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Transcriptional Modulation of Enterotoxigenic *Escherichia coli* Virulence Genes in Response to Epithelial Cell Interactions

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Enterotoxigenic *Escherichia coli* (ETEC) strains are a leading cause of morbidity and mortality due to diarrheal illness in developing countries. There is currently no effective vaccine against these important pathogens. Because genes modulated by pathogen-host interactions potentially encode putative vaccine targets, we investigated changes in gene expression and surface morphology of ETEC upon interaction with intestinal epithelial cells *in vitro*. Pan-genome microarrays, quantitative reverse transcriptase PCR (qRT-PCR), and transcriptional reporter fusions of selected promoters were used to study changes in ETEC transcriptomes. Flow cytometry, immunofluorescence microscopy, and scanning electron microscopy were used to investigate alterations in surface antigen expression and morphology following pathogen-host interactions. Following host cell contact, genes for motility, adhesion, toxin production, immunodominant peptides, and key regulatory molecules, including cyclic AMP (cAMP) receptor protein (CRP) and c-di-GMP, were substantially modulated. These changes were accompanied by visible changes in both ETEC architecture and the expression of surface antigens, including a novel highly conserved adhesin molecule, EaeH. The studies reported here suggest that pathogen-host interactions are finely orchestrated by ETEC and are characterized by coordinated responses involving the sequential deployment of multiple virulence molecules. Elucidation of the molecular details of these interactions could highlight novel strategies for development of vaccines for these important pathogens.

The enterotoxigenic *Escherichia coli* (ETEC) strains are among the leading causes of diarrheal illness in developing countries. Each year, these organisms account for hundreds of thousands of deaths, particularly in young nonimmune children (1–3). Presently there is no vaccine for ETEC that offers sustained broad-based protection (4).

While these organisms share the ability to produce and effectively deliver heat-stable (ST) and/or heat-labile (LT) enterotoxins, many aspects of the pathogenesis of these organisms remain unexplored. Much of the work on ETEC pathogenesis, and consequently ETEC vaccine development, has focused intensively on the known enterotoxins, and a heterogeneous collection of plasmid-encoded colonization factors (CFs) (5). More recent studies have suggested that the pathogenesis of ETEC is considerably more complex than previously appreciated, involving additional virulence molecules. These include the EtpA exoprotein adhesin (6–8), and EatA (9), a member of the serine protease autotransporter of the *Enterobacteriaceae* (SPATE) family, which has recently been shown to moderate EtpA-mediated adhesion and accelerate delivery of LT (10). Moreover, many proteins, including EtpA and EatA are recognized following infection, suggesting that there may be additional vaccine targets in addition to LT and CFs (11).

While modulation of virulence genes following host cell contact is well described for a number of pathogens (12, 13), and pilus-mediated adherence has been shown to induce gene expression in *E. coli* (14), little is known regarding transcriptional or translational modification of ETEC on interaction with the intestinal epithelium. Because the identification of genes modulated during pathogen-host interactions can potentially be used to identify additional previously unheralded targets for vaccine development (15–17), we investigated transcriptional changes in ETEC following attachment to host cells. The studies reported here demonstrate that in response to interactions with intestinal epithelial cells, ETEC strains modulate a large number of genes, including those encoding recently described novel virulence proteins, putative virulence factors, and important virulence regulators, including cyclic AMP (cAMP) receptor protein-cAMP complex (CRP-cAMP), and cyclic-di-GMP. Paralleling these changes, we observed significant alteration in surface molecules and the architecture of ETEC following host cell attachment.

MATERIALS AND METHODS

Bacterial strains and plasmids. A complete list of the strains and plasmids used in these studies is included in Table 1. ETEC strain E24377A was kindly provided by Stephen Savarino at the National Naval Medical Center and was obtained from good manufacturing practice (GMP) lots of

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TABLE 1 Plasmids and bacterial strains used in these studies

Strain or plasmid	Description[a]	Reference
Strains		
H10407	ETEC (origin, Bangladesh) O78:H11 CFA/I LT/ST-1a/ST-1b	61
E24377A	ETEC (origin, Egypt) O139:H28 CFA/II (CS1/CS3) LT/ST	62
BL21-AI	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm araB*::T7 RNAP-*tetA*	
GPM1746a	H10407 *hsdR*1::*lacZ*::Tn*10*	G. P. Munson, unpublished data
jf2624	GPM1746a containing single-copy integrant of *eaeH* promoter-*lacZYA* transcriptional fusion at $attB_{HK022}$	This study
jf2575	GPM1746a containing promoterless *lacZYA* reporter integrated at $attB_{HK022}$	This study
jf2450	H10407(pGFPmut3.1)	This study
Plasmids		
pAH69	CRIM integrase expression helper plasmid	23
pHKLac1	Reporter with promoterless *lacZYA*, R6Kγ, *aadA*, $attP_{HK022}$	22
pQL002	*eaeH* promoter cloned into BamHI/EcoRI sites of pHKLac1	This study
pGFPmut3.1	pUC18-based gfpmut3* expression plasmid, Amp^r	63
pDONOR221	Entry cloning plasmid containing *ccdB* negative-selection gene and Cm^r cassette between lambda *attP* sites for recombination with PCR products containing *attB* regions, Km^r	Invitrogen
pET-DEST42	T7 *lac* promoter/IPTG-inducible expression plasmid, *ccdB*, Cm^r genes flanked by *attR* sites for recombination with entry plasmid, C-terminal V5 and 6-His tags	Invitrogen
pSS001	4,254-bp *eaeH* amplicon cloned into pDONOR221	This study
pSS002	EaeH expression plasmid derived from LR recombination reaction of pSS001 and pET-DEST42 placing *eaeH* in frame with V5 and 6-His epitope tags	This study

[a] Amp^r, ampicillin resistance; Km^r, kanamycin resistance; Cm^r, chloramphenicol resistance; *aadA*, aminoglycoside-3-adenyltransferase gene encoding streptomycin and spectinomycin resistance; *attB*, bacterial chromosome phage integration site; *attP*, complementary phage-derived integration site; R6Kγ, *pir*-dependent origin of replication.

bacteria maintained at Walter Reed Army Institute of Research (WRAIR). The ETEC H10407 isolate used in these studies was also originally obtained from Marcia Wolf at WRAIR from a GMP lot used in volunteer studies.

Caco-2 cell culture conditions. Caco-2 intestinal epithelial cells (ATCC HTB-37) were propagated in Eagle's minimal essential medium supplemented with fetal bovine serum to a final concentration of 20% and maintained in a 5% CO_2 atmosphere at 37°C. Caco-2 cell monolayers were grown to near confluence in 20- by 100-mm dishes.

Bacterial growth conditions and infection of intestinal epithelial cells. Cultures of ETEC strains H10407 and E24377A were grown from frozen glycerol stocks overnight in 2 ml Luria broth (LB) at 37°C with shaking at 225 rpm. The following morning, cultures were diluted 1:100 in fresh LB and grown at 37°C at 225 rpm for an additional 90 min. Bacteria were then centrifuged at 10,000 × *g* for 1 min and resuspended in tissue culture medium at a concentration of ~2×10^8 CFU/ml. Caco-2 monolayer dishes were each inoculated with ~10^9 bacteria and allowed to adhere for 15, 30, 60, or 120 min. At the end of the incubation period, nonadherent (planktonic) bacteria were collected from the supernatant media by centrifugation at 10,000 × *g* and washed once in ice-cold Hanks' balanced salt solution (HBSS), followed by HBSS containing 1% saponin, and after centrifugation, bacterial pellets were saved at −80°C for further processing. Monolayers with adherent bacteria were washed twice with 5 ml of ice-cold Hanks' balanced salt solution (HBSS) and then lysed with 5 ml of 1% saponin in HBSS for 5 min on ice to release adherent bacteria. Bacteria released by lysis of Caco-2 cells were recovered as pellets following centrifugation at 4°C and then saved at −80°C for subsequent RNA extraction.

