Proteomic Analysis of the Intestinal Epithelial Cell Response to Enteropathogenic Escherichia coli*

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We present the first large scale proteomic analysis of a human cellular response to a pathogen. Enteropathogenic Escherichia coli (EPEC) is an enteric human pathogen responsible for much childhood morbidity and mortality worldwide. EPEC uses a type III secretion system (TTSS) to inject bacterial proteins into the cytosol of intestinal epithelial cells, resulting in diarrhea. We analyzed the host response to TTSS-delivered EPEC effector proteins by infecting polarized intestinal epithelial monolayers with either wild-type or TTSS-deficient EPEC. Host proteins were isolated and subjected to quantitative profiling using isotope-coded affinity tagging (ICAT) combined with electrospray ionization tandem mass spectrometry. We identified over 2000 unique proteins from infected Caco-2 monolayers, of which ~13% are expressed differentially in the presence of TTSS-delivered EPEC effector proteins. We validated these data in silico and through immunoblotting and immunofluorescence microscopy. The identified changes extend cytoskeletal observations made in less relevant cell types and generate testable hypotheses with regard to host proteins potentially involved in EPEC-induced diarrhea. These data provide a framework for future biochemical analyses of host-pathogen interactions.

Enteropathogenic Escherichia coli (EPEC) is the leading cause of bacterial-mediated diarrhea in children and is a major endemic health threat in the developing world. EPEC binds to intestinal epithelial cells, forming a characteristic lesion (attaching/effacing (A/E)) resulting from localized microvilli destruction and the formation of an underlying pedestal-like projection composed of epithelial-derived cytoskeletal components (1). Bacteria remain adherent on these cup-like projections, rarely penetrating the intestinal barrier.

The bacterial factors responsible for the formation of attaching/effacing lesions and diarrheal disease are produced and regulated by a pathogenicity island described as the locus of enterocyte effacement (2). The locus of enterocyte effacement (LEE) encodes a Type III secretion system (TTSS), a cellular receptor, numerous secreted/translocated proteins, and over 20 open reading frames of unknown function. The TTSS is a molecular syringe that directs the active transport of proteins from the bacterial cytoplasm across the inner and outer bacterial membranes, directly into the cytoplasm of an associated eukaryotic cell (3). TTSSs are widely conserved among a diverse array of animal and plant pathogens and critical for their virulence.

Of major interest is the TTSS-specific response of human intestinal epithelial cells to EPEC infection. Previous research has focused primarily on morphological changes to the host as inferred from immunostaining. Particular interest has developed in the elucidation of host components contributing to pedestal formation. Transcriptional profiling of the host has been attempted, although not yet in a relevant cell type (4). A fundamental understanding of how the intestinal epithelial proteome is altered by this pathogen and its TTSS effector proteins is needed, as few host pathways have been examined.

We therefore undertook a quantitative analysis of the proteome of polarized Caco-2 intestinal epithelial cells infected with either wild-type or TTSS-deficient EPEC. Microcapillary liquid chromatography combined with electrospray ionization tandem mass spectrometry (ESI μLC-MS/MS) identifies proteins from mixtures without prior electrophoretic separation. Labeling of protein lysates prior to μLC-MS/MS with isotope-coded affinity tags (ICAT) specific to sulfhydryl groups greatly reduces sample complexity, allows detection of low abundance proteins, and aids in quantification of relative protein abundance between samples (5). We employed ICAT technology to both identify and quantify TTSS-dependent alterations to host protein expression, performed in silico validation, and further confirmed our results through immunoblotting and immunofluorescence microscopy. We discuss the predominant functional categories of differentially regulated proteins and their implications to the future study of EPEC-host interactions and present novel testable hypotheses about newly identified host proteins and their potential role in diarrhea.

EXPERIMENTAL PROCEDURES

Bacterial Strains—The bacterial strains used in this study were wild-type EPEC E2348/69 (6) and EPEC E2348/69ΔescN (7).

Cell Culture—Human Caco-2 cells (8) were grown at 37 °C, 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% de-
complemented fetal calf serum, and 1% non-essential amino acids. Cells were cultured in 24-mm diameter polyester Transwell plates (Costar) for at least 21 days prior to infection.

**Infections and Protein Preparation**—Bacterial cultures grown overnight were subcultured 1:50 into Dulbecco’s modified Eagle’s medium and grown for 3 h at 37 °C, 5% CO₂, without shaking. Bacteria were applied to Caco-2 cells for 4 h at a multiplicity of infection of 50:1. Following infection, the media was aspirated and cells were washed 8 times with PBS. Cells were lysed in 500 μl of 1% Triton X-100 in PBS and centrifuged at 12,000 × g for 5 min. The supernatant was transferred to 5 volumes of cold acetone, precipitated, and resuspended in 0.1% SDS, 6 M urea.

**ICAT Labeling and Analysis**—Caco-2 lysates were labeled as described in the Supplementary Materials, SEQUEST™ (Thermo Finngan) was used to sequence the peptides and XPRESS (9) software was used to perform relative quantitation between light and heavy ICAT-tagged peptides. PeptideProphet™ (10) was used to verify correctness of peptide assignments.

