Immunoproteomic analysis to identify Shiga toxin–producing *Escherichia coli* outer membrane proteins expressed during human infection.

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Running Title: STEC antigen candidates identified by immunoproteomic

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ABSTRACT

Shiga-toxin producing *Escherichia coli* (STEC) are etiologic agents of acute diarrhea, dysentery, and hemolytic uremic syndrome (HUS). There is no approved vaccine for STEC infection in humans, and antibiotic use is contraindicated as it promotes Shiga toxin production. In order to identify STEC-associated antigens and immunogenic proteins, outer membrane proteins (OMPs) were extracted from STEC strains O26:H11, O103, O113:H21, and O157:H7, with commensal *E. coli* strain HS used as a control. SDS-PAGE, 2D-PAGE analysis, Western blot assays using sera from pediatric HUS patients and controls, and MALDI-TOF/TOF analyses were used to identify 12 immunogenic OMPs, some of which were not reactive with control sera. Importantly, seven of these proteins have not been previously reported to be immunogenic in STEC. Among these seven proteins, OmpT and Cah displayed IgG and IgA reactivity with sera from HUS patients. Genes encoding for these two proteins were present in a majority of STEC strains. Knowledge of the antigens produced during infection of the host and the immune response to those antigens will be important for future vaccine development.

INTRODUCTION

Shigatoxin producing *Escherichia coli* (STEC) is emerging globally as a leading zoonotic pathogen associated with food-borne illnesses. STEC is an etiologic agent of acute diarrhea, dysentery, and hemolytic uremic syndrome (HUS). The primary animal reservoir of STEC is cattle, and infection can result from contaminated water or food such as meat.
products, prepared leafy green salads, unpasteurized milk and fruit juices (1-3). O157:H7 is the most common serotype associated with sporadic outbreaks of dysenteric diarrhea and severe HUS cases. Nevertheless, other STEC serogroups such as O26, O103 and O113 have been also implicated in such outbreaks (4). The presence of virulence factors, such as the outer membrane protein intimin and its receptor Tir, both coded by the locus of enterocyte effacement (LEE), are considered a risk factors for developing HUS (1, 5).

HUS, which has a high incidence in children less than 5 years of age (6), is the most severe consequence of STEC infection, and is characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal insufficiency, which can progress to chronic renal failure (7). HUS treatment is primarily supportive, as there is no specific therapy for STEC. Antibiotics are contraindicated, as they may promote Shiga toxin (Stx) production and release (8), increasing the risk of developing HUS (9). Therefore, because the most deleterious effects of STEC are those resulting from Stx, efforts to develop therapies have focused on compounds that bind to Stx and block its action. However, treatments have been unsuccessful to date or have yet to be tested in humans for clinical efficacy (10-12).

Although the incidence of STEC infection appears low, economic losses (at the individual or national level) associated with community outbreaks, significant hospitalization costs required for the management of HUS and its possible sequelae, in addition to a few cases of death, justify the effort to find suitable targets against this pathogen (6). In this context, vaccine candidates have been tested in animal models with varying levels of success, albeit the lack of an animal model that accurately reproduces the clinical profile of infection is a considerable barrier (13). Some candidates have included recombinant
Stx, intimin, EspA (14), chimeric proteins constructed by fusing A and B subunits of Stx1 and Stx2 (15), and avirulent O157:H7 strains (16). The most promising vaccine candidate tested in humans was based on the covalent fusion of the \textit{E. coli} O157:H7 O-specific polysaccharide and \textit{Pseudomonas aeruginosa} exotoxin A (O157-rEPA). A phase I trial conducted in adults and phase II trial conducted in children were completed in 2006 and showed the candidate to be safe and immunogenic but to date phase III trials have not been initiated (17). Overall, these efforts have targeted mainly O157, disregarding non-O157 serogroups, and as a result would provide incomplete coverage against STEC infections. Moreover, characterization of STEC antigens has focused on the O157 serogroup. Reports have described a humoral immune response in patients infected with these bacteria against the lipopolysaccharide (LPS), Stx (18), flagellin (FliC - H7) (19), and antigens coded in the LEE locus which apart from intimin and Tir include EspA, EspB, and EspD (20-22). While identifying these proteins may lead to a vaccine with improved coverage, protection would still be limited mainly to the STEC-LEE-positive serogroups.

Therefore, it is important to identify antigens and immunogenic proteins conserved in a wide variety of STEC and capable of providing broader coverage. Immunoproteomics (2D-PAGE, mass spectrometry, and Western blot), using serum from infected patients, has proved useful in the identification of proteins synthesized \textit{in vivo} during infection that stimulate the host's humoral immune response, some of which have shown long-lasting effects (23-26).

In the present study we were able to identify STEC-associated immunogenic antigens recognized by human IgG and IgA by means of immunoproteomic analysis of OMP
extracts. A total of 12 immunogenic STEC proteins were identified, seven of which are novel antigen candidates. These seven proteins include OmpT and Cah, which displayed IgG and IgA reactivity with sera from HUS patients.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. The bacterial strains and plasmids used in this study are listed in Supplementary Table 1. The STEC strains were clinical isolates characterized using PCR and serotyped by the Chilean Institute of Public Health and the Enteropathogen Laboratory, Microbiology and Mycology Program, School of Medicine at the University of Chile. In order to exclude proteins that might generate cross-reactivity with commensal microbiota, the reference commensal E. coli HS strain was used (kindly provided by Dr. Myron Levine, University of Maryland). A collection of 170 STEC isolates and 11 commensal E. coli isolates from human feces (PCR-negative for known diarrheagenic pathotypes virulence factors), described in Supplementary Table 2, were used for ompT, ag43, and cah gene detection frequency.

