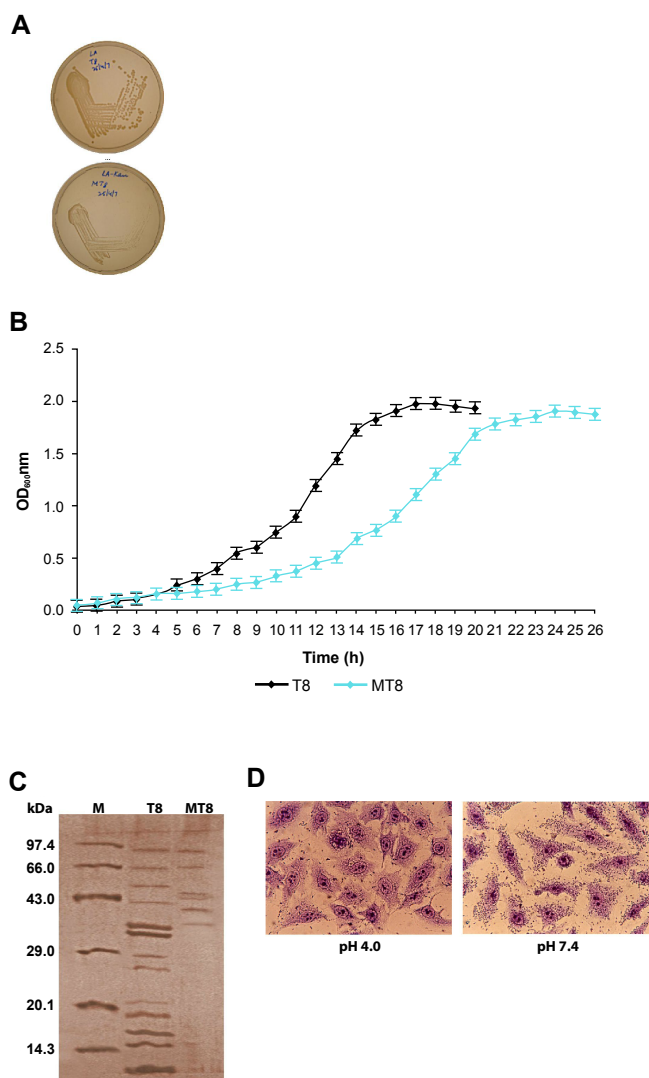
**Figure 3:** Construction and confirmation of *rpoS* gene mutant. DNA from EAEC (T8) was isolated as described in Materials and Methods section. 1.4 Kb *rpoS* gene was PCR amplified with gene specific primers and resolved on agarose gel (1%) (A). Lane M: 100 bp ladder. Further, PCR amplification of 1.4 Kb *rpoS* amplicon as a template with intron specific primers for mutation showed three amplicons on agarose gel (4%) (B). Lane M: 100 bp ladder. The 350 bp amplicon was PCR purified and digested as mentioned in Materials and Methods section for ligation. The blue-white colony selection was performed by replica plating after transformation. The white (recombinant) colonies were selected and plasmid DNA was prepared. The recombinant plasmid was further sub-cloned in the wild type strain EAEC (T8). Again after white (recombinant) colony selection in transformation, DNA was prepared. Confirmation of MT8 by PCR amplification with primers flanking the gene target site of *rpoS* gene and intron region showed increase in the amplicon sizes by 200 bp (C). Lane M:  $\phi$ X174 HindIII and HaeIII double digest. Notably, experiments shown above and below were repeated at least three times with reproducible results, and a representative one is presented. MW marker (M) (base pairs) is indicated on the left. The major PCR products are marked by arrows on the right.