**Western Blot Analysis**—Samples for Western blot analysis were resolved by SDS-PAGE and analyzed as described in Ref. 7. The following antibodies were utilized at a dilution of 1:1000: calpain-5 (BD Biosciences), caspase-7 (New England Biolabs), dynactin (BD Biosciences), dynamin (BD Biosciences), espin (BD Biosciences), integrin-linked protein kinase (New England Biolabs), Nod2 (Immunologicalsdirect.com), Rac1 (New England Biolabs), and talin (Sigma).

**Immunofluorescence**—HeLa cells were grown on glass coverslips in 24-well tissue culture plates and infected for 4 h with 5.0 μl of bacterial overnight culture. After infection, cells were washed 3 times in PBS containing Ca²⁺ and Mg²⁺ and fixed in 2.5% paraformaldehyde in PBS for 10 min at room temperature. Cells were permeabilized in 0.1% saponin in PBS, blocked in 5% goat serum in PBS + 0.1% saponin, and incubated with the following primary antibodies diluted 1:1000 in blocking solution for 1 h at room temperature: integrin-linked protein kinase, talin, and calpain-5.

**RESULTS**

We utilized the mammalian Caco-2 cell culture line as a model for EPEC pathogenesis in the small bowel. Caco-2 cells provide an ideal infection model, as they are derived from the human intestine, able to polarize, develop microvilli, form tight junctions, and are infected by EPEC (11). To study host responses specific to the EPEC TTSS, we compared the effect of a well characterized wild-type strain (E2348/69; wt) to a strain deficient in the TTSS (E2348/69ΔescN; N⁻). Caco-2 lysates prepared after 4 h infection were differentially ICAT labeled (Fig. 1 and supporting text), and analyzed by microcapillary high performance liquid chromatography-tandem mass spectrometry (μLC-MS/MS) (9). Relative protein abundance was determined by the ratio of signal intensities of peptide pairs using the XPRESS software tool (9). The sequence identity of the proteins in the sample was determined by correlating collision-induced dissociation mass spectra with the NCBI protein data base using the SEQUEST algorithm (12).

2,090 proteins were identified from 10,921 tandem mass spectra of peptides matched to peptide sequences in a data base using SEQUEST (see Supplementary Materials Fig. S1 for the complete dataset). 264 proteins with an annotated biological function (~13%) displayed at least 2-fold expression differences between wt- and N⁻-infected cells (Table I). Approximately equal numbers of proteins were up- versus down-regulated. We used the bioinformatics tool GoMiner (13) to classify the differentially regulated Caco-2 proteins into functional categories based on the functional annotations assigned to these proteins (14) (Table II).

Research into EPEC interactions with epithelial cells has focused primarily on host cytoskeletal rearrangements and signaling pathways mediating such rearrangements. Indeed, the major functional categories contributing most to the total of differentially regulated proteins are those involved in the cytoskeleton, cell adhesion, and G-protein signaling (Fig. 2). Proteins involved in ion transport and ion channel function were over-represented in the set of up-regulated proteins, potentially contributing to diarrhea. We validated in silico our results by searching all differentially expressed proteins against all previous publications in bacterial pathogenesis. The subset of the proteins previously implicated in bacterial pathogenesis and likely to be involved in host-EPEC interplay is bolded in Table II and discussed in detail below.

The most striking change in the host epithelial cell during EPEC infection is the formation of actin-rich pedestals, upon which EPEC attaches. The primary components of the EPEC pedestal have been characterized with immunofluorescence microscopy (15). We identified 12 of the 20 proteins catalogued, 11 (92%) of which are up-regulated in a TTSS-dependent manner, detected the differential expression of an integrin-linked kinase (see below), and also confirmed increased expression of the host tyrosine kinase potentially responsible for Tir phosphorylation (16).

Protein identification, abundance analysis, and validation are performed by searching each MS/MS spectrum against a data base of human protein sequences using SEQUEST. Supplementary Materials Fig. 2A displays MS/MS data derived from the fragmentation of a peptide ion unique to the cysteine protease calpain-5. Calpain-5 is instrumental for brush border effacement during EPEC pathogenesis and recruits ezrin to the brush border (17). Confidences assignments were made in PeptideProphet™ based on how closely the observed mass spectra matched the theoretical fragmentation profile (18). XPRESS software then separates the single-ion current profile of the d₃- and d₆-labeled peptides and calculates a peptide relative abundance ratio based on integrated peak areas. For calpain-5, the abundance ratio was 4.6 (wt:N⁻; Supplemental Materials Fig. 2B).

To assess the correspondence of these data with independent assays of protein abundance, we performed Western blot analysis of numerous host proteins for which we detected TTSS-dependent differential expression (Fig. 3). Caco-2 monolayers were infected with either wt or N⁻ for 2–6 h and subjected to immunoblotting. Calpain-5 expression increased significantly between 2 and 4 h in cells infected with wt, whereas expression remained low in cells infected with N⁻ (Fig. 3A). The relative difference in expression was estimated to be 6–8-fold, similar to the abundance ratio derived from ICAT analysis. We also examined the expression profile of ADAMTS-1, a metalloproteinase involved in proteoglycan degradation (19). ADAMTS-1 expression was not detected in wt cells by immunoblotting, but was strongly expressed in N⁻-infected cells (Fig. 3B).