E. coli were grown in Luria Bertani broth (LB, Tryptone 10 g/L, NaCl 10 g/L and yeast extract 5 g/L) at 37 °C for 18 h with no agitation. Bacteriological agar in a final concentration of 1.5% (w/v) was added to prepare the solid media. The culture media were supplemented as needed with ampicillin (100 µg/mL) and 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG). To perform alpha complementation, ampicillin (100 µg/mL), IPTG (50 µg/mL), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (50 µg/mL) were added to the culture media.
Outer membrane protein extraction. OMP extracts were obtained as described in Rivas et al., 2008 (31) with minor modifications. Briefly, two liters of bacterial culture grown in LB medium were centrifuged at 9,000 g for 10 min at 4 °C. The supernatant was discarded and the pellet was washed and resuspended in a final volume of 5 mL solution containing 10 mM Tris-HCl pH 8.0 supplemented with 1mM phenylmethylsulfonylfluoride (PMSF) (Sigma Aldrich Co., St. Louis, MO). Each suspension was sonicated on ice (40 cycles for 30 s with 30 s intervals, which allow cooling). To remove cells and cell debris, cell extracts were deposited in new tubes and centrifuged at 12,000 g for 10 min at 4 °C. The supernatant (total protein extract) was treated with DNase / RNase at room temperature for 20 min and centrifuged at 12,000 g for 10 min at 4 °C. The cytoplasmic membrane was solubilized incubating the supernatant with Sarkosyl at 2% for 30 min at 25 °C (N - lauroyl sarcosine, Sigma Aldrich Co., St. Louis, MO) and centrifuged at 20,500 g for 80 min at 4 °C. Next, the pellet was washed with 1 mL of sterile milliQ water plus PMSF (1mM) and centrifuged at 20,500 g for 50 min at 4 °C. Finally, the pellet was resuspended in 200 µL of sterile milli-Q water. The protein concentration in the outer membrane fraction was measured using the reactive “Bradford protein assay dye reagent” (Bio-Rad, USA) and standard bovine serum albumin (BSA) according to manufacturer’s directions. The OMP extracts were stored at -80 °C until use.

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE). 8 µg of OMP samples from each strain were separated using 12% sodium dodecyl sulfate polyacrylamide gel
electrophoresis (SDS-PAGE) according to the method described by Laemmli (32), using a
SE 600 Ruby Standard Dual Cool Vertical Unit (Amersham Biosciences, GE). Precision Plus
Protein™ Kaleidoscope™ Standards (Bio-Rad) were used as molecular weight markers. Proteins were stained with either the Coomassie Brilliant Blue G-250 (Bio-Rad, USA) or the
Silver Stain Plus Kit (Bio-Rad, USA).

Two-dimensional electrophoresis (2D-PAGE). Precast IPG strips (13 cm, pH 4-7, linear, GE
Healthcare) were rehydrated with 250 µL of DeStreak rehydration solution (GE
Healthcare) containing IPG buffer pH 4-7 (1%) (GE Healthcare), 10 mM DTT and 200 µg of
OMPs, for 16 h at room temperature according to the manufacturer’s directions. The
rehydrated strips were then subjected to Isoelectric focusing (IEF) using an Ettan IPGphor
Isoelectric Focusing System (GE Healthcare) at 20 °C with a current limit of 50 µA/strip
according to the following protocol: 200 V/1 h, 500 V/1 h, 1000 V gradient/1 h, 8000 V
gradient /3:30 h and finally 8000 V until the focusing reached 20 kVh. After IEF, the strips
were equilibrated for 15 min in 3 mL of buffer I [Tris-HCl 50 mM pH 8.8, urea 6 M, glycerol
30% (w/v), SDS 2% (w/v) and DTT 10 mg/mL (w/v)] and then in 3 mL of buffer II for 15 min.
Buffer II differed from the first in that it contained iodoacetamide (25 mg/mL) instead of
DTT. Then, the strips were directly applied to 12% polyacrylamide gels. Second dimension
was performed using SE 600 Ruby Standard Dual Cool Vertical Unit (Amersham
Biosciences, GE) according to the manufacturer’s directions. Electrophoresis was
performed at a constant current of 5 mA/gel for 20 min followed by 15 mA/gel for 9 h
until the bromophenol dye had run out of the gel. Following separation in the second
dimension, the gels were stained with Coomassie Brilliant Blue G-250 (Bio-Rad, USA), photographed, and the images were analyzed using BioNumerics 2D software version 6.6 (Applied-Maths, Belgium). Precision Plus Protein™ Kaleidoscope™ Standards (Bio-Rad, USA) were used as molecular weight markers.