Isolation of ETEC RNA. Total RNA was isolated from adherent and planktonic (nonadherent) bacterial fractions using an RNeasy minikit (Qiagen; catalog no. 74104) and was treated with DNase (Ambion; catalog no. AM1907). Conventional PCR for *arcA* (a housekeeping gene) was used to confirm the removal of DNA (data not shown). The RNA obtained from the adherent bacterial population is significantly contaminated with host RNA. Using 10 μg total RNA as the starting material, samples were processed with MICROBEnrich (Ambion; catalog no. AM1901) to remove host RNA, followed by depletion of the bacterial rRNA using MICROBExpress (Ambion; catalog no. AM1905). The remaining RNA, primarily bacterial mRNA, was ethanol precipitated and dissolved in 30 μl RNase-free water.

Microarray hybridization. To compare the transcriptomes of adherent and planktonic ETEC E24377A following infection of Caco-2 intestinal epithelial cells, we used the FDA-*E. coli-Shigella* (FDA-ECSG) Affymetrix array (http://pfgrc.jcvi.org/index.php/microarray/affy_array_description/ecoli_shigella/version1.html), which represents genes from multiple *E. coli* pathogens, including those from ETEC strain E24377A (18). Array hybridizations were carried out as previously described (19). Briefly, cDNA was synthesized with random hexamers and partially digested with DNase to obtain 20- to 200-bp fragments, end labeled with biotin-11-ddATP, and hybridized for 16 h at 45°C. Microarrays for processing were allowed to warm to room temperature for 30 min. The OligoB2 mixture (Affymetrix; catalog no. P/N 900301) is heated to 65°C for 5 min then mixed with the labeled and verified gDNA described above, dimethyl sulfoxide (DMSO), and hybridization buffer included in the Affymetrix hybridization, wash, and stain kit (Affymetrix; catalog no. P/N 900720). This solution is further denatured at 94°C for 5 min. During this incubation, the microarrays are equilibrated with prehybridization buffer and placed in the Affymetrix hybridization oven at 45°C for 10 min with rotation at 60 rpm. The labeled DNA is then hybridized to the microarray for 16 h at 45°C with rotation at 60 rpm. Microarrays were then washed and stained according to manufacturer's specifications using two stain cycles and the Affymetrix prokaryotic washing protocol (http://www.affymetrix.com).

Microarray data analysis. Array analysis was carried out a previously described (19). Briefly, array scans were acquired in Affymetrix GCOS software, and expression was normalized using the simpleaffy Bioconductor R package (20). Comparisons were completed by double filtering using fold change values of ≥2 and Student's *t* test values with $P \leq 0.05$. Two biological replicates for the different conditions were obtained for each biological condition. Custom Perl and Python scripts were then used to identify transcriptionally altered genes. To construct a heat map representing genes of interest with significant changes in transcription, data were imported into and analyzed in R (version 2.15.1; http://www.R-project.org/) (21).

TABLE 2 Primers used in quantitative reverse transcriptase PCR

Gene	Primer sequence	
	Forward	Reverse
crp	CCGTCAGGAAATCGGTCAGA	TGCGTCCCACGGTTTCA
fimH	GATGCGGGCAACTCGATT	GCCCTGCGCTGGTGAA
fimA	TGCGGGTAGCGCAACAA	
fimB	GGAGATTCATCCGCACATGTT	TCCCATATTCGCCAAAGCA
fimC	CGTTGCCACCCGATCAG	AATTCGCGCTACGACGAAA
fimD	CAGTGCCAGCTACAGCATGTC	TACCGGCCAGATTGGTCATC
fimE	CGCGGGAGTCGGCTTT	ACCGGCATCACGAATAATGC
fimF	CCCTGTGGTAACGCCGTTT	GGCTATCTGCAACGCCAGTAA
fimG	AATCAGCGCACTTCCCGTTA	TGAGTGGCTCCGCCATTTAC
fimH	GATGCGGGCAACTCGATT	GCCCTGCGCTGGTGAA
fimI	CGGAAACTTGCCGGATTG	GCCCCATATTGACCGTCATT
cfaA	CAGGAGCGAGTAAATCAATACGTTT	CTATCTGGTTTTACCGCCTCAAA
cfaB	AAATGGCGTATCATCTTCTCAAGAG	GGCGGTACCGGCAGTTT
cfaC	GGTTGGAGTGGATGCTACGAA	CAGAGTACTTGTCCATCCTAATAAAGGA
eatA	AAAGCTCCAATGCCTGATTTTAGTA	GTGTATGGTCTCCTGGTGGTAATG
etpB	GAACAGGGATAATGCCACAAA	TCCCAGATTCACCTCGTTTT
etpA	ATGCCGGTGGCTATATTGTG	GTACGGATGGTGCCACTGTT
etpC	CCACCTGGTCTGAGCATATTG	CCGGGTCTCCCAGGTAGT
eaeH	GAAGGATGCGTACGGGAAC	CCGCTAAACACTGGTGCAT
tia	ACAGGCTTTTATGTGACCGGTAA	GACGGAAGCGCTGGTCAGT
arcA	ATCAATCTGCCGGGTAAGAACGGT	TCCAGATCACCGCAGAAGCGATAA
cexE	GCGTGTAATGAGACAGTTGCTAAAG	CCTCATAATTTACAGTCCGATGCA
aatD	CAAAACCACGGAGGAACTGTACT	GCGATGTTGCGAATACGCTAT
aatP	CAACTTTTTTGGATAGCCTTGGA	GCGCCGTTTCTAATGGGATA
aatB	CAGCGGTATGGAGGGTGTAAA	AGATCTGATGATAGGTTTCCATTGTTT
aatA	CTTGAGGAGGCGAATATTAATTTTGA	GGCGTCAGCCCCAAAGA
aatC	TGGCATATCTCCAGAAGCATCA	CTTGCAAGCGCAACTCGTT
gspC	GTCGCCACGCCGGTAA	GACGCGTTTCTGCCACAGA
gspD	CGGATACCAACGGCGATCT	CCGCTAAAGCCGGAAAGAA
gspE	TGCGCGCGATTTTGC	CGGATTTCACCGACCATCA
gspF	AAAGAGCTTATCCCCGTGCAT	AACATCCCCCCTGACGAAGT
gspG	GCAAGTCTGGTGGTGCCTAAC	TTTTGCCGATCCGCTTTCT
gspH	CAGATCCGTTTTTCGCCTTTT	CGAGTAGAAGCGCAGCGTAA
gspI	GCTCCAGGCGGTGGATATT	TGACTGAATGACCGACGAAAAG
gspJ	GCCGACAATGCAAAAGTTGA	CCGTCGTAGAACTGCAAACG
gspK	TGCTGGTAACCATCACGCTTT	GCGTTCGCCCAAGTTGTT
gspL	CTCCAGCAATGGCTGCAA	GATCCATTCGCCGGGAAT
gspM	CGCTGCGGGTGACACAA	ACATTCACCATCCCAGTCTTCTC
eltA	TTCATCAAGAACAATTACAGGTGATA	TGATATTTCCTGAGATATATTGTGCTC
yebT	TCGTAAACTCACCAGCAAAGG	AGAAACTCAACGCCATCCAG

qRT-PCR. For quantitative reverse transcriptase PCR (qRT-PCR) analysis, total RNA was converted to cDNA by use of reverse transcriptase and random hexamers using the Transcriptor first-strand cDNA synthesis kit from Roche (catalog no. 04379012001). Gene expression was quantified on an ABI-PRISM 7900HT sequence detection system with gene-specific primers in PCR buffer containing SYBR green. Gene-specific transcripts were normalized to the housekeeping gene *arcA*. Gene-specific primers were designed using Primer Express Software v3.0 (Applied Biosystems) and are shown in Table 2.