We extended this comparative analysis to a larger subset of TTSS-dependent differentially expressed proteins using cell lysates prepared from independent infections. We analyzed the
expression of nine different proteins and plotted ICAT abundance ratios versus expression ratios computed from Western blotting (Fig. 3C). Significant correlation was observed between the two assays ($r^2 = 0.80$). Similar results were observed with immunofluorescence microscopy (discussed below) and in previous ICAT experiments (20). We also used immunofluorescence microscopy to visualize the expression of Caco-2 proteins measured by ICAT to be differentially regulated during infection. HeLa cells were infected with either wt or N$^-$, fixed, and stained against calpain-5, integrin-linked kinase (ILK), and talin (Supplementary Materials Fig. 3). After staining, exposure under identical conditions revealed that, as expected, calpain-5 expression was increased in wt-relative to N$^-$-infected cells. Surprisingly, we observed co-localization of ILK with actin (middle row), implicating this important signaling molecule as part of the EPEC pedestal. We also observed the expected increased expression and co-localization of talin with actin pedestals (bottom row).

**DISCUSSION**

We present the first proteomic analysis of the TTSS-specific human response to a pathogen. We used ICAT labeling technology to examine changes in the host proteome in response to wt- and TTSS-deficient EPEC and experimentally verified key findings with more classical analyses. The use of ICAT to compare mammalian proteomes has several advantages over classical two-dimensional gel analyses (5). Biotinylated cysteine-containing peptides are selectively isolated for mass spectrometry, greatly reducing sample complexity. Two-dimensional gels are limited to analysis of high abundance, dye-reactive amounts of protein, whereas ICAT is suitable for lower abundance analysis. ICAT has a larger dynamic range and the two isotope labels serve as mutual internal standards for quantitation. However, the small fraction of proteins lacking cysteine is transparent to analysis and only relative changes in protein abundance are interrogated; aspects of protein regulation such as post-translational modification, trafficking, and protein-protein interactions are assessed through additional experimentation. Several aspects of EPEC-host cell interactions that both validate our findings and implicate new host proteins in induced cytoskeletal rearrangement, ion transport, and innate immunity are discussed.

**EPEC Pedestal**—Tir, a TTSS-translocated protein, initiates pedestal formation by binding the Nck adaptor protein. N-WASP is recruited, which then binds Arp2/3, resulting in de novo actin polymerization (21). We identified 12 of the 20 identified pedestal components (15). It is striking that 92% of identified pedestal proteins are expressed more highly in wt-versus N$^-$-infected cells, underscoring the importance of TTSS-encoded effector proteins in establishing a cytoskeletal framework for EPEC.

The cytoskeletal protein talin couples integrins to F-actin, and also binds vinculin, a protein implicated in the negative regulation of cell motility. The attachment of Shigella flexneri to Chinese hamster ovary cells (22) elicits localized accumulation of talin, and the Yersinia enterocolitica invasin protein modulates reorganization of talin (23). In our experiments, talin expression was strongly up-regulated in wt-infected cells, and was recruited to EPEC pedestals (Supplemental Materials Fig. 3).

Cofilin and gelsolin are actin-severing components of the EPEC pedestal that were overexpressed in wt-infected cells. Cofilin has been implicated in phagocytosis (24) and both interact with and enhance the activity of the Na$^+$,K$^+$-ATPase, providing a link between the cytoskeleton and ion transporter activity (25). The increased abundance of actin depolymerizing agents in the pedestal suggests a complex molecular warfare between the host and a TTSS-effector protein capable of inactivating these potent depolymerizing activities.

We detected numerous other actin-binding proteins not previously implicated in EPEC pedestals whose expression was regulated in a TTSS-dependent manner (Table II). Notably, the expression of the integrin-linked kinase 2 (ILK2) was localized to pedestals, but down-regulated in wt-infected cells (Table II and Supplemental Materials Fig. 3). ILK is a phosphatidylinositol 3-kinase-dependent regulator of integrin-mediated cell adhesion and has recently been implicated in tumor angiogenesis (26). The host tyrosine kinase that EPEC recruits in actin pedestal formation has recently been suggested (16). It is of note that this kinase, c-Abl, was overexpressed in wt-infected cells.

**Ion Transport/Diarrhea**—The molecular mechanism(s) by which EPEC induces diarrhea are unknown, although several are proposed. Microvilli effacement may reduce nutrient absorption, but cannot explain the rapid kinetics of diarrheal onset (27). We found that calpain-5, a host protease involved in brush border effacement (17), was strongly up-regulated (Supplemental Materials Figs. 2 and 3), whereas the brush border dipeptidylpeptidase IV (28) was down-regulated. Sequestration of tight junctional proteins (e.g. ezrin) to the pedestal may contribute to the disruption of tight junction integrity and decrease in transepithelial resistance (29).

Host cells infected with EPEC release nucleotide mediators capable of triggering chloride secretion from neighboring cells through interaction with purinergic receptors (30). Nucleotide release is not because of apoptosis and is TTSS-dependent, probably because of the effector protein EspF (31). We hypothesized that host nucleotide biosynthesis and degradation pathways would therefore be differentially expressed in a TTSS-dependent manner during wt infection, but were unable to find significant difference between wt- and N$^-$-infected cells.

Other studies have proposed a greater dependence of EPEC-induced diarrhea upon HCO$_3^-$ secretion (32), and have also implicated calcium fluxes (33). We detected large increases in expression of both the bile salt export pump (34) and calcium-activated chloride channels implicated in regulation of mucin (35).