on LA. The growth pattern of MT8 at pH 4.0 in LB was slower than the wild type EAEC (T8) with a doubling time of 15 h (Figure 4B). The wild type EAEC (T8), however, attained log phase in 9 h. However, there was no change in the growth pattern in the mutant strain at control pH 7.4 (data not shown). OMP were prepared at pH 4.0 in MT8 strain and the protein expression was studied by 12.5% SDS-PAGE. There were significant differences in the expression of OMP in MT8 as compared to the wild type EAEC (T8) at pH 4.0 (Figure 4C) during log phase ( $OD_{600}$  nm 0.6). The expression of *de novo* OMP (41 kDa and 48 kDa) as well as 34 kDa porin was lacking in MT8. Besides these, the other OMP of sizes 12 kDa, 15 kDa, 17 kDa, 19 kDa, 26 kDa and 28 kDa were also not detectable in MT8 under similar conditions (Figure 4C). However, there was no change in the expression of high kDa OMP at pH 4.0 in MT8. HEP-2 adherence assay, which is the gold standard for EAEC identification (Fig. 4D; right panel) was absent in MT8. However, diffusely adherent pattern (DA) was observed in MT8 with HEP-2 cells at pH 4.0 (Figure 4D; left panel).

## Discussion

To date, the mechanism by which Enteroaggregative *Escheri-*  
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*chia coli* (EAEC) sense and respond to acidic pH have not been fully elucidated. Here we identify the acid-induced OMP responsible for its adaptation and survival and the effect of stress regulator on the expression of acid-induced OMP to correlate with EAEC-induced pathogenesis. In our study, EAEC (T8) demonstrated growth at pH 4.0, almost 3-fold slower than the control pH 7.4 (Figure 1A and B). EAEC (T8) was sensitive to killing in acidified media at pH 3.0 (Figure 1A). Our results are similar to the previous reports that some strains of *E. coli* like EHEC and *S. flexneri* are able to survive at pH values as low as 2.5 but it does not grow at pH values less than 4.4 (Small et al., 1994; Benjamin and Datta, 1995) because of growth inhibition by both low pH<sub>i</sub> and the differential ability of anions to inhibit metabolism. *E. faecalis* showed growth within a range of pH<sub>i</sub> 4.5-9.5 (Kobayashi, 1985). Recently, acute acid stress in EHEC at pH 3 preceded by acid adaptation at pH 5 significantly enhanced the adhesion of surviving EHEC to epithelial cells and bacterial induction of host cell apoptosis (House et al., 2009). However, *N.*



**Figure 4:** Characterization of MT8. (A) The smaller colonies of MT8 on Luria Agar (LA) with kanamycin (50 mg/ml) were obtained (bottom panel) than the wild type strain (T8) on LA (top panel). (B) The slow growth in MT8 was observed at pH 4.0 by  $OD_{600}$ nm. (C) The changes in the expression of OMP during log phase in MT8 at pH 4.0 were confirmed by silver staining. Lane M: Protein MW marker. (D) Typical aggregative adherence pattern with HEP-2 cells, a gold standard for the identification for EAEC, was absent in MT8. However, MT8 showed DA pattern (left panel) while AA in the wild type strain (right panel).

*gonorrhoeae* had survival peak at pH 4.6-4.7 (Pettit et al., 1999) and *Y. enterocolitica* showed survival peak at pH 4.5 (De Koning-Ward and Robins-Browne, 1995). The capacity to invoke ATR in *S. mutans* on teeth ensures increased survival at pH 4.3 (Bowden and Hamilton, 1998). In EAEC (T8) log phase was attained in 7 h when measured on spectrophotometer at OD<sub>600nm</sub> in which the optical density might include viable cells with some of the dead cells (Figure 1A). However, the cell viability of EAEC (T8) by CFU count was observed at 9 h (Figure 1B). This result is consistent with earlier observations when exposed to a sublethal physical environment i.e., cells failed to grow where CFU count clearly differentiated the viable cells from dead cells in a sensitive method for cell survival (Mothersill et al., 1998).

Despite the altered OMP expression at pH 4.0 (Figure 2A), the protein expression in LB showed two *de novo* OMP of sizes 41 kDa and 48 kDa which were identified as elongation factor chain to chain G and hypothetical proteins (data not shown). This might explain why many genes regulating amino acid metabolism were differentially expressed under acid stress conditions that adapt to new demands for protein synthesis. This finding is consistent with the finding that proteins, which cannot be folded by molecular chaperones, may be degraded in order to recycle amino acids for *de novo* protein synthesis (Jenal and Hengge-Aronis, 2003). Thus, our results for the expression of elongation factor might have similar function in rapid synthesis of a specific set of protective proteins in EAEC (T8) during acid stress (pH 4.0).