Sera and IgG and IgA concentrations. Sera were obtained from 10 pediatric patients (ID patients, Supplementary Table 3) in the convalescent phase, who presented with prodromal diarrhea within 20 days prior to HUS diagnosis (HUS sera), collected from 1990 – 1993 and 1999 – 2003 (kindly provided by Dr. Valeria Prado). The sera were tested individually and were pooled, designated HUS antiserum. As a control, a mixture was created from the sera of three patients with no history of HUS or episodes of STEC-associated diarrhea (C, control patient, Supplementary Table 3). The sera were obtained from various health centers in Santiago with the informed consent of the parents or legal guardians of all subjects. All procedures were approved by the local Ethics Committee of the University of Chile, School of Medicine. Supplementary Table 3 shows the clinical history of each patient who donated serum used in this study. Furthermore, IgG and IgA concentrations in HUS and control sera were determined by ELISA with the Protein Detector ELISA Kit (Kirkegaard & Perry Laboratories), according to the manufacturer’s directions.

Protein transfer and immunodetection. OMPs separated by SDS-PAGE and 2D-PAGE were transferred to nitrocellulose membranes (Millipore, Germany) for 60 min at 100 V / 4 °C
using the Mini Trans-Blot Cell system (Bio-Rad), according to the manufacturer’s directions.

After transfer was completed, the non-specific binding sites available on the membrane were saturated using a blocking solution [Tris buffer saline 1X (TBS-1X), 0.0003% Tween-20 and 2% BSA] for 1 h at room temperature. Next, the membranes were rinsed three times with TBS-T solution (TBS 1X, 0.003% Tween-20) for 5 min each. Then the membranes were incubated with agitation for 1 h in a blocking solution that contained the HUS or control sera, diluted to 1:3500. After 2 rinses with TBS-T solution for 10 minutes each time, the membranes were incubated with blocking solution containing anti-human IgG conjugated with alkaline phosphatase (Invitrogen, USA) diluted to 1:5000 for 1 h at room temperature with agitation. The images were revealed with Nitro blue tetrazolium / 5-bromo-4-chloro-3-indolyl phosphate chromogenic substrate kit (NBT/BCIP) (Invitrogen, USA), and the reaction was stopped with distilled water.

The seroreactivity of the recombinant proteins OmpT (rOmpT) and Cah (rCah) to IgG and IgA of the HUS and control sera was evaluated following the above protocol. However, when anti-human IgA secondary antibody conjugated with alkaline phosphatase (1:2000) was used, the HUS and control sera were diluted to 1:1000.

Identification of immunogenic proteins using mass spectrometry. The proteins recognized by antibodies from HUS but not control sera, or those only present in the STEC strains, were cut from the SDS-PAGE and 2D-PAGE gels stained with Coomassie Brilliant Blue G-250. Other proteins that demonstrated immunodominance were also cut from the
gels. These proteins were sent to the Mass Spectrometry Core at the University of Texas Medical Branch at Galveston (Galveston, Texas, United States) for identification using MALDI-TOF/TOF mass spectrometry. Each peptide fragment fingerprint was submitted to the MASCOT server (http://www.matrixscience.com) in order to identify the protein by comparing it with the fragments of other proteins included in the database. The identities of the rOmpT and rCah were also confirmed by MALDI-TOF/TOF.

Bioinformatic analyses of the immunogenic proteins identified. The subcellular localization of proteins was predicted using pSORTb version 3.0 (http://psort.org/). Theoretical isoelectric points and molecular weights were determined by using the ExPASy Proteomics Server UniProt Knowledgebase (http://us.expasy.org/). The BLASTN algorithm (http://blast.ncbi.nlm.nih.gov) was used to evaluate the distribution of coding genes for each protein in other E. coli strains as well as in other bacterial species.

Gene detection frequencies for ompT, ag43, and cah. Polymerase chain reaction (monoplex PCR) was used to determine the presence of ompT, ag43, and cah genes. Specific primers were designed using nucleotide sequences available in the GenBank database using the ClustalW algorithm for alignment (http://www.ebi.ac.uk) as well as NCBI/Primer-BLAST (http://www.ncbi.nlm.nih.gov). The specificity of each primer and their predicted PCR products were verified by comparison to the GenBank database using BLASTN (http://www.ncbi.nlm.nih.gov) and were also tested using reference strains (Supplementary Table 1). In addition, considering that the main difference between ag43
and cah is a 270 bp deletion in the coding region of Cah’s α domain, primers were designed targeting conserved regions that flank this deletion in order to obtain two amplicons with differing molecular weights. Supplementary Table 4 shows the list of primers used in this study. The amplification reactions were performed in a final volume of 25 µL that contained DNA template, 0.4 µM of each primer, 5 µL of 5X GoTaq DNA-polymerase buffer, 0.2 µM of each dNTP (Fermentas, Lithuania), and 1.25 U de GoTaq® DNA Polymerase (Promega, USA). The PCR conditions were specific for each pair of primers. The hybridization temperature was set as a function of the melting temperature of the primer, and the duration of the extension stage as a function of the DNA fragment length, generally 1min/kb.

The frequency data obtained were subjected to a two-tailed Fisher’s exact test with the significant level set at 95%.