The Nedd4 binding protein 1 was up-regulated by a TTSS-dependent factor. Nedd4 is an E3 ubiquitin ligase important to the targeting of the epithelial sodium channels for endocytosis and degradation (36). The epithelial sodium channel regulates salt and fluid homeostasis and interacts with the WW domains of Nedd4 (37). Grb10, an adaptor protein known to associate with Nedd4 and sodium channels, was also up-regulated at both the transcriptional (38) and translational level. Thus, the

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**Table I**

|        | wt$^a$ | N$^-$ | Up$^b$ | Down$^c$ | Unchanged |
|--------|--------|-------|--------|----------|-----------|
|        | 3      | 7     | 125 (138) | 139 (141) | 1486      |

$^a$ Identified only in Caco-2 cells infected with wt.

$^b$ Identified only in Caco-2 cells infected with N$^-$.  

$^c$ Expression ratios in which [wt > N$^-$] is greater than 2-fold. Hypothetical proteins are shown in parentheses.

$^d$ Expression ratios in which [wt < N$^-$] is greater than 2-fold. Hypothetical proteins are shown in parentheses.
**TABLE II**

Differentially regulated Caco-2 proteins (>2-fold) from ICAT experimentation

Data are sorted primarily by biological function as annotated by GoMiner (13) and secondarily by average wt/N; expression ratio. Bolded proteins are discussed in text and previously implicated in bacterial pathogenesis.

| Category | Protein | wt/N | Notes |
|----------|---------|------|-------|
| actin-binding | talin 2 | 11.11 | couples integrins to F-actin |
| actin-binding | gelsolin | 3.57 | actin filament severing |
| actin-binding | espin | 3.03 | microvillar actin bundling |
| actin-binding | coronin | 2.95 | Arp2/3 regulation |
| actin-binding | coflin, non-muscle isoform | 2.50 | |
| actin-binding | glia maturation factor beta | 2.08 | actin filament severing |
| actin-binding | ARP3 actin-related protein 3 homolog | 0.48 | |
| actin-binding | plastin 1 | 0.47 | F-actin stabilization |
| actin-binding | myristoylated alanine-rich protein kinase C substrate | 0.43 | actin polymerization |
| actin-binding | similar to beta actin | 0.16 | |
| actin-binding | steprin2 protein | 0.09 | |
| actin-binding | nesprin 2 | 0.23 | actin-bundling |
| CHO metabolism | N-acetylgalactosaminyltransferase 1 | 7.14 | O-linked glycosylation |
| CHO metabolism | phosphorylase, glycogen | 0.46 | |
| CHO metabolism | N-acetylgalactosaminyltransferase | 0.45 | |
| CHO metabolism | alpha-mannosidase II | 0.43 | |
| CHO metabolism | glucose transporter type 5, small intestine | 0.42 | |
| CHO metabolism | protein phosphatase 1, regulatory (inhibitor) subunit 2 | 0.39 | |
| CHO metabolism | sucrose-isomaltase | 0.39 | |
| CHO metabolism | 4-alpha-glucanotransferase isomerase | 0.37 | glycogen debranching |
| CHO metabolism | glucosamine-6-phosphate isomerase | 0.29 | |
| CHO metabolism | activity-dependent neuroprotector | 0.19 | |
| CHO metabolism | lysozyme precursor | 0.08 | peptidoglycan degradation |
| cell cycle/cell death | vasopressin-activated calcium-mobilizing receptor | 2.15 | |
| cell cycle/cell death | NuMA protein | 4.17 | microtubule crosslinking |
| cell cycle/cell death | caspase-7 precursor | 3.23 | apoptosis induction |
| cell cycle/cell death | FAT tumor suppressor 2 precursor | 3.03 | cadherin |
| cell cycle/cell death | anaphase-promoting complex 1 | 2.63 | |
| cell cycle/cell death | minichromosome maintenance deficient protein 5 | 0.43 | cell cycle regulation |
| cell cycle/cell death | cystatin B | 0.18 | inhibits lysosomal proteases |
| cytochrome | cytochrome c oxidase subunit VIIb | 2.22 | |
| cytochrome | cytochrome P-450 II C | 2.13 | |
| cytochrome | cytochrome P450, subfamily A, polypeptide 1 | 2.04 | |
| cytochrome | cytochrome P450, subfamily F, polypeptide 12 | 0.46 | |
| cytoskeleton | putative collagen homolog protein-a | 4.17 | junctional connectivity |
| cytoskeleton | epilplakin | 3.85 | membrane fusion |
| cytoskeleton | dynamin-1 | 3.70 | caveolae formation |
| cytoskeleton | dysferlin | 3.33 | |
| cytoskeleton | obscurin | 2.08 | binds ankyrin 1 |
| cytoskeleton | kinesin family member 13B | 0.50 | |
| cytoskeleton | adaptor-related protein complex 1, mu 2 subunit | 0.48 | |
| cytoskeleton | cadherin 17 precursor | 0.47 | |
| cytoskeleton | adaptor-related protein complex 3 delta 1 subunit | 0.44 | |
| cytoskeleton | keratin 1 | 0.44 | |
| cytoskeleton | pinin | 0.43 | desmosome associated |
| cytoskeleton | dynein, axonemal, heavy polypeptide 8 | 0.43 | vesicular trafficking |
| cytoskeleton | protocadherin alpha 6 precursor | 0.33 | |
| cytoskeleton | keratin 10 | 0.29 | |
| cytoskeleton | dynacin 4 (p62) | 0.26 | |
| cytoskeleton | plakophilin 3 | 0.23 | desmosomal plaque |
| cytoskeleton | dynein, axonemal, heavy polypeptide 5 | 0.19 | vesicular trafficking |
| DNA repair | mutS homolog 6 | 7.14 | mismatch repair |
| DNA repair | ATP-dependent DNA helicase Q1 | 5.26 | |
Proteomic Analysis of EPEC Pathogenesis