Bacterial attachment to host cells represents the first opportunity to disrupt the bacteria-host interaction. An 18 kDa fimbrial adhesin from an Indian strain of EAEC (T7) inhibited HEp-2 cell adherence and agglutinated human blood group A erythrocytes in presence of 5 mM Ca<sup>2+</sup> at 25°C and pH 6.5 (Grover et al., 2001). However, to our surprise, no such adhesin was identified under low pH (data not shown). One report demonstrated that OmpU homolog in *V. fischeri* is 32.5 kDa OMP; the mutant grew normally in both complex and minimal media (M9) but had a decreased ability to colonize the host cells (Aeckersberg et al., 2001). It is reported in Enteropathogenic *Escherichia coli* (EPEC) that enterotoxins activate adenylate cyclase leading to cAMP accumulation, which causes the reorganization of F-actin in HEp-2 cells (Fabbri et al., 1999). However, our data showed at pH 4.0, a DA pattern was observed with HEp-2 cells in EAEC (T8) (data not shown). Also, pro-inflammatory mediators like TNF- $\alpha$  induce necrotic and apoptotic cell death in intestinal epithelial cells *via* activation of protein kinase C (PKC) activators which rapidly induce a variety of cellular responses including cell shape changes, cytoskeletal remodeling, decreased cell-cell communication and increased exocytosis (Chang and Tepperman, 2003). The impact of acid stress on virulence properties including survival, host adhesion, Shiga toxin production, motility and induction of host cell apoptosis has been recently reported in EHEC (House et al., 2009). The discrepancy is still unknown. It is possible that membrane probable protein and hypothetical proteins (Table 1) expressed at pH 4.0, might have some adhesion function in EAEC (T8).

A large part of the enterobacterial function exposed to an 'extracellular' pH takes place in the OMP and envelope, and thus it is not surprising that several envelope components have pH-dependent expression (Maurer et al., 2005). The studies are con-

sistent with our findings that acid survival of *E. coli*, *S. enterica* and *S. flexneri* demonstrated different types of ATR systems that exist in both log and stationary phase cells (Lin et al., 1996) and numerous ASP were synthesized when cells adapted at pH below 5.0. It is suggested that these proteins are needed to efficiently protect cells from subsequent exposure to otherwise lethal levels of acid (Bearson et al., 1997). In our study in EAEC (T8), six clearly delineated spots whose intensity was significantly altered were found to be the membrane associated, fatty acid metabolism and hypothetical proteins (Table 1). These proteins belong to different groups, including cytoplasmic enzymes in amino acid catabolism, membrane-bound transporters, periplasmic proteins and extra cellular components, which were consistent to the earlier reports and the exact function for many of them is not known (Blankenhorn et al., 1999; Rowbury et al., 1999; Stancik et al., 2002). In a separate study, 2D PAGE analysis of the log ATR in *S. mutans* H7 involving multiple stresses revealed significant responses to starvation, acid (from pH 7.5-5.5 for 2 h) and heat stress with the synthesis of 52, 36 and 54 proteins, respectively (Svensater et al., 2000). These proteins could be classified as general stress proteins, stress-specific proteins (acid-10) and a variable number of shared proteins common to other stresses, which included 16 proteins with unknown function, 11 proteins involved in protein synthesis, 4 proteins involved in transport, 2 proteins involved in cell envelope development and 1 protein involved in fatty acid biosynthesis. On the other hand, 2D PAGE of *S. typhimurium* during the stationary phase in LB media with glucose at pH 5.0 revealed 5 proteins including FliC, FljB, DnaK, AtpB and GroEL, which have not previously been identified as ASP (Table 1). In addition, in *S. flexneri*, most of the proteins induced showed involvement in energy production and conversion at pH 4.5 (Cheng et al., 2007). Interestingly, EHEC did not secrete periplasmic levels of Shiga toxin during acid shock. However, *de novo* protein synthesis was shown to be required for the enhanced adhesion of acid-shocked in erythromycin treated group in EHEC (House et al., 2009). Besides these, upregulation of some genes induced by acid stress additionally encoded hypothetical proteins with an unknown function, which is consistent to our findings (Table 1).