Construction of single-copy transcriptional fusions. To construct transcriptional reporter fusions, regions upstream of the gene of interest were amplified by PCR and cloned into pHKLac1, a promoterless *lacZYA* reporter plasmid (22) containing an R6Kγ origin of replication and HK022 phage *attP* site for integration into the respective HK022 chromosomal *attB* site (23). Briefly, a 935-bp amplicon containing the *eaeH* promoter region (from −594 to +341 of *eaeH*) was PCR-amplified using primers jf032812.1 (5′-GATCGGATCCGATCCCATAGTTTATCCGG-3′) and jf032812.2 (5′-GATCGAATTCTTCCCGAGCCACTCCTG-3′) and directionally cloned into the corresponding BamHI and EcoRI sites (respectively underlined in sequences listed above) of pHKLac1, resulting in plasmid pQL002. pQL002 was then introduced by electroporation into strain GPM1746a containing the pAH69 CRIM integrase helper plasmid. After initial growth at 30°C on plates containing ampicillin (100 μg/ml), individual colonies were streak purified onto Luria agar plates containing streptomycin (Sm) (30 μg/ml) and spectinomycin (Sp) (30 μg/ml) and incubated overnight at 37°C. Ampicillin-sensitive, Sm^r/Sp^r colonies were then tested by PCR to verify the presence of stable single-copy integrants containing the reporter as previously described (23).

β-Galactosidase assays. Assessment of β-galactosidase activity in intestinal epithelial cell monolayers infected with reporter strains was adopted from methodology previously reported by Klarsfeld, et al. (24). Briefly, Caco-2 cells were seeded in black-walled 96-well tissue culture plates and grown to semiconfluent monolayers. Cultures of reporter strains were grown overnight in Luria broth containing Sm (30 μg/ml) and Sp (30 μg/ml), diluted 1:100 into fresh LB the following morning, and then grown for an additional 2 h at 37°C at 225 rpm. Monolayers were then infected at a multiplicity of infection (MOI) of ~100 and incubated for the indicated time period. Planktonic bacteria were resuspended in

200 μl of assay buffer containing $Na_2HPO_4 \cdot 7H_2O$ (60 mM), NaH_2PO_4 (40 mM), KCl (10 mM), $MgSO_4$ (1 mM), and β-mercaptoethanol (50 mM). Twenty microliters of resuspended bacteria was saved from each sample to determine the number of CFU present by plating dilutions. One hundred microliters of the remaining suspension was saved for analysis. Monolayers with attached bacteria were washed four times with phosphate-buffered saline (PBS), and 100 μl of assay buffer was added to each well. To quantitate the number of attached bacteria, parallel wells containing infected monolayers were treated in an identical fashion, with 100 μl of MUG (4-methylumbelliferyl β-D-galactopyranoside) assay buffer containing 0.1% Triton X-100. Dilutions of these cell lysates were plated onto Luria agar and incubated overnight at 37°C. To detect β-galactosidase activity in planktonic and attached bacteria, MUG was added at a final concentration of 0.2 mg/ml and the mixture was incubated for 1 h at 37°C. One hundred microliters of 1 M Na_2CO_3 stop buffer was added, the plate was read at absorbance and emission wavelengths of 365 and 450 nm, respectively, and endpoint data were acquired using Gen5 v 2.0 software. β-Galactosidase activity was then expressed as units of fluorescence/min/10^4 CFU.

Cloning, expression, and purification of recombinant EaeH. In constructing an *eaeH* expression plasmid, primers jf051010.1 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTGGGAAGGAGATAGAACCATGTCACATTATAAAACAGGT-3′) and jf051010.2 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTGGCATCTCCTCCTCGCCATT-3′) were first used to amplify a 4,254-bp *eaeH* gene fragment (lacking only the stop codon) from H10407 genomic DNA. The resulting amplicon, containing 5′- and 3′-terminal *attB* sites, was then cloned by bacteriophage λ-mediated recombination with corresponding *attP* sites on pDONR221, yielding pSS001. After confirmation of the construction of pSS001 by restriction digests and DNA sequencing, this plasmid was recombined with pET-DEST42, placing the *eaeH* gene in frame with C-terminal V5 and polyhistidine tags to create the pSS002 expression plasmid. The pSS002 expression plasmid encoding the EaeH–V5–6-His fusion protein was introduced into BL21A1 (Table 1), and recombinants were selected on ampicillin (100 μg/ml). Following induction of BL21A1(pSS002) with IPTG (isopropyl-β-D-1-thiogalactopyranoside), recombinant polyhistidine-tagged protein was recovered from bacterial lysates (bacterial protein extraction reagent [B-PER]; Pierce/Thermo Scientific) by nickel metal affinity chromatography. Western blotting using monoclonal antibody against the V5 epitope (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr) was then used to confirm expression of the fusion protein.

Production and purification of polyclonal anti-EaeH antibodies. Polyclonal antiserum was produced in rabbits as previously described (6). Briefly, two New Zealand White rabbits were immunized with recombinant V5 polyhistidine-tagged EaeH. The resulting polyclonal antisera were preabsorbed using an *E. coli* lysate column (Pierce) and lyophilized strain AAEC191-A (25). Protein A agarose (Protein A Plus; Thermo Scientific) was used to separate antibodies from serum components, and antibodies were affinity purified against rEaeH-V5.

Confocal immunofluorescence microscopy of ETEC-infected Caco-2 cells. Caco-2 cells were seeded onto poly-D-lysine-coated glass coverslips, incubated overnight, and infected with cultures of H10407 expressing green fluorescent protein (GFP). At 5 h postinfection, samples were fixed with 2% paraformaldehyde for 30 min, permeabilized with 0.1% saponin for 10 min, and blocked with PBS supplemented with 1% bovine serum albumin (BSA) for 30 min. Samples were incubated with affinity-purified anti-EaeH primary antibody for 1 h at 37°C and then goat anti-rabbit secondary antibody conjugated to Alexa Fluor 594 (Invitrogen), DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen), and phalloidin 647 (Invitrogen) for 1 h at 37°C. Samples were mounted in ProLong reagent (Invitrogen). Images were captured with a Zeiss LSM 510 META confocal laser scanning microscope using Axiovision software.

Scanning electron microscopy. To examine the surface of ETEC cells adherent to cultured epithelial cells, bacteria were added to nonconfluent Caco-2 cells seeded onto glass coverslips at a multiplicity of infection (MOI) of ~100 organisms/cell and incubated for 15 to 60 min. At the end of the indicated time period, infected supernatants were removed, and the cells were washed with PBS and then processed for scanning electron microscopy (SEM) as described previously (7). All images were acquired on a Philips environmental scanning electron microscope (FEIC Philips XL30 ESEM) and saved as TIFF files. Images were then processed in Image J (v1.45s) augmented with the DeconvolutionLab plug-in (26).