### TABLE II—continued

| Category                        | Protein                                                        | wTNS | Notes                                      |
|---------------------------------|----------------------------------------------------------------|------|--------------------------------------------|
| DNA repair                      | DNA damage binding protein 1                                   | 0.50 |                                            |
| DNA repair                      | RNase 8, placental                                             | 0.18 |                                            |
| DNA replication                 | cell division cycle protein 27                                | 5.88 | anaphase-promoting                         |
| DNA replication                 | Elb                                                             | 3.70 | DNA helicase                               |
| DNA replication                 | PCAF-associated factor 400                                     | 0.46 |                                            |
| DNA replication                 | DNA polymerase epsilon, catalytic subunit A                    | 0.34 |                                            |
| extracellular matrix            | sidekick 2                                                     | 50.00| laminar adhesion                           |
| extracellular matrix            | enamelin                                                       | 0.42 |                                            |
| extracellular matrix            | ADAMTS-14 precursor                                            | 0.36 | matrix metalloprotease                     |
| extracellular matrix            | lamina-associated polypeptide 1B                               | 0.15 |                                            |
| extracellular matrix            | similar to ADAM 25 precursor                                   | 0.08 |                                            |
| extracellular matrix            | ADAMTS-1 precursor                                             | 0.05 | matrix metalloprotease                     |
| general metabolism              | glutamate decarboxylase 2                                      | 7.69 |                                            |
| general metabolism              | formimidoyltransferase-cyclodeaminase                          | 6.67 |                                            |
| general metabolism              | gamma-glutamyl carboxylase                                     | 5.00 |                                            |
| general metabolism              | cysteine sulfenic acid decarboxylase-related protein 1         | 3.70 |                                            |
| general metabolism              | phosphoribosylformylglycinamidine synthase                     | 3.21 |                                            |
| general metabolism              | nucleoredoxin                                                  | 0.50 |                                            |
| general metabolism              | aldelyde dehydrogenase 9A1                                     | 0.49 |                                            |
| general metabolism              | antiptitin                                                     | 0.49 |                                            |
| general metabolism              | aldelyde dehydrogenase                                         | 0.48 |                                            |
| general metabolism              | transketolase                                                  | 0.47 |                                            |
| general metabolism              | thionylate sulfutransferase                                    | 0.47 | cyanide detoxification                     |
| general metabolism              | steroid dehydrogenase homolog                                 | 0.46 |                                            |
| general metabolism              | retinal short-chain dehydrogenase/rodductase 3                 | 0.45 |                                            |
| general metabolism              | transglutaminase 2                                             | 0.43 |                                            |
| general metabolism              | leukotriene 12-hydroxydehydrogenase                           | 0.42 |                                            |
| general metabolism              | mitochondrial carrier homolog 2                               | 0.39 |                                            |
| general metabolism              | retinal short-chain dehydrogenase/rodductase 2                 | 0.38 |                                            |
| general metabolism              | alanine aminotransferase                                       | 0.36 |                                            |
| general metabolism              | lysosomal acid phosphatase 2 precursor                         | 0.36 |                                            |
| general metabolism              | similar to oxidoreductase UCPA                                | 0.30 |                                            |
| general metabolism              | membrane-associated protein HEM-1                             | 0.19 |                                            |
| G-protein signalling            | facioskeletal dysplasia protein                                | wfe  | cdc42 activation                           |
| G-protein signalling            | putative GTP-binding protein PTD004                            | 3.75 |                                            |
| G-protein signalling            | G protein-coupled receptor SNSR2                              | 3.57 |                                            |
| G-protein signalling            | RanBP1                                                         | 3.57 | centrosome cohesion                        |
| G-protein signalling            | cadherin EGF LAG seven-pass G-type receptor 2                  | 3.33 | cell adhesion                              |
| G-protein signalling            | similar to small GTPase Rac1                                  | 2.22 |                                            |
| G-protein signalling            | guanylate cyclase soluble, beta-1 chain                        | 2.04 |                                            |
| G-protein signalling            | TC10-like Rho GTPase                                           | 0.49 | F-actin regulation                         |
| G-protein signalling            | GDP dissociation inhibitor 1                                   | 0.48 | GTPase regulation                          |
| G-protein signalling            | ras-related protein Rab-7                                      | 0.47 | GTPase                                     |
| G-protein signalling            | ras homolog gene family, member C                             | 0.47 | Ras-like small GTPase                      |
| G-protein signalling            | rho GDP dissociation inhibitor (GDI) alpha                    | 0.44 | regulation of Rho/Rac                     |
| G-protein signalling            | alsin                                                           | 0.44 | nucleotide exchange fac                    |
| G-protein signalling            | rho GTPase activating protein 4                                | 0.44 |                                            |
| G-protein signalling            | GDP binding protein, alpha z polypeptide                       | 0.36 |                                            |
| G-protein signalling            | alpha-2C-adrenergic receptor                                   | 0.29 |                                            |
| G-protein signalling            | Rac1                                                            | 0.21 | membrane ruffling                          |
| G-protein signalling            | Rho GEF                                                         | 0.12 | regulates Rho activity                     |
| immunity                        | collectin sub-family member 12 isoform 1                       | 7.14 | binds microbial CHOs                       |
| immunity                        | ficolin 1 precursor                                            | 3.70 | complement activation                      |
| immunity                        | leukocyte immunoglobulin-like receptor                        | 3.57 |                                            |
| immunity                        | complement receptor type 2 precursor                          | 2.67 |                                            |
| immunity                        | mannose receptor C type 1 precursor                            | 2.07 | binds microbial CHOs                       |
### TABLE II—continued