**Generation of recombinant proteins OmpT and Cah.** The coding sequences for OmpT and Cah were amplified from the reference *E. coli* strain O157:H7 EDL933, using the primers described in Supplementary Table 4, with recognition sites for the restriction enzymes NdeI and XhoI at the 5’ and 3’ gene terminals, respectively. The PCR products were directly ligated to the vector pTZ57R/T (Fermentas, Lithuania), according to the manufacturer’s directions to construct the vectors pTZ57R/T_ompT and pTZ57R/T_cah. These vectors were used to transform the laboratory strain *E. coli* DH5α, and clones were selected according to ampicillin resistance and α-complementation. Sequencing was performed to confirm the correct cloning of the coding sequences (Macrogen, USA).
Plasmid DNA was extracted from transformed DH5α *E. coli* strains and digested with restriction enzymes *Ndel* and *Xhol*. The products of digestion were analyzed in 1% agarose gels, and *ompT* and *cah* were purified. These sequences were ligated to the expression vector pET15C (30), allowing the production of recombinant proteins under the control of the T7 Phage promoter and with the histidine tag at the C-terminus of each protein, to construct the vectors pET15C_ompT and pET15C_cah. These vectors were used to transform the *E. coli* DH5α and *E. coli* BL21(DE3) strains. *E. coli* BL21(DE3) strain, as a control, was also transformed with an empty pET15C vector. The bacterial clones were selected according to ampicillin resistance and confirmed by PCR. The transformed *E. coli* BL21(DE3) strains were cultivated in LB broth supplemented with 100 µg/mL of ampicillin for 10 h at 37 °C with agitation, and then synthesis of the recombinant proteins was induced for 4 h, by supplementing the culture media with 1mM of IPTG.

The recombinant proteins, rOmpT and rCah, were partially purified using immunoprecipitation by μMACSTM Epitope Tag Protein Isolation Kits (Miltenyi Biotec, GmbH, Germany), according to the manufacturer’s directions. Briefly, 5 µg of OMPs extracted from *E. coli* BL21(DE3)/pET15C_ompT or *E. coli* BL21(DE3)/pET15C_cah, respectively were suspended in 1mL of Lysis Buffer [1M NaCl, 1% Triton X-100, 50 mM Tris HCl (pH 8.0)] and mixed with 50 µL of Anti-His Tag MicroBeads. Each μ Column was placed in the magnetic field of μMACS Separator and was prepared by applying 200 µL of Lysis Buffer. The solution (containing proteins) was incubated for 30 min on ice, and then loaded to the μ Column. Later the columns were rinsed 4X200 µL with Wash Buffer 1 [4M NaCl, 1% Igepal CA-630 (formerly NP-40), 0.5% sodium deoxycholate, 0.1% SDS, 50 mM...
Tris HCl (pH 8.0]) and 1X100 µL with Wash Buffer 2 [20 mM Tris HCl (pH 7.5)]. The µ Column was added with 70 µL pre-heated Elution Buffer at 95 °C [50 mM Tris HCl (pH 6.8), 50 mM DTT, 1% SDS, 1 mM EDTA, 0.005% bromophenol blue, 10% glycerol]. The elute was collected, analyzed by SDS-PAGE and verified by MALDI-TOF/TOF.

RESULTS

Differences between OMP profiles of STEC and *E. coli* HS strains. Exposed bacterial proteins are recognized as foreign antigens by the host immune system, and thus OMPs may play an important role in immunogenicity and immunoprotection. Figure 1A shows protein bands in STEC strains (lanes 1 - 4) that are apparently absent in *E. coli* HS (lane 5). These proteins are in the range of 37 to 75 kDa. In contrast, in all OMP profiles, the most abundant proteins are located close to 35 kDa with minor variations in their electrophoretic mobility. Due to limitations in resolution, shown in the Fig 1A, it was difficult to identify each protein. Therefore, we performed 2D-PAGE to better discriminate OMPs based not only on the molecular mass but also by isoelectric point.

Because the literature suggests that most *E. coli* OMPs are best resolved at a pH of 4 to 7 (34, 35), the proteins were separated within this pH range on 2D gels, stained with Coomassie blue G-250, photographed, and analyzed using BioNumerics 2D software, with an average of 47 spots detected per strain (Fig. 2A-E). The software also provided a global OMPs profile of the STEC strains analyzed, which was then superimposed on the OMP
profile of the commensal *E. coli* HS strain in order to distinguish proteins unique to the STEC strains (Fig. 2F).

**Immunogenic OMPs associated with STEC.** ELISA showed that IgG and IgA concentrations did not differ significantly between HUS and control sera (unpublished observations). Pooled HUS sera recognized many of the OMPs, while the pooled control sera recognized only a minority of the OMPs or showed weak reactivity (Fig. 1B and 1C, respectively). Some of the STEC-associated proteins numbered 4, 5, 6 and 11 in Fig. 1B, were recognized by the HUS sera and not by the control sera, while other, numbered 1, 2, 3, 7, 8 and 9, were reactive to both sera (Table 1). STEC-associated proteins that were seroreactive with HUS sera were extracted from the SDS-PAGE and 2D-PAGE gels for MALDI-TOF/TOF identification. Other proteins with molecular weights between 32 and 37 kDa were also extracted, because despite their presence in all OMP profiles, they were strongly seroreactive with the HUS sera.