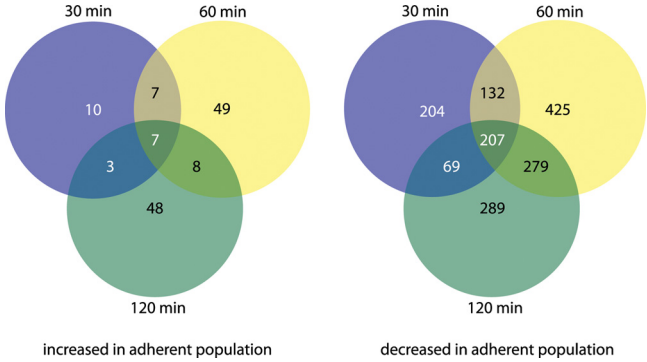

FIG 1 Global alteration in ETEC transcriptome over time, comparing planktonic and attached organisms. The total number of genes with significantly increased transcripts in adherent organisms at each time point is shown to the left. Gene transcripts that were increased in planktonic organisms (decreased in adherent organisms) are shown to the right. Complete lists of individual genes modulated at each time point are contained in Tables S1 to S3 in the supplemental material.

Flow cytometry. To examine the quantity of flagellin associated with the bacterial surface of H10407 (O78:H11), we performed flow cytometry as previously described (27). Briefly, suspensions of bacteria were fixed with 2% paraformaldehyde for 15 min, washed twice phosphate-buffered saline (PBS), pH 7.2, blocked with 1% BSA in PBS for 30 min, followed by flagellin detection with affinity-purified mouse polyclonal anti-H11 antisera (28), and labeled with Alexa Fluor 488 anti-mouse (IgG) secondary antibody (Invitrogen). Data were acquired on a BD FACSCalibur 4-color dual-laser flow cytometer and processed using FlowJo software (v9.4.1).

Supernatant protein precipitation and immunoblotting. Proteins in culture supernatants were precipitated as previously described (6) with trichloroacetic acid (TCA). Briefly, 750 μl of ice-cold TCA was added to an equal volume of bacterial culture supernatant, incubated on ice for 30 min, centrifuged at 15,000 × *g* at 4°C, acetone washed, dried, and resuspended in 1 M Tris, prior to separation by SDS-PAGE and immunoblotting for EtpA and flagellin (6, 7).

Microarray data accession number. All microarray data for this project have been deposited in the GEO (Gene Expression Omnibus) repository (http://www.ncbi.nlm.nih.gov/projects/geo/) under accession no. GSE40427.

RESULTS

Global changes in the ETEC transcriptome with pathogen-cell contact. Using the *E. coli-Shigella* pan-genome array, we interrogated the transcriptomes of planktonic and adherent ETEC strains. Comparative analysis of the genes that were transcriptionally altered >2-fold and had a *P* value of <0.05 are presented in Fig. 1. A total of 214 gene features on the array displayed a consistently altered transcriptional pattern at all three time points examined, suggesting an early and sustained response to the interaction of *E. coli* E24377A with epithelial cells (Fig. 1; see Tables S1 to S3 in the supplemental material). Fewer genes exhibited demonstrable increases in transcription with cell contact relative to the number that were negatively modulated. Figure 2 illustrates transcrip-

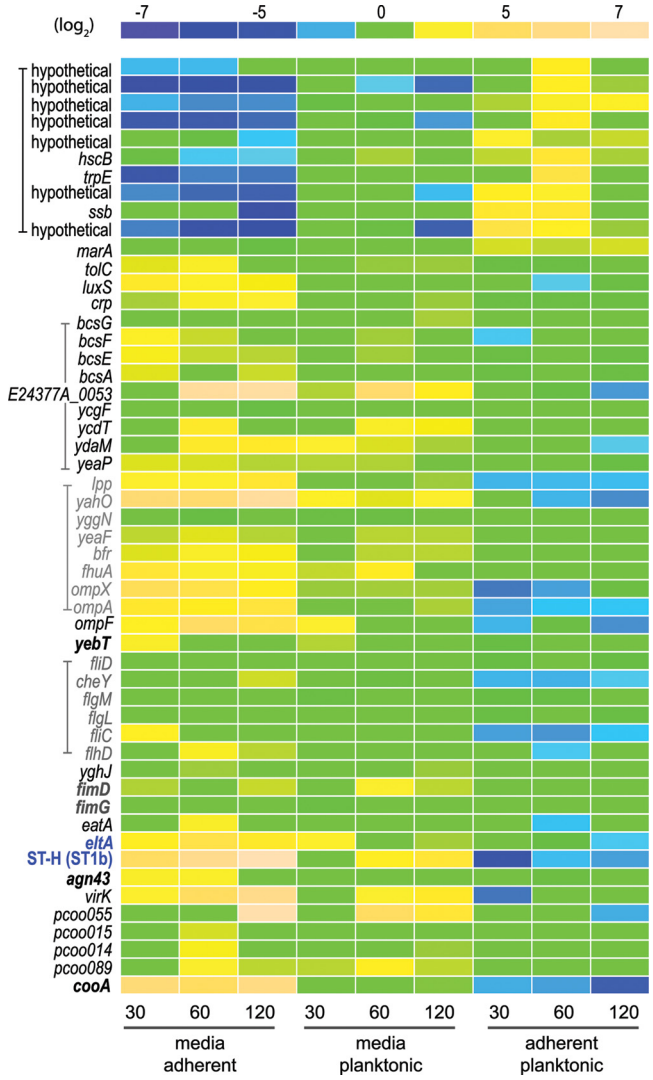

FIG 2 Modulation of ETEC gene expression upon interaction with intestinal epithelial cells. A relative expression intensity scale ($\log_2$ values) appears at the top of the figure. Gene features are shown to the left, with related features depicted with brackets/color. Included are known/candidate virulence genes, immunogens, or regulatory genes. The genes most responsive ($n = 10$) to bacterial adhesion are depicted at the top of the figure. The toxin gene *eltA*, encoding the LT-A subunit, and the gene coding for heat-stable toxin ST-H (ST-1b) are shown in blue print. Adhesins or putative adhesins are depicted in boldface letters. pCoo plasmid gene designations refer to plasmid-encoded sequences from the pCoo plasmid (64), for which the complete sequence is available at NCBI accession no. NC_007635.1 The conditions and time points (minutes) for each column of data are shown at the bottom of the figure. Data in the far left column depict the fold changes in transcription for bacteria grown in unconditioned tissue culture media relative to the adherent population at 30 min, while those in the far right column represent changes in adherent bacteria relative to planktonic organisms after 120 min. Elements for which there was no significant difference in the microarray data are depicted as 0-fold change (green).

tional regulation of known and potential virulence genes following attachment and encompasses a number of gene features exhibiting significant responses at early, middle, and late time points following cellular attachment. As depicted in Fig. 2, the majority of genes demonstrating significant increases in transcription following attachment are currently annotated as hypothetical genes. However, included among genes upregulated on attachment was *marA*, which encodes a regulatory protein involved in *E. coli* stress responses (29). In contrast, the majority of genes for known and/or putative virulence factors or those encoding immunodominant antigens were transcriptionally repressed following cell contact.