| Category                | Protein                                               | wtN^a | Notes                               |
|-------------------------|-------------------------------------------------------|-------|-------------------------------------|
| immunity                | stabilin 2                                            | 2.04  | hyaluronan receptor                 |
| immunity                | hyaluronan binding protein 4                          | 0.49  |                                    |
| immunity                | NOD2 protein                                          | 0.42  | binds muramyl dipeptide             |
| immunity                | lymphocyte antigen 75                                 | 0.42  |                                    |
| immunity                | natural killer cells protein 4 precursor              | 0.36  | cellular adhesion                   |
| immunity                | leukocyte immunoglobulin-like receptor 1             | 0.15  |                                    |
| immunity                | DMBT1                                                 | 0.13  | immune recognition                  |
| ion transport           | calcium activated chloride channel 4                  | 25.00 | chloride transport                  |
| ion transport           | bile salt export pump                                 | 11.11 | excretion of bile acids             |
| ion transport           | similar to ferritin, heavy polypeptide-like 17        | 8.33  |                                    |
| ion transport           | solute carrier family 12 member 8                     | 8.33  |                                    |
| ion transport           | chloride channel protein, skeletal muscle             | 7.14  | chloride transport                  |
| ion transport           | ATPase, Na+/K+ transporting, alpha 3 polypeptide      | 3.45  |                                    |
| ion transport           | potassium channel subfamily K member 17              | 2.50  |                                    |
| ion transport           | solute carrier family 27                              | 2.12  |                                    |
| ion transport           | ferritin light chain                                  | 2.08  |                                    |
| ion transport           | putative S100 calcium-binding protein                | 0.50  |                                    |
| ion transport           | ceruloplasmin (ferroxidase)                           | 0.50  | binds copper                        |
| ion transport           | ATPase, Na+/K+ transporting, alpha 1 polypeptide      | 0.49  |                                    |
| ion transport           | chloride intracellular channel 6                     | 0.45  | chloride transport                  |
| ion transport           | endoplasmic reticulum calcium ATPase 2               | 0.45  |                                    |
| ion transport           | plasma memb. calcium-transporting ATPase 1            | 0.41  | calcium transport                   |
| ion transport           | sodium- and chloride-dependent glycine transporter 1 | 0.37  |                                    |
| ion transport           | K⁺ voltage-gated channel, subfamily H, member 6      | 0.37  |                                    |
| ion transport           | Na⁺/K⁺-transporting ATPase alpha-1 chain precursor    | 0.36  |                                    |
| ion transport           | ALFY (androgen-induced leucine zipper)               | 0.23  |                                    |
| ion transport           | membrane glycoprotein HP59                            | 0.19  |                                    |
| ion transport           | ATPase, H⁺/K⁺ exchanging, alpha polypeptide           | 0.16  |                                    |
| ion transport           | cyclic nucleotide gated channel beta 3               | 0.11  |                                    |
| ion transport           | similar to Sodium/hydrogen exchanger 1               | 0.09  | sodium transport                    |
| lipid metabolism        | paraoxonase 2                                         | 4.55  |                                    |
| lipid metabolism        | choline phosphotransferase 1                          | 2.94  |                                    |
| lipid metabolism        | copine 7                                              | 2.56  |                                    |
| lipid metabolism        | geranylgeranyl diphosphate synthase 1                 | 0.48  | protein prenylation                 |
| lipid metabolism        | fatty acid amide hydrolase                           | 0.28  |                                    |
| protein degradation     | peptidase (mitochondrial processing) beta            | 25.00 |                                    |
| protein degradation     | similar to polyubiquitin                              | 6.67  |                                    |
| protein degradation     | Nedd4 binding protein 1                               | 5.56  | Na⁺ channel ubiquitination          |
| protein degradation     | calpain 5                                             | 4.55  | microvillar assembly                |
| protein degradation     | ubiquitin-conjugating enzyme E2 variant 1 isoform a  | 4.35  |                                    |
| protein degradation     | similar to peptidylprolyl isomerase Λ                | 3.58  |                                    |
| protein degradation     | HDJ2 protein,Dna1 (Hsp40) homolog                    | 3.50  |                                    |
| protein degradation     | similar to Heat shock protein HSP-2 (hsp-2)          | 3.13  |                                    |
| protein degradation     | S-phase kinase-associated protein 2                   | 3.12  | protein degradation                 |
| protein degradation     | peptidyl-Pro cis trans isomerase                      | 3.03  |                                    |
| protein degradation     | ubiquitin carboxyl-terminal hydrolase 25             | 2.94  |                                    |
| protein degradation     | vesicular inhibitory amino acid transporter          | 2.56  |                                    |
| protein degradation     | 26S proteasome regulatory subunit 1                   | 2.49  | protein degradation                 |
| protein degradation     | glutamine-rich tetrapeptide repeat-containing         | 2.08  |                                    |
| protein degradation     | dipeptidylpeptidase 1                                 | 0.50  | microvillar peptidase               |
| protein degradation     | AFG3-like protein 2                                   | 0.48  |                                    |
| protein degradation     | placental thrombin inhibitor                          | 0.47  |                                    |
| protein degradation     | aminopeptidase B                                      | 0.43  |                                    |
| protein degradation     | signal recognition particle 68kDa                    | 0.41  |                                    |
| protein degradation     | karyopherin alpha 3                                   | 0.39  |                                    |
| protein degradation     | DNA2-like homolog                                     | 0.33  |                                    |
| protein degradation     | disintegrin and metalloproteinase domain 18          | 0.32  | matrix metalloprotease              |