Immunogenic proteins identified by MALDI-TOF/TOF are shown in Table 1. A total of 12 immunogenic proteins were identified, some of which were present in multiple STEC OMP profiles. The porins OmpC, OmpF, and OmpA, ubiquitous in *E. coli* (Fig 1A, and Fig. 2) were recognized by both HUS and control sera, although seroreactivity was weak in the latter group (Fig 1B and 1C). The protein L-asparaginase II was observed only in the O103 and O113:H21 OMPs profiles (Fig. 2B – C, and Fig. 3); however, its molecular weight and isoelectric point coincided with the OmpA protein present in the other studied strains, possibly masking its presence. Recent results in our laboratory using anti-L-asparaginase II
antibodies suggest that this protein is present in all OMP profiles (result not shown). However, it is noteworthy that this protein was seroreactive only with HUS sera (Fig. 3B). Flagellar proteins (FliC) were strongly recognized by both HUS and control sera numbered 1, 2 and 3 in Fig. 1B and 1C. Proteins Ag43 (α43), NmpC, OmpT, EF-Tu (numbered 4, 5, 6 and 11, respectively in Fig. 1B) and Hek (Fig. 3B) present in the STEC OMP profiles and not in the E. coli HS strain, were only recognized by HUS sera and therefore were classified as STEC-associated immunogenic proteins. The quantity of proteins/spots observed and identified in OMPs profiles is consistent with other proteomics studies conducted in E. coli (24, 36, 37).

In silico bioinformatics analysis of identified immunogenic proteins. Bioinformatic analysis using PSORTb to predict subcellular localization of immunogenic proteins indicated that EF-Tu and L-asparaginase II are cytoplasmic and periplasmic proteins, respectively. All other proteins were localized in the outer membrane (OM) or bacterial surface. In addition, cah, a homologue of ag43, was identified in some STEC genomes, but not in commensal E. coli genomes by BLAST analysis. This analysis suggests that cah is present in pathogenic E. coli but not in commensal E. coli (Table 2). However, this finding may be an artifact due to the low numbers of E. coli genomes sequenced to date.

Presence of ompT, ag43 and cah in STEC vs. commensal E. coli strains. To determine the presence of ompT, ag43 and cah genes among STEC and commensal E. coli strains, PCR gene detection analysis was conducted using the isolates listed in Supplementary Table 2.
The *ompT* gene was present in 98% and 36% of STEC and commensal *E. coli* respectively, while *cah* was present in 64% and 9% of STEC and commensal *E. coli*, respectively. *ompT* (p < 0.0001) and *cah* (p < 0.001) were more frequently detected in STEC as compared to commensal isolates. Detection frequency for *ag43* was significantly greater (p < 0.01) in non-O157 human isolates as compared to commensal isolates (Table 3). These data demonstrate that *ompT* and *cah* are highly conserved in STEC strains and mostly absent in commensal *E. coli* strains.

**Recombinant proteins rOmpT and rCah are reactive for IgG and IgA in HUS patient sera.**

To confirm that the antiserum was recognizing OmpT and Cah, the genes encoding these proteins were cloned into expression vectors and expressed in BL21(DE3). As shown in the Figure 4A (Line 5), we observed three different protein bands (arrows 13, 14 and 15) in the OMP profile for the strain expressing *cah* that were not present in the OMP profile obtained from the BL21(DE3) strain carrying the empty plasmid. These three protein bands were identified by MALDI-TOF/TOF as Cah and one of them had the expected size (92kDa) (Table 4). OmpT and Cah were identified as STEC associated antigens using the pooled antisera, and it was important to determine the response to these antigens by HUS patients. Therefore, the individual antisera were tested for reactivity with recombinant OmpT and Cah (Fig. 4 and Supplemental Fig. 1). By using each of the sera of 10 HUS individuals and three controls, Western blot determined that rOmpT (arrow 16) was strongly recognized by IgG and IgA of HUS sera and weakly by control sera. Two other proteins co-purified with rOmpT and rCah, were identified by MALDI-TOF/TOF as PhoE
DISCUSSION

The aim of this study was to identify immunogenic OMPs broadly conserved among STEC strains, considering for that the most common isolates associated with dysenteric diarrhea and HUS like STEC O26:H11, O103, and O157:H7 serotypes (38, 39). Furthermore, to broaden the search for antigens, we included the LEE-negative O113:H11 serotype, which has also been associated with HUS (40, 41). Prior studies have reported that exposure to enterobacterial OMPs stimulates a humoral immune response in infected patients (42 - 44), therefore making them suitable candidates for vaccination against Gram-negative pathogens (45 - 48).

In this study, sera from children with STEC infections had an IgG immune response to FliC, expressed in serotypes O103, O113, and O157, that was strongly recognized by both HUS and control sera, consistent with its characterization as a pathogen-associated molecular pattern (49). FliC was not detected in STEC O26, possibly because FliC was not synthesized in our experimental culture conditions. Other detected proteins, such as OmpC, OmpF, and OmpA, have also been reported to be immunogenic in E. coli, Salmonella and Shigella (24, 50 - 52). The pooled HUS antiserum also reacted with L-asparaginase II, an enzyme that catalyzes asparagine hydrolysis (53), and EF-Tu, one of the most abundant and best-characterized cytoplasmic proteins with a role in protein synthesis (54 - 56). Similar to our
findings, L-asparaginase II and EF-Tu have been reported to be immunogenic in other pathogenic bacteria (52, 57 - 62). In addition, other studies have described the presence of EF-Tu in *E. coli* OM or OMP extracts (35, 63 - 67), as well as interaction with fibronectin (68) or indicating participation in the adhesion to human intestinal cells and mucin (69 - 71). It is also possible that EF-Tu belongs to the group of “moonlighting proteins” (72, 73), which can be found in various locations within the bacterial cell and fulfill specific functions that vary by position.