***crp* and cAMP-CRP-dependent virulence genes are modulated following host contact.** Among the genes transcriptionally altered in ETEC strain E24377A upon contact with epithelial cells was *crp*, encoding the cAMP receptor protein (CRP), a global regulator involved in modulating the transcription of many *E. coli* genes (30), including a number of known ETEC virulence factors, such as the colonization factor antigens and the heat-labile and heat-stable enterotoxin genes (31). Likewise, genes previously shown to be *crp* dependent, including those governing flagellar assembly and chemotaxis and those coding for CFs, LT, and the heat-stable ST-1b toxin (ST-H), were significantly altered relative to planktonic organisms following attachment (Table 3), with both toxins transcriptionally repressed in the attached bacteria. Similarly, qRT-PCR studies of ETEC H10407 demonstrated that in comparison to planktonic organisms, *crp* expression was depressed in attached bacteria (Fig. 3). However, because an earlier analysis of H10407 demonstrated that the cAMP-CRP complex represses transcription of heat-labile toxin genes (31), we predicted that in contrast to E24377A, cell contact by H10407 should enhance transcription genes encoding LT. Indeed, consistent with the repression of CRP following cell contact in this strain, we found that transcription of the gene encoding the A subunit of the toxin (*eltA*) was increased relative to planktonic organisms (average, 7.2 ± 3.8-fold; $P = 0.047$). Together, these findings suggest that while the cAMP-CRP complex plays a substantial role in governing ETEC bacterium-host interactions, there are likely to be significant interstrain differences in transcription of essential virulence genes modulated by this key regulator.

Influence of pathogen-host interaction on c-di-GMP signaling. In addition to cAMP, gene expression in *E. coli* and other bacteria is also known to be governed by another second messenger, cyclic-di(3′→5′)-guanylic acid (c-di-GMP) (32). Microarray data (Fig. 2) indicated that multiple genes associated with c-di-GMP signaling, including several known or putative diguanylate cyclase proteins containing GGDEF domains, as well as known and a novel putative phosphodiesterase encoded on the pETEC_74 virulence plasmid from E24377A (E24377A_0053) were transcriptionally altered on ETEC-host interaction (Table 4). Likewise, we noted modulation of several genes associated with cellulose biosynthesis, which has been demonstrated to be influenced by alteration in c-di-GMP levels (33). Collectively, these data are congruent with previous studies in other pathogens that have demonstrated that c-di-GMP-coordinated changes are associated with the transition between the planktonic and adherent states (32).

Temporal activation of multiple bacterial adhesins. Data from the DNA microarray studies suggested that the transcription of multiple virulence genes, including a number of adhesin genes, was modulated either upon exposure to media conditioned by host cells or by host cell contact. Therefore, we conducted additional experiments with ETEC H10407 to examine modulation of adhesion-related genes. These studies, summarized in Fig. 4, demonstrated that at early time points following attachment of ETEC to Caco-2 cells, multiple genes associated with the biogenesis of

TABLE 3 Alteration of *crp*- and cAMP-CRP-dependent genes on cell contact[a]

Gene name	Protein annotation	Time (min)	Fold change (log_2)[b]	*P* value
crp	cAMP regulatory protein	30	−1.60	0.019
		60	−1.84	0.036
		120	−2.16	0.038
Flagellar genes				
flgK	Flagellar hook-associated protein 1	30	−1.22	0.042
		60	−	NS[c]
		120		NS
fliD	Flagellar hook-associated protein 2	30	−1.169	0.015
		60		NS
		120	−1.106	0.0017
flit	Flagellar biosynthesis protein	30		NS
		60		NS
		120	−1.12	0.028
flgL	Flagellar hook-associated protein 3	30	−1.55	0.017
		60		NS
		120	−1.44	0.016
fliC	Flagellin	30	−3.19	0.02
		60	−3.33	0.0006
		120	−2.61	0.0019
flgM	Anti-sigma 28 factor	30	−1.096	0.0082
		60	−1.488	0.0310
		120		NS
cheY	Chemotaxis regulatory protein	30	−2.894	0.0495
		60	−2.979	0.0004
		120	−2.379	0.0051
cheW	Purine-binding chemotaxis protein	30		NS
		60	−1.176	0.0011
		120		NS
luxS	*S*-Ribosylhomocysteine lyase	30		NS
		60	−2.3	0.0003
		120	−1.93	0.016
cooA	CS1 type fimbrial major subunit	30	−3.008	0.0241
		60	−3.212	0.0001
		120	−4.580	0.0043
ompA	Outer membrane protein A	30	−3.137	0.0106
		60	−2.71	0.0040
		120	−2.64	0.016
tolC	TolC outer membrane channel protein	30	−1.74	0.022
		60	−1.8267	0.013
		120		NS
eltA	Heat-labile toxin, A subunit	30		NS
		60	−1.19	0.017
		120	−2.46	0.003
ST-1b	Heat-stable toxin (ST-H)	30	−5.14	0.036
		60	−2.79	0.015
		120		NS

[a] The data reported in the table depict all genes with previously established CRP-cAMP dependence for which there were statistically significant changes in gene expression comparing attached and planktonic organisms using the FDA-*E. coli-Shigella* (FDA-ECSG) Affymetrix array.
[b] Adherent versus planktonic (log_2 values).
[c] NS, nonsignificant change.

type 1 fimbriae were transcriptionally activated (Fig. 4a). In contrast, transcription of *cfaABC* genes involved in assembly of the plasmid-encoded colonization factor antigen 1 fimbriae (CFA/I), as well as those involved in synthesis and export of the EtpA two-partner exoprotein adhesin, was repressed following contact. We also examined the transcription of the gene encoding EatA, a secreted autotransporter recently shown to accelerate delivery of the heat-labile toxin by preventing accumulation of the EtpA adhesin (10). Interestingly, consistent with its function in modulating EtpA-mediated adhesion, *eatA* transcription was activated at early time points following cell contact.

Intriguingly, *eaeH*, a putative adhesin gene originally discovered by subtractive hybridization of ETEC H10407 and *E. coli* K-12 (34), which is highly conserved in pathogenic *E. coli*, was strongly upregulated at later time points following contact with intestinal epithelial cells (Fig. 4b). The predicted EaeH peptide shares a number of features in common with known adhesins, such as intimin from enteropathogenic *E. coli* (EPEC), as well as

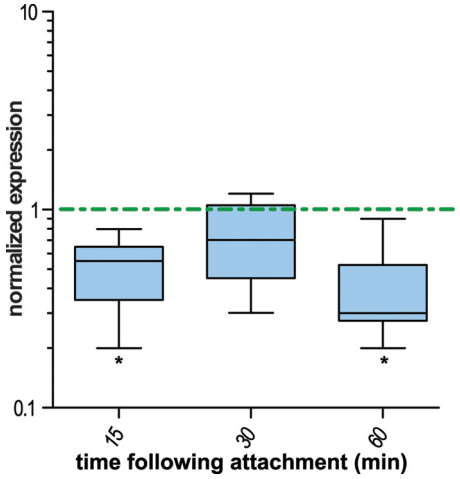

FIG 3 *crp* expression is depressed in ETEC H10407 following contact with epithelial cells. Data represent the summary of six independent experiments in which ETEC H10407 was used to inoculate Caco-2 intestinal epithelial cell cultures and then harvested from either the supernatant (planktonic organisms) or from the cell surface (attached), followed by comparison of *crp* transcripts determined by qRT-PCR for attached bacteria relative to planktonic organisms. All data are normalized to the housekeeping gene *arcA* and represent the ratio of attached to planktonic organisms. Whisker plots depict the range of data obtained over 6 experiments, with the horizontal line representing the mean value. A dashed horizontal line is drawn at a normalized expression value of 1 (no difference between attached and planktonic bacteria). *, $P \leq 0.05$ by Wilcoxon's signed-rank test.