 expanded some text here
| Category                        | Protein                                | wtN-a | Notes                      |
|--------------------------------|----------------------------------------|-------|---------------------------|
| protein degradation            | secretory leukocyte protease inhibitor | 0.29  | protease inhibition       |
| protein degradation            | karyopherin beta 1                     | 0.23  |                          |
| signal transduction            | vitamin D receptor-interacting protein | χ-b   |                          |
| signal transduction            | retinoid X receptor interacting protein | 20.00 |                          |
| signal transduction            | growth factor receptor-bound protein 10| 5.88  | adaptor protein           |
| signal transduction            | transmembrane-type protein tyrosine phosphatase H | 5.88 |                          |
| signal transduction            | C-jun-amo-terminal kinase interacting protein 2 | 4.00 |                          |
| signal transduction            | retinitis pigmentosa RPI protein       | 2.63  |                          |
| signal transduction            | retinoid-acid induced protein 1        | 2.63  |                          |
| signal transduction            | integrin alpha-1b precursor            | 2.50  |                          |
| signal transduction            | desert hedgehog preproprotein          | 2.38  | morphogenesis regulation  |
| signal transduction            | protein-kinase, interferon-inducible  | 2.38  |                          |
| signal transduction            | DNA-dependent protein kinase catalytic subunit | 2.38 |                          |
| signal transduction            | I-kappa-B-related protein              | 2.34  |                          |
| signal transduction            | cholinergic receptor, nicotinic, gamma polypeptide | 2.33 |                          |
| signal transduction            | tyrosine-protein kinase ABL2           | 2.22  |                          |
| signal transduction            | N-methyl-D-aspartate receptor subunit 2B precursor | 2.22 |                          |
| signal transduction            | calcium/calmodulin-dependent protein kinase II | 2.17 |  |
| signal transduction            | paired basic amino acid cleaving enzyme 4 precursor | 2.01 | serine endoprotease   |
| signal transduction            | serine/threonine,protein phosphatase 5 | 0.48  |                          |
| signal transduction            | integrin, beta 6                       | 0.42  |                          |
| signal transduction            | interferon, omega 1                    | 0.39  |                          |
| signal transduction            | integrin-linked protein kinase 2       | 0.32  |                          |
| signal transduction            | DNA-dependent protein kinase catalytic subunit | 0.30 |                          |
| transcription                   | zinc finger protein 1                  | 33.33 |                          |
| transcription                   | nuclear receptor co-repressor 1        | 33.33 |                          |
| transcription                   | homeobox even-skipped homolog protein 2| 14.29|                          |
| transcription                   | estrogen-related receptor gamma        | 7.69  |                          |
| transcription                   | similar to Trip230                     | 6.25  |                          |
| transcription                   | zinc finger protein 345                | 5.88  |                          |
| transcription                   | STAT 1-alpha/beta                     | 3.85  |                          |
| transcription                   | zinc finger protein 9                  | 3.62  |                          |
| transcription                   | zinc finger protein 441                | 3.33  |                          |
| transcription                   | PGC-1-related estrogen receptor alpha coactivator | 3.23 |  |
| transcription                   | trinucleotide repeat containing 11    | 2.86  |                          |
| transcription                   | similar to zinc finger protein 443     | 2.78  |                          |
| transcription                   | DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 9 | 2.50 | RNA helicase          |
| transcription                   | zinc finger protein 91 (HPF7, HTF10)   | 2.44  |                          |
| transcription                   | similar to nuclear receptor binding factor-2 | 2.13 |                          |
| transcription                   | SET binding protein 1                  | 0.50  |                          |
| transcription                   | ELL gene (11-19 lysine-rich leukemia gene) | 0.45|                          |
| transcription                   | similar to Zinc finger protein ZFD25    | 0.42  |                          |
| transcription                   | zinc finger protein 41-like            | 0.38  |                          |
| transcription                   | similar to VENT-like homebox 2         | 0.35  |                          |
| transcription                   | similar to transcription factor TFII/1-alpha | 0.34|  |
| transcription                   | chromodomain helicase DNA binding protein 6 | 0.34|  |
| transcription                   | host cell factor 2                     | 0.18  |                          |
| transcription                   | BTAF1 RNA polymerase II, 170kDa        | 0.07  |                          |
| translation                    | ribosomal protein L18a                 | 5.26  |                          |
| translation                    | phenylalanyl-tRNA synthetase beta chain | 5.00 |                          |
| translation                    | eEF1A2 binding protein                 | 3.85  |                          |
| translation                    | ribosomal protein L8                   | 2.94  |                          |
| translation                    | histidyl-tRNA synthetase               | 0.50  |                          |
| translation                    | selenocysteine-specific elongation factor | 0.42 |  |
| translation                    | tRNA splicing 2' phosphotransferase 1  | 0.27  |                          |
| translation                    | ribosomal protein S3a                  | 0.11  |                          |
| unclassified                    | similar to RCC1-like protein           | χ-b   |                          |
proteasome-mediated degradation of specific ion transporters potentially contributes to EPEC-induced diarrhea.