Other proteins identified in O113:H11 (LEE-negative), but not in the LEE-positive STEC strains studied, include Hek and NmpC. It is clear that within LEE-negative STEC, there must be unique virulence factors that mediate colonization and infection in the absence of the proteins coded in the LEE locus. Hek is an important virulence factor in *E. coli* strains associated with neonatal meningitis-causing *E. coli* (NMEC). In these cases, it causes autoaggregation and facilitates epithelial cell adherence and invasion (74). NmpC has been reported to be immunogenic in uropathogenic *E. coli* (UPEC) (24) and to increases *E. coli* survival by a factor of 50 to 1000 when exposed to the bacteria at 60°C (75). It is likely that both proteins, Hek and NmpC, may be relevant and conserved antigens in other LEE-negative STEC.

Ag43 was another immunogenic protein that participates in autoaggregation, biofilm formation and also may act as an adhesin with certain extracellular matrix proteins (76, 77). Other studies demonstrate that this protein is an autotransporter with an N-proximal passenger domain (α43), which is processed by yet an unidentified protease and a C-terminal β-barrel domain (β43) that forms an integral outer membrane protein (78). Our
results show that in O26:H11, both domains were identified, but only α43 was recognized by the HUS sera. Two phylogenetic subfamilies have been classified in Ag43 based on the variable region located on the C- and N-terminals of α43 and β43, respectively. Subfamily I include proteins coded for by the allele present in E. coli K-12 and other variants of UPEC (E. coli CFT073). Subfamily II is characterized by a deletion of 72 amino acids in the C-terminal of the passenger domain α43 and includes the allele present in E. coli O157:H7 EDL933 (79). Interestingly, other studies have classified the allele present in O157:H7 as a homologue gene, called cah (calcium binding antigen 43 homologue) that encodes a protein that has a 68.5% amino acid identity and 72% similarity with Ag43 of E. coli K-12.

Cah protein binds calcium ions and also participates in autoaggregation and biofilm formation (80). Analysis of the genome sequences available for STEC and commensal E. coli strains revealed that cah is associated primarily with STEC strains, whereas ag43 is not. We investigated further using PCR in a collection of 170 STEC isolates (including 4 STEC strains analyzed using immunoproteomics) and 11 fecal commensal isolates of E. coli (plus E. coli HS strain). The frequency of detection of cah was significantly greater in human STEC isolates as compared to commensal strains (p<0.0001), whereas this was not the case for ag43. However, Cah was not detected in the OMP profiles of O26:H11 and O157:H7, both of which are positive for the cah gene. One possible explanation of this finding is that Cah is observed and detect by SDS-PAGE and Western blot only when the bacteria are cultivated in DMEM supplemented with 3mM of EDTA (80). Therefore, in order to evaluate reactivity in HUS and control sera we cloned the gene and obtained a
recombinant Cah protein. After obtaining rCah (92 kDa), we observed two additional bands with molecular weights of 78 kDa and 97 kDa, also identified as Cah.

One possible explanation for this finding is that the protease responsible for protein processing is not expressed in this bacterium. rCah was reactive for IgG and IgA in the majority of HUS sera (Fig 4B, Supplementary Figure 1). Therefore, Cah was included in the group of STEC-associated immunogenic proteins and is one of the most promising antigens for future studies. Interestingly, rCah was not seroreactive in 2/10 HUS sera, indicating that the STEC strains responsible for these HUS cases are probably not able to express Cah, or they do not carry the cah gene, a finding that is consistent with our detection frequency analysis of cah in STEC (Table 3).

OmpT is another immunogenic protein present in all STEC OMP profiles. OmpT is a serine protease well characterized in NMEC and UPEC strains as an important virulence factor (81-85). In STEC, OmpT degrades LL-37 (86), an antimicrobial peptide secreted by epithelial cells of the stomach and colon (87,88). Therefore, OmpT could play a defensive role against the host’s immune response. Moreover, a role for OmpT in outer membrane vesicle (OMV) biogenesis in STEC has been described recently (89). The ompT gene is abundant and widely distributed in E. coli. However, epidemiological studies seem to indicate that it is associated mainly with pathogenic strains. ompT detection frequencies of 96% have been described in NMEC (n=70) (82) and over 80% in UPEC (83,85). For commensal fecal isolates, ompT detection frequency is unclear, as reports have described frequencies from 67.7% (n=318) (85) to 19% (n=135) (83). These divergent results may be attributable to the fact that the fecal isolates used as intestinal commensal controls may...
have contained NMEC or UPEC (opportunistic pathogens), making it difficult to confirm that the strains are truly commensal intestinal types. To our knowledge, there have been no previous reports on ompT detection frequency in STEC. The presence of ompT in our STEC collection and fecal commensal isolates confirms that ompT detection frequency is significantly greater (p<0.0001) in STEC as compared with human intestinal commensal strains (Table 3).