the Air protein from enteroaggregative *E. coli* (EAEC) (35), including multiple bacterial immunoglobulin-like (Big) domains (pfam02369). In support of the data obtained by quantitative PCR, we examined gene expression using single-copy *eaeH* promoter β-galactosidase fusion constructs. Again, these data demonstrated a significant increase in *eaeH* expression in attached bacteria relative to planktonic cells (Fig. 5a). We also identified EaeH on the surface of ETEC H10407 attached to intestinal cells *in vitro*, both at the point of cell contact (Fig. 5b to e) and diffusely distributed on the surface of H10407 (Fig. 5f to i).

Interestingly, we observed comparatively little transcriptional modulation following adhesion in *yebT*, a gene encoding another putative outer membrane protein adhesin highly conserved in *E. coli* and a number of other pathogens. Real-time quantitative PCR to examine *yebT* expression in ETEC H10407 suggested that this gene is similarly expressed in both planktonic and adherent ETEC strains (Fig. 4a and b). This is consistent with the proposed constitutive expression of *yebT*, potentially making its gene product, Mam7, available for participation in early phases of adhesion in a number of pathogenic bacteria, including other *E. coli* pathovars (36).

Collectively, these data suggest that ETEC strains engage host cells by sequentially altering multiple adhesin molecules as the interaction matures. Furthermore, they suggest that pathovar-specific virulence factors, such as the CFs and EtpA, function within a contextual framework of highly conserved adhesins, including type 1 fimbriae, EaeH, and Mam7.

ETEC undergoes morphological changes following cell contact. In the course of recent studies to investigate the involvement of flagella in attachment of ETEC to epithelial cells (7), we noted that at very early time points (5 to 15 min), ETEC cells appear to engage host cells at a distance via their long peritrichous flagella (Fig. 6a), while at later time points (Fig. 6b and c), flagella appear shortened and/or engulfed by the host cell, accompanied by the emergence of bleb- or vesicle-like structures on the bacterial surface. Consistent with these apparent changes in bacterial surface structures, we demonstrated that the production of two secreted proteins, flagellin (the major subunit of flagella), and EtpA, a flagellin-binding adhesin, were both reduced following exposure to host cell-conditioned media (Fig. 6d), as demonstrated by immunoblotting. In support of these data, flow cytometry studies also indicated that flagellin on the surface of H10407 decreased with exposure to conditioned media (Fig. 6e).

The appearance of bleb-like structures on the surface of ETEC is intriguing in that previous studies have demonstrated that

TABLE 4 Transcriptional modulation of genes related to c-di-GMP metabolism and sensing[a]

Gene	Product description[b]	Transcriptional modulation comparison[c]	Time point (min)	Maximal difference ($\log_2$)[d]	*P* value
Cyclic-di-GMP metabolism					
yeaP	GAF/GGDEF-domain diguanylate cyclase	Medium vs adherent	30	2.44	0.0001
ydaM	Putative GGDEF-domain protein, *cgsD* regulator	Medium vs adherent	60	3.781	0.028
ycdT	Putative GGDEF-domain protein diguanylate cyclase	Medium vs adherent	60	3.748	0.002
ycgF	Cyclic-di-GMP phosphodiesterase	Medium vs adherent	120	−1.56	0.02
Locus tag *E0053*	Novel EAL-domain protein pETEC_74 virulence plasmid	Medium vs adherent	60	6.64	5.32×10^{-6}
Cellulose biosynthesis					
bcsA	Cellulose synthase catalytic subunit	Medium vs adherent	30	2.50	0.002
bcsE	Cellulose biosynthesis protein	Medium vs adherent	30	2.67	3.90×10^{-5}
bcsF	Cellulose biosynthesis protein	Medium vs adherent	30	3.33	2.49×10^{-5}
bcsG	Cellulose biosynthesis protein (predicted endoglucanase)	Medium vs planktonic	60	1.399	0.012

[a] The data reported in the table depict all genes with previously established or putative association with c-di-GMP metabolism/sensing for which there were statistically significant changes in gene expression under the conditions reported using the FDA-*E. coli-Shigella* (FDA-ECSG) Affymetrix array.
[b] The GAF domain refers to pfam01590, a domain present in cGMP-specific phosphodiesterases, the GGDEF domain refers to cd01949, a domain present diguanylate cyclases, and the EAL domain refers to cd01948, a second domain present in c-di-GMP phosphodiesterases.
[c] "Medium" refers to unconditioned tissue culture medium used to grow Caco-2 epithelial cells.
[d] A negative value reflects a decrease in transcription under the first condition relative to the comparison group.

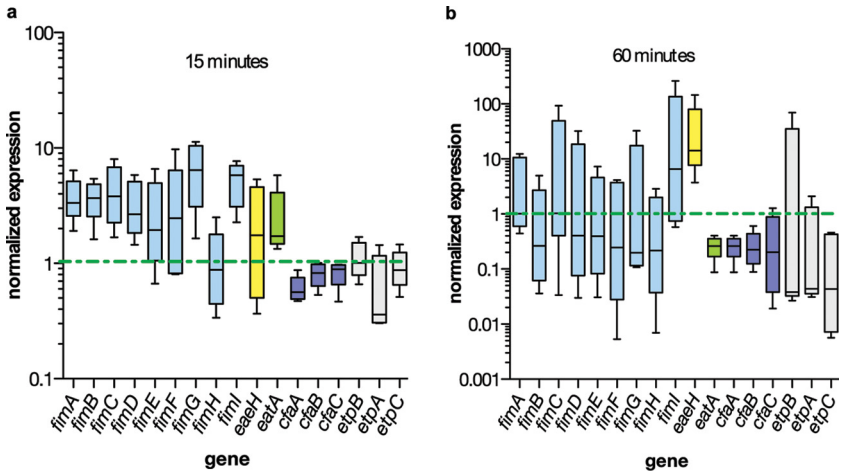

FIG 4 Modulation of ETEC H10407 adhesion-related genes on cell contact qRT-PCR for transcripts of adhesion-related genes in attached bacteria relative to planktonic organisms. Whisker plots depict the range of data obtained over five experiments, with the horizontal line representing the mean value. In each panel, a dashed horizontal line is drawn at normalized expression value of 1 (no difference between attached and planktonic bacteria). Panels a and b show the relative transcription at 15 min (a) and 60 min (b) following infection of Caco-2 cells with ETEC H10407.

much of the LT produced by ETEC is associated with these outer membrane vesicles (37, 38) and that these structures can effectively deliver toxin (39) to target epithelial cells. Consistent with vesicle structure formation and the membrane remodeling observed following attachment, we also observed concomitant transcriptional modulation of *hns* encoding the global DNA binding transcriptional repressor H-NS, previously associated with vesicle formation in *E. coli*. Interestingly, mutation of *hns* has been associated with an increase in vesicle formation (37), and following attachment *hns* transcription was significantly repressed (over 12-fold; $P = 0.002$) relative to that of planktonic organisms (see Table S2 in the supplemental material), suggesting that attachment of ETEC to the host cells may promote the formation of vesicles.