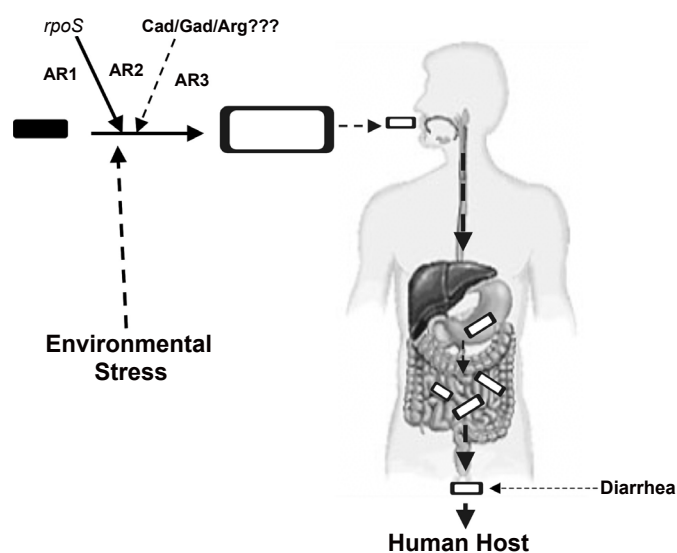
We demonstrated that acid stress induces the synthesis of fatty acid metabolism, membrane protein and lipoprotein biosynthesis at pH 4.0 in EAEC (T8). Our results are inconsistent in number with the report in *L. lactis* (Hartke et al., 1994) where carbon starvation showed the expression of genes involved in fatty acid and phospholipid metabolism (8 genes), membrane biosynthesis (5 genes) and cell wall biosynthesis (17 genes) and in the cell division process (8 genes) were under expressed. However, the decrease in expression of genes involved in processes of growing cells (transcription, translation, nucleotide biosynthesis, cell envelope metabolism and cell division) can be compared to a stringent response in *E. coli* or *B. subtilis* during starvation (Vogel et al., 1992; Eymann et al., 2002), which lead to growth arrest. However, in our study, the growth arrest at pH 4.0 was not observed, rather EAEC (T8) cells were growing slow with increased lag phase, which might be necessarily linked to new protein synthesis required for adaptation during acid stress and therefore, the proteins with function showing metabolism and transcription were upregulated.

The identified nitrate transporter in EAEC (T8) (Table 1) is

similar to highly basic Asr protein serving as a proton sink in the periplasm sequestering protons and protecting essential proteins from denaturation at an extremely low pH (2.0) (Seputiene et al., 2003). However, the expression of porins of sizes 35-38 kDa was downregulated at pH 4.0 in EAEC (T8) (Fig. 2A), which might be related to the inhibition of H<sup>+</sup> influx by lowering down their expression. Sugar transporters such as OmpF and the maltose regulon are downregulated at low pH, as sugar fermentation generates short-chain acids. *M. tuberculosis* K<sup>+</sup> and Na<sup>+</sup> transport systems, which exchange protons for cations in association with ATP hydrolysis in phagosomes are reported to be highly expressed in mild acidic environment at pH 6.1-6.5 (Sturgill-Koszycki et al., 1994). IldP encodes the membrane transport carrier L-lactate permease (LldP), which was also shown to be acid upregulated (Maurer et al., 2005). Indeed, upregulation of genes during log phase ATR at pH 5.5 in LB media of F1F0-ATPase has been demonstrated to be a key mechanism for extrusion of protons in the response and tolerance to low pH in *L. acidophilus* (Kullen and Klaenhammer, 1999).

The identification of probable membrane protein with unknown function in EAEC (T8) was observed at pH 4.0 (Table 1). A recent report demonstrates that the gene, hdeB, encodes a periplasmic protein of 12.5 kDa, which combats the deleterious effects of acid on periplasmic proteins in *E. coli* (Gajiwala and Burley, 2000). However, the hdeB, encodes a putative periplasmic protein and hdeD encodes an integral membrane protein of unknown function. The results further implied that modification of the cell envelope is important for AR.