Moreover, Western blot assays showed that rOmpT was strongly recognized by IgG and IgA antibodies present in HUS sera and was weak in one control sera (Table 1; Supplementary Figure 1). One possible explanation for OmpT reactivity in control sera could be cross-reactivity. OmpT has been described in other pathogenic E. coli, which could have generated a previous immunogenic cross-reaction. Despite the heterogeneity in the humoral immune response observed for each HUS serum through Western blot assays (Lane 1 in each panel of Supplementary Figure 1), both rOmpT and rCah were immunoreactive. These results indicate that OmpT and Cah are proteins synthesized in vivo during STEC infection in humans that generate a strong humoral immune response.

Although the immunoproteomic focus used in this study allowed us to identify STEC antigen proteins, we cannot rule out the presence of others immunogenic proteins, due to limitations inherent in the experimental design. First, OMP profiles were obtained by growing strains in a single culture medium. Second, low-abundance proteins that can be immunogenic are usually hidden by high-abundance proteins in complex samples analyzed by 2D-PAGE. Furthermore, because the proteins were subjected to denaturing conditions...
before Western blot analysis, it is likely that some conformational epitopes were not recognized by the sera.

Overall, our principal finding in this study was to identify Ag43, Cah, OmpT, Hek, NmpC, EF-Tu, and L-asparaginase II proteins as immunogenic proteins produced during STEC infection. These had not been characterized previously as immunogenic in STEC, even after extensive studies aimed at identifying antigens expressed in vivo during infection in humans (90). Several lines of evidence suggest that two of these antigens, Cah and OmpT, deserve further study to determine their potential role as protective antigens for STEC-related infections. Because STEC colonizes the mucosa, humoral IgA response is required in order to reduce the chances of intestinal colonization by this pathogen, and both Cah and OmpT react with IgA from HUS sera. The cah and ompT genes are conserved in STEC but not in fecal commensal E. coli, suggesting that Cah and OmpT could have a role in protection against a wide range of STEC serogroups with minimal cross-reactivity with commensal microbiota. Overall, our findings are important in the understanding of the antigens produced by STEC during human infection and the immune response against these pathogens in HUS.

ACKNOWLEDGMENTS

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REFERENCES


Figure 1. OMP profiles, and OMP IgG seroreactivity to pooled sera from HUS patients and controls. (A) SDS-PAGE of OMPs. 12% polyacrylamide gel and silver stained. (B and C) Western blot of SDS-PAGE on 12% polyacrylamide gels using pooled sera from HUS patients (B) and controls (C) (1:3500 dilution). Anti-human IgG secondary antibodies diluted 1:5000. (M) Protein Ladder, indicates molecular weights of the various proteins.

(Line 1) STEC O26:H11. (Line 2) STEC O103. (Line 3) STEC O113:H21. (Line 4) STEC O157:H7. (Line 5) E. coli HS. Arrows with numbers correspond to the proteins shown in Table 1.

Figure 2. 2D-PAGE OMP profiles from STEC and E. coli HS. pH range 4 - 7. 12% polyacrylamide gels and Coomassie blue G-250 stained. The images show the immunogenic proteins identified using MALDI-TOF/TOF. (A) STEC O26:H11. (B) STEC O103. (C) STEC O113:H21. (D) STEC O157:H7. (E) E. coli HS. (F) Differences between OMP profiles of STEC strains and E. coli HS. A unique OMP profile was identified for the STEC strains (in blue) and was superimposed on the OMP profile of the E. coli HS strain (in orange). Analysis was performed using BioNumerics 2D software (version 6.6). The scale bar indicates molecular weights.
Figure 3. Immunogenic proteins identified with 2D-PAGE Western blot analysis. O113:H11 OMP profile on 12% polyacrylamide (A) and Western blot (B) using pooled sera from HUS patients (1:3500 dilution). Anti-human IgG secondary antibodies diluted 1:5000. The scale bar indicates molecular weights. The arrow at the bottom of the figure indicates the pH range of the separation.

Figure 4. OMP profiles of transformed *E. coli* BL21(DE3) strains and Western blot of the recombinant proteins with HUS and control sera. (A) SDS-PAGE of OMPs on 12% polyacrylamide and Coomassie blue G-250 stained. (1) *E. coli* BL21(DE3)/pET15C. (2) *E. coli* BL21(DE3)/pET15C_ompT (3) rOmpT (4) *E. coli* BL21(DE3)/pET15C_cah (5) rCah. The arrows and numbers correspond to proteins shown in Table 4. (B) Representative Western blot using serum obtained from patient indicated as HUS - 15 (1:3500 dilution) and anti-human IgG secondary antibodies (1:5000 dilution). (C) Representative Western blot using serum obtained from patient indicated as HUS - 15 (1:1000 dilution) and anti-human IgA secondary antibodies (1:2000 dilution). (D) Western blot representative using control - 19 serum (1:3500 dilution) and anti-human IgG secondary antibodies (1:5000 dilution). (E) Western blot representative using control - 19 serum (1:1000 dilution) and anti-human IgA secondary antibodies (1:2000). (1) *E. coli* BL21(DE3)/pET15C. (2) rCah (3) rOmpT. (M) Molecular weight ladder. The molecular weights of the standards are indicated. * rCah and ** rOmpT.
Figure 3
Figure 4
<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein</th>
<th>Best match</th>
<th>E-value</th>
<th>Theoretical pI/Mw</th>
<th>Experimental pI/Mw</th>
<th>Location</th>
<th>Strain</th>
<th>Reactivity</th>
<th>HUS Control</th>
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<td>4.6 / 65000</td>
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<td>O103</td>
<td>+</td>
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<td>gi</td>
<td>15831916</td>
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<td>4.70 / 59953</td>
<td>4.8 / 62000</td>
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</table>