The plasma membrane calcium-transporting ATPase was down-regulated by a TTSS-encoded factor. Bovine ileal loop experiments with *Salmonella typhimurium* have suggested that plasma membrane calcium-transporting ATPase down-regulation contributes to inflammation and diarrhea (39).

**G-protein Signaling**—G-proteins play critical roles in cell signaling and cytoskeletal rearrangement and were over-represented in our dataset of TTSS-regulated host responses. Rho GTPases regulate actin structure, primarily at sites of membrane ruffling. EspH induces Cdc42-dependent filopodia formation (40) and the mitochondrial targeted effector, Map, has also been implicated in Cdc42-dependent processes (41). However, agents that inhibit Rho, Rac, and Cdc42 do not block pedestal formation (42). Therefore, other GTPases that have yet to be identified are likely important to EPEC pathogenesis. We identified numerous G proteins differentially regulated by TTSS-dependent factors that have not previously been implicated in bacterial pathogenesis.

Fgd1 was observed only in cells infected with wt, and encodes a guanine nucleotide exchange factor that activates Cdc42, a Rho GTPase that controls the organization of the actin cytoskeleton. Fgd1 also interacts directly with cortactin, a protein essential to pedestal formation. Abnormal Fgd1 localization results in actin cytoskeletal abnormalities and significant changes in cell shape and viability (43).

TC10, a down-regulated Rho GTPase, is involved in regulating the insulin-stimulated translocation of the glucose transporter GLUT4 (44). TC10 is also able to disrupt cortical actin and regulates actin polymerization on membrane transport vesicles.

**Innate Immunity**—We discovered that a large number of proteins important to innate immune function are differentially regulated by TTSS-dependent factors. In cultured epithelial cells, EPEC activates NF-κB independent lipopolysaccha-
ride stimulation (45). This factor then stimulates the transcription of interleukin-8, to recruit polymorphonuclear leukocytes to the site of infection. Microarray analysis of EPEC-infected HeLa cells demonstrated induction of the Egr-1 transcription factor, involved in mitogen-activated protein kinase activation (4). However, few other studies have examined TTSS-dependent stimulation of innate immunity during EPEC infection.

Collectin and ficolin expression were strongly up-regulated. Collectins recognize pathogen-associated molecular patterns (46) and assist in bacterial killing through complement activation and phagocytosis. Ficolins have affinity for N-acetylglucosamine, act as opsonins, and activate complement via the lectin pathway (47).

We hypothesized that EPEC effectors modulate the innate immune response by down-regulating expression of host proteins involved in bacterial recognition. Accordingly, we observed that Nod2 is down-regulated during wt infection. Nod2 regulates inflammatory responses (48) through recognition of the muramyl dipeptide of bacterial peptidoglycan (49). Transfection of a Nod2 expression plasmid into Caco-2 cells reduces the number of viable internalized *S. typhimurium*, suggesting an additional role for Nod2 as an antibacterial factor (50). Our data indicate that a TTSS-delivered EPEC effector may modulate the expression of this important innate immunity protein.

Our proteomic analysis of the Caco-2 host response to TTSS-encoded EPEC effectors has generated novel testable hypotheses about how this enteric human pathogen causes disease. The use of quantitative mass spectrometry obviates the need for cell fractionation and increases greatly the number of proteins that can be interrogated. Our results correspond closely with more traditional analyses of protein expression, as well as previous studies of the host response to enteric pathogens. It is important to emphasize that our experiments define expression ratios in cells infected with TTSS-competent versus -deficient EPEC strains. Direct quantitative comparison of host protein expression between infection conditions controls for nonspecific epithelial interactions with bacteria (e.g. lipopolysaccharide detection), although differences in adhesion and infection kinetics may contribute to a subset of the identified epithelial responses. Post-translational regulation of protein function must also be evaluated through other means. ICAT will facilitate comparison of the host response among both diverse pathogens and specific bacterial effector molecules. We are currently...
conducting RNA interference experiments to assess the importance of the differentially regulated Caco-2 proteins to EPEC pathogenesis.

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REFERENCES

1. Donnenberg, M. S., Kaper, J. B., and Finlay, B. B. (1997) Trends Microbiol. 5, 109–114
2. Elliott, S. J., Wainwright, L. A., McDaniel, T. K., Jarvis, K. G., Deng, Y. K., Lai, L.-C., McNamara, B. P., Donnenberg, M. S., and Kaper, J. B. (1998) Mol. Microbiol. 28, 1–4
3. de Grado, M., Rosenberger, C. M., Gauthier, A., Vallance, B. A., and Finlay, B. B. (1993) Biochem. J. 292, 377–385
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