The our study showed that *rpoS* mutant in MT8 did not induce AR 1 when grown at pH 4.0. This supports the notion that they



**Figure 5:** Schematic representation of the working hypothesis for EAEC (T8) infection under acid stress. Briefly, gastric juice is the first line of bactericidal barrier (pH 1.5), but in our study EAEC (T8) has shown survival *in vitro* at pH 4.0, which is comparatively higher than the gastric pH. During infection in the stomach, the bacteria are already in the stationary phase (non-dividing) and it is well reported that the survival potential of stationary phase or acid-adapted cells is greater than that of exponentially growing cells over the initial period of acid challenge. Our results showed the expression of acid-induced OMP, which could provide a protective effect increases at pH 4.0. Therefore, once induced, the AR system will remain active until cells reenter log phase during the course of infection. Then, it might be possible that these bacteria could survive *in vivo* extreme acidic conditions at pH 1.5. Thus, EAEC (T8) might be already adapted outside when it encounters low pH in the stomach.

do not possess a functional *rpoS* and a fully operational AR system. Our results are in agreement that the MT8 strain was found to have a significantly lower survival than the wild type and affects all the three stationary phase AR systems (Cheville et al., 1996). Acid-sensitive cells showed no induction or a reduced level of *rpoS* (Jorgensen et al., 2000). It is found that  $\sigma^S$  mutation has an impact on the survival and adaptation of EAEC at low pH by growth characteristics (4B). Also the screening tests for the induction of log phase acid response in MT8 did not show the expression of ASP at pH 4.0 (Figure 4C). The growth phase sigma factor *rpoS* regulates AR and plays a key role in the survival of bacteria under stress (Hengge-Aronis, 2002; Weber et al., 2005). The *rpoS*-dependent AR 1 enables stationary phase *Shigella* species to survive at a pH below 2.5 for 2h (Waterman and Small, 1996a, 1996b). The chromosomal copy of *rpoS* from strains 251 and 258 was inefficient in supporting host cell adherence functions (in addition to a defect in temperature tolerance), which is in agreement with our finding loss of typical aggregative adherence to HEp-2 cells (Figure 4D; left panel) at pH 4.0 in MT8 strain.

## Conclusions and Perspectives

The working hypothesis is that the gastric juice (pH 1.5) is the first line of bactericidal barrier yet in our study EAEC (T8) has shown survival *in vitro* at pH 4.0, which is comparatively higher than the gastric pH (Figure 5). The acid challenge at pH lower than 4.0 (lowest pH for *in vitro* survival) can be correlated with an infectious dose (ID), which is a percentage of highly acid tolerant population (Brandl, 2006). EHEC can survive pH 2.0 for 5 hours whereas the nontoxicogenic strain can survive pH 3.0 only (Jordan et al., 1999). This could be explained in that when food products serve as the vehicle of infection, the ingested inoculum may either have quite a high number of bacteria or may have the opportunity to replicate in food to high titers before consumption and passage through the stomach (Figure 5). During infection in the stomach, the bacteria is already in stationary phase (non-dividing) and it is well reported that the survival potential of stationary phase or acid-adapted cells is greater than that of exponentially growing cells over the initial period of acid challenge (Jordan et al., 1999). Our results showed the expression of acid-induced OMP, which could provide a protective effect increases at pH 4.0. Therefore, once induced, the AR system including *rpoS* will remain active until cells reenter log phase during the course of infection. Thus, the bacteria might be adapted when it encounters low pH in the stomach. Thus, EAEC (T8) might be already adapted outside when it encounters low pH in the stomach. To date, no report is available on acid stress response in EAEC or on its proteome, therefore, our model system correlates well in adaptation and protection of EAEC (T8) under acid stress conditions (Figure 5). This would be an incremental step in advancing our understanding of its pathogenic mechanism and further characterization of molecular events involved in the regulation of stress proteins need to be explored.

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