ETEC suppresses production of immunogenic proteins on cell contact. Recent immunoproteomic studies of ETEC using human sera obtained following natural infections or from experimentally infected mice demonstrated that multiple proteins are recognized during ETEC infections (11). Interestingly, we found that genes encoding many of these immunoreactive proteins were transcriptionally repressed upon interaction with host cells or conditioned media (Table 5), suggesting that ETEC may transcriptionally modulate these genes as a strategy to avoid elimination by the host.

DISCUSSION

The enterotoxigenic *E. coli* strains are an important focus of current enteric vaccine development efforts. ETEC vaccine attempts to date have been largely directed at heat-labile toxin and/or colonization factor antigens (2). Unfortunately, these approaches have been hindered by poor conservation of colonization factor antigens and the lack of complete, sustained protection afforded by LT immunization (5). The more recent discovery of additional novel virulence genes, even among prototypical ETEC strains, implies that our understanding of the pathogenesis of these organ-

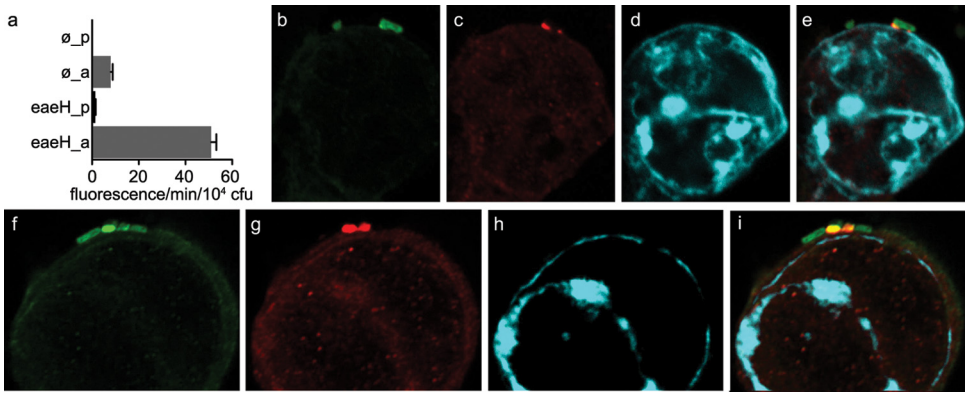

FIG 5 The putative adhesin gene *eaeH* is preferentially expressed in the context of epithelial cell attachment. (a) Activation of the *eaeH* promoter in attached bacteria relative to planktonic ETEC strains carrying an integrated single-copy *eaeH* promoter-*lacZYA* transcriptional fusion. ø_p and ø_a refer to control strain jf2575 with promoterless *lacZYA* in planktonic and adherent states, respectively, while eaeH_p and eaeH_a refer to data generated with strain jf2624 carrying the *eaeH* promoter-*lacZYA* fusion. MUG–β-galactosidase determinations are expressed in fluorescence units/min/10^4 CFU, with bars representing the mean of data from triplicate wells. EaeH expression following bacterial attachment was identified at the interface of ETEC H10407 and Caco-2 epithelial cells (b to e) and in a diffuse distribution on the surface of adherent bacteria (f to i). Confocal microscopy image panels from left to right show GFP-expressing ETEC (green), EaeH immunofluorescence (red), host cell actin (phalloidin; far-red, cyan), and the composite merged image.

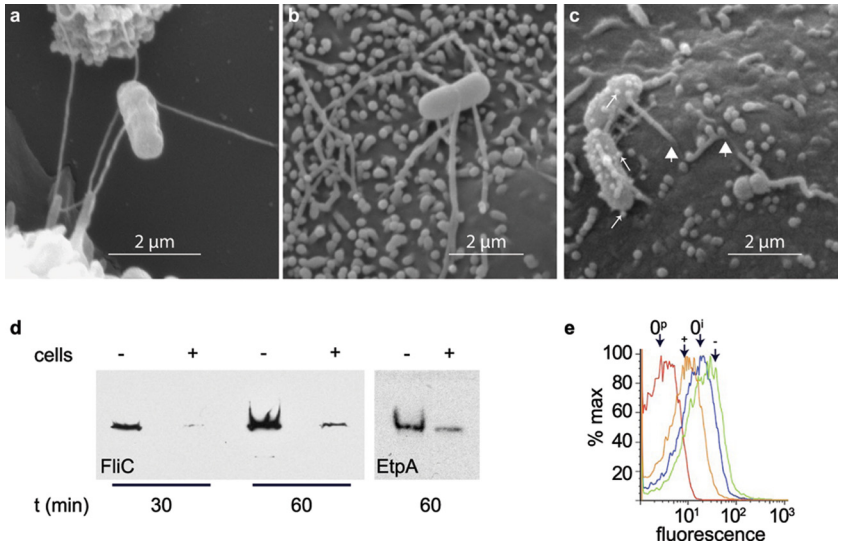

FIG 6 ETEC strains undergo changes in surface architecture after engaging intestinal epithelial cells. Scanning electron micrographs obtained at 15 (a), 30 (b), and 60 (c) min following addition of ETEC H10407 to intestinal epithelial cells *in vitro*. Small arrows in panel c indicate the appearance of blebs on the surface of H10407, while large arrowheads show the course of flagellum that has been partially engulfed by the cell surface. Panels b and c were enhanced using deconvolution. (d) Treatment of ETEC with Caco-2 cell-conditioned media leads to decreased amounts of flagellin (FliC) and EtpA in bacterial culture supernatants. FliC and EtpA immunoblots are shown to the left and right, respectively. −, tissue culture medium alone; +, medium that has been conditioned by growth of Caco-2 epithelial cells. (e) Flow cytometry used to detect FliC H11 on the surface of ETEC H10407 (serotype O78:H11) following growth in ordinary Luria broth prior to incubation with tissue culture media. 0^i, detection of FliC expression using anti-FliC antibody at time zero prior to incubation in media; 0^P, preimmune sera control at time zero; +, FliC expression after 60 min in conditioned media; −, FliC expression after 60 min in tissue culture media not preconditioned with Caco-2 cells.

isms is incomplete (40) and also raises the possibility that recent technologic advances may be applied to the discovery of previously unheralded vaccine targets. One approach that has been applied to other pathogens is the use of transcriptional profiling to identify genes modulated during host-pathogen interactions (16).

Here we demonstrate that ETEC-host interactions promote pronounced changes in the ETEC transcriptome that are accompanied by significant modification of the architecture of these organisms at the pathogen-host interface. Collectively, the data suggest that ETEC interactions with host cells are finely orchestrated, with the sequential deployment of multiple adhesins as the bacteria engage their epithelial targets culminating in effective toxin delivery.