Numbers correspond with proteins shown in Figure 1.

Probability of erroneously assigning the protein identity.

The isoelectric point and theoretical molecular weight were determined using the ExPASy tool from the Proteomics Server UniProt Knowledgebase (http://us.expasy.org/).

Prediction performed using the PSORTb program v3.0 (http://www.psort.org/psortb/index.html).

Reactivity with HUS or control sera

OM, Outer membrane; F, Flagellum; C, Cytoplasm; P, Periplasm, pl, Isoelectric point; Mw, Molecular weight.

Recognition of OmpT by only one control sera using the recombinant protein (rOmpT).
TABLE 2. Presence of *ag43* and *cah* in STEC and avirulent *E. coli* strains according to genome sequence analysis

<table>
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<th>Strain</th>
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<th>Gene</th>
<th>Length (AA)</th>
<th>% Identity/Similarity</th>
<th>Score</th>
<th><em>a</em></th>
<th><em>b</em></th>
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<td>O157:H7 str. EDL933</td>
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<td><em>cah</em></td>
<td>1005</td>
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<td>-</td>
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<tr>
<td></td>
<td>AAG55766.1</td>
<td><em>cah</em></td>
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<td>O26:H11 str. 11368</td>
<td>BAI24655.1</td>
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<td>BAI26616.1</td>
<td><em>cah</em></td>
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<td>90 / 93</td>
<td>3855</td>
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<tr>
<td></td>
<td>BAI26126.1</td>
<td><em>ag43</em></td>
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<td>BAI28612.1</td>
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<td>O103:H2 str. 12009</td>
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<td>72 / 72</td>
<td>1640</td>
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<td>O111:H- str. 11128</td>
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<td>69 / 76</td>
<td>1633</td>
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<td><em>E. coli</em> H5</td>
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<td>82 / 87</td>
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<td><em>E. coli</em> SE15</td>
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<td></td>
<td>BAI57742.1</td>
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<td><em>E. coli</em> SE11</td>
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<td><em>E. coli</em> ED1A</td>
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<td>1042</td>
<td>75 / 75</td>
<td>1703</td>
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</table>

*a* EMBOSS 6.3.1: matcher (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::matcher).

*b* BLASTN algorithm


* indicates gene with a deletion; AA, amino acids.
**TABLE 3.** Detection frequency for *ag43*, *cah* and *ompT* in 182 *E. coli* isolates of different origins, determined using PCR

<table>
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<tr>
<th>Origin of isolate</th>
<th>Number of isolates</th>
<th><em>ag43</em></th>
<th><em>cah</em></th>
<th><em>ompT</em></th>
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</thead>
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<tr>
<td>Commensal <em>E. coli</em> *</td>
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<td>3 (25)</td>
<td>1 (8)</td>
<td>4 (33)</td>
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<tr>
<td>STEC O157:H7 from humans</td>
<td>48</td>
<td>1(2)</td>
<td>48 (98)</td>
<td>48 (100)</td>
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<tr>
<td>STEC O26:H11 from humans</td>
<td>28</td>
<td>28 (100)</td>
<td>28 (100)</td>
<td>28 (100)</td>
</tr>
<tr>
<td>Other non-O157 from humans</td>
<td>49</td>
<td>32 (65)</td>
<td>16 (33)</td>
<td>49 (100)</td>
</tr>
<tr>
<td>Total non-O157</td>
<td>77</td>
<td>60 (78)</td>
<td>44 (57)</td>
<td>77 (100)</td>
</tr>
<tr>
<td>Total STEC from humans</td>
<td>125</td>
<td>61 (49)</td>
<td>92 (74)</td>
<td>125 (100)</td>
</tr>
<tr>
<td>STEC from animals</td>
<td>45</td>
<td>26 (58)</td>
<td>17 (38)</td>
<td>41 (91)</td>
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<tr>
<td>Total STEC isolates</td>
<td>170</td>
<td>87 (51)</td>
<td>109 (64)</td>
<td>166 (98)</td>
</tr>
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</table>

*P < 0.05; *b* *P < 0.01; *c* *P < 0.001; *d* *P < 0.0001. Two-tailed Fisher’s exact test (as compared with commensal isolates). * Commensal *E. coli* from human feces including *E. coli* HS.
TABLE 4. Recombinant proteins identified using MALDI-TOF/TOF

<table>
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<tr>
<th>Band No.</th>
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*Numbers correspond with the proteins shown in Figure 4A.*

*Probability of erroneously assigning the protein identity.*

*Highest score for protein.*