The precise stimuli for these changes remain to be elucidated. However, the studies do appear to affirm a central role of cAMP and the cAMP receptor protein (CRP) in modulation (41) of

TABLE 5 Transcriptional modulation of previously identified ETEC immunoreactive proteins[a]

Gene	Product description	Transcriptional modulation comparison	Time point (min)	Maximal difference ($\log_2$)[b]	*P* value
fliC	Flagellin monomer	Adherent vs planktonic	60	−3.33	0.0006
eatA	Secreted ETEC autotransporter virulence protein	Adherent vs planktonic	60	−2.71	0.002
agn43.2	Surface autotransporter	Adherent vs medium	60	−2.96	0.0007
cooA[c]	CS1 major fimbrial subunit	Medium vs adherent	120	5.98	1.8×10^{-5}
fimD	Fimbrial usher, type 1 fimbriae	Medium vs planktonic	60	3.13	0.003
eltA	Heat-labile toxin subunit	Medium vs adherent	60	4.66	0.0002
ompA	OmpA porin	Medium vs adherent	120	4.16	0.0007
fecA	Iron transport membrane receptor	Medium vs adherent	60	1.06	0.03
ompX	OmpX porin	Medium vs adherent	30	4.84	0.001
fhuA	Ferrichrome outer membrane transporter	Medium vs adherent	30	4.06	0.009
bfr	Bacterioferritin	Medium vs adherent	60	2.88	0.017
yggN	Hypothetical	Medium vs planktonic	60	1.92	0.0009
yahO	Hypothetical	Medium vs adherent	120	6.60	7.42×10^{-5}
lpp	Hypothetical	Medium vs adherent	30	3.22	0.001
yghJ	Secreted type II secretion effector	Medium vs adherent	60	1.14	0.006
yeaF	Hypothetical; MipA domain protein/envelope biogenesis	Medium vs adherent	60	2.483	0.03

[a] The data reported in the table depict all genes identified in prior immunoproteomic analysis (11) for which there were statistically significant changes in gene expression under the conditions reported using the FDA-*E. coli-Shigella* (FDA-ECSG) Affymetrix array.
[b] Negative values reflect decreases in transcription under the first condition relative to the comparison group.
[c] Earlier immunoproteomic analysis performed with H10407 identified the CfaB protein as the major structural subunit of CFA/I fimbriae.

known (22) and putative (42) virulence genes, including genes involved in flagellar biosynthesis (7), whose expression is known to be under the control of *crp* (43).

Interestingly, the *crp* gene is itself negatively autoregulated by the cAMP-CRP complex (44). One possible explanation for the modulation of genes under the control of cAMP-CRP and *crp* in response to interaction with host cells is that ETEC strains are able to sense cAMP generated by host epithelial cells in response to stimulation by heat-labile toxin. Bacteria have long been known to respond to extracellular cAMP, and more recently, host cyclic nucleotide efflux systems that are activated in response to increasing intracellular concentrations of cAMP have been described in some detail (45, 46). Therefore, it is plausible to suggest that ETEC strains may not only provoke increases in cAMP as they successfully deliver heat-labile toxin, but respond to pathogen-induced extracellular increases in this critical second messenger by modulating the expression of essential virulence genes.

Among the genes whose expression was significantly affected by cell contact were those associated with bacterial cell signaling, including the autoinducer 2 (AI-2) synthase gene *luxS*, which has previously been shown to be dependent on cAMP-CRP (47). Interestingly, we also noted significant alteration of other bacterial cell signaling genes, including a number of genes coding for known or putative diguanylate cyclases (e.g., *yeaP*) needed for synthesis of cyclic di-GMP (48, 49), a highly conserved bacterial second messenger involved in regulating a host of virulence traits, including biofilm formation, motility, and expression of virulence genes (50). In addition, there was appreciable modulation of genes encoding di-GMP phosphodiesterases that typically contain EAL domains (51). These included *ycgF*, which encodes a known *E. coli* c-di-GMP phosphodiesterase as well as a novel, yet uncharacterized gene located on an E24377A virulence plasmid encoding a putative EAL domain protein. Likewise, we noted the modulation of putative and known c-di-GMP binding proteins, including YcgR, which has been shown to be essential for *E. coli* motility (50). These findings are in keeping with the known central role of c-di-GMP sensing in orchestrating adaptation of bacteria from a planktonic to an attached or sessile lifestyle (32).

Interestingly, we found significant overlap in the assemblage of genes that were negatively modulated by either direct cell contact or exposure to media conditioned by host cells and genes encoding proteins previously identified in recent immunoproteomic studies of ETEC using convalescent-phase sera (11). This may suggest that ETEC strains are programmed to specifically extinguish production of immunodominant proteins once they have reached their epithelial targets, a phenomenon that could have significant implications for the design of effective mucosal vaccine strategies for ETEC.

The modulation of multiple genes involved in formation of outer membrane vesicles (OMV) following successful attachment is also intriguing for several reasons. Enhancement of the production of vesicles in the context of intimate interaction with the host may provide an important means of countering innate defenses as most Gram-negative OMV serve as potent stimuli of innate immune responses (52, 53). More importantly, the apparent formation of outer membrane vesicles following attachment would potentially provide an important link between earlier studies demonstrating that ETEC vesicles are laden with LT and that purified vesicles can serve as effective means of toxin delivery (38). Programmed vesicle formation upon pathogen-host interaction could theoretically enhance delivery of OMV-associated virulence proteins such as LT. While vesicle biogenesis in the context of epithelial cells requires additional study, the formation of vesicle-like structures by other *E. coli* pathotypes (54) upon cell contact might suggest that ETEC strains have coopted a more general response to host-pathogen interactions for toxin delivery.

One of the gene transcripts that significantly increased following adhesion was *marA*, which encodes an AraC-type transcriptional regulator involved in the modulation of a variety of genes which impact virulence in uropathogenic *E. coli*, including the ability to form biofilms, and effectively colonize the urinary tract (55). MarA acts to indirectly reduce levels of OmpF (56); correspondingly, *ompF* transcription was noted to be decreased in bacteria adherent to host cells. Interestingly, MarA also governs resistance to cationic antimicrobial peptides (CAMPs), thought to be part of the innate immune response to enteric pathogens, such as ETEC. Human β-defensin 1 (HBD-1), which is constitutively expressed by Caco-2 intestinal epithelial cells (57) and was used here as the target for bacterial adhesion, has been shown to induce *marA* transcription (58).

While the modulation of genes following cell contact has been described for a number of other pathogens (12–14, 59), these are the first studies to systematically examine alteration in ETEC gene transcription upon interaction with host epithelial cells. Most prior studies have focused narrowly on transcriptional changes in genes encoding colonization factors or the toxins. We should note that one recent genome-wide transcriptome study of ETEC that examined the effect of exposure to bile salts (19) revealed significant interstrain variation in transcriptional responses of key virulence genes. Indeed, the demonstration here that transcription of genes encoding heat-labile toxin is differentially modulated in response to cell contact in E24377A and H10407 appears to support this concept and add another layer of complexity to the study of this genetically diverse population of pathogens.

Collectively, however, the data presented here would suggest that ETEC interactions with host cells are finely orchestrated events during which the bacteria engage their epithelial targets by concerted action of a number of different bacterial adhesins. Early predominance of pathoype-specific adhesins, including EtpA, may set the stage for more intimate adhesion events mediated by more highly conserved structures, including type 1 fimbriae, and outer membrane proteins, including EaeH.

Ultimately, ETEC strains must be able to effectively deliver their toxin payload to epithelial cells. Remarkably, the essential elements required for toxin delivery by these complex pathogens to host cells are still very poorly understood. Although we have demonstrated that the EtpA adhesin (60) and the EatA protease that modulates EtpA-mediated adhesion (10) are both essential for optimal delivery of LT to the target intestinal epithelial cells, the studies included here intimate a complex series of events that would appear to include significant alteration of bacterial surface structures. Unraveling the molecular details underlying these events could provide novel approaches to the rational design of ETEC vaccines.

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