Identification of Escherichia Coli O157:H7 Outer Membrane Proteins Which Mediate Adherence to Bovine Endothelial Cells

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ABSTRACT: Escherichia coli (E. coli) O157:H7 is a human pathogenic bacterium known to cause foodborne outbreaks of bloody diarrhea and hemolytic uremic syndrome. Cattle are a primary reservoir of E. coli, which are shed in the feces and transmitted to humans through contaminated dairy products and undercooked or raw meat. The purpose of this study is to identify bacterial outer membrane proteins (OMPs) involved in the colonization of bovine endothelial cells. It was hypothesized that similar E. coli O157:H7 OMPs would be involved in adhesion to both human and bovine endothelial cells. An optimized "pull-down" technique was developed to selectively anchor biotin-labeled bovine endothelial cell surface proteins (CSP) onto a streptavidin bead matrix followed by incubation with E. coli OMPs. After washing the beads, bound OMPs were eluted and subsequently analyzed by peptide sequencing. A total of 90 proteins were identified, including significant hits of OmpA, OmpX, OmpSlp and flagellin, in addition to several chaperone proteins which represented a group of previously well-characterized mediators of E. coli adhesion to human cells. This is the first study in the literature to demonstrate the role of these OMPs for attachment of E. coli to bovine endothelial cells..

KEYWORDS: E. coli; O15:H7; outer membrane proteins; bovine; OmpA

Introduction. The bacterium Escherichia coli is a normal resident of humans and animal intestines. Most E. coli do not cause any harm, actually helping in food digestion and preventing colonization of harmful bacteria within the intestinal tract. O157:H7 serotype of E. coli belongs to the family of enterohemorrhagic zoonotic bacteria is one of the leading causes of food-borne disease outbreaks in the United States1. Infection is transmitted from livestock to humans via consumption of uncooked and contaminated dairy and meat products. E. coli O15:H7 strain produces Shiga toxin which results in severe systemic infections that can lead to symptoms such as bloody diarrhea and hemolytic uremic syndrome[1].

E. coli O157:H7 attach to the human intestinal epithelial cells and flatten the finger-like projections of the epithelial cells described as effacement[2]. The attachment and effacement lesions on epithelial cells are a result of outer membrane proteins (OMPs) binding to host cells and releasing bacterial toxins and virulence factors. Proteins such as OmpA and OmpX, in addition to flagellins, may be critical for bacterial binding to human epithelial cells. Bacterial binding causes rearrangement of the host's cytoskeleton and forms pedestals under the surface of attached bacteria resulting in irreversible damage to the intestinal epithelial cells. E. coli O157:H7 inserts an intimin receptor into the epithelial cells which binds to intimin on bacteria resulting in tight attachment and efficient host colonization[2]. Next, Shiga toxins are released, causing death of the epithelial cells, breakdown of intestinal barrier, and hemolytic diarrhea. Tissue damage can stimulate the host's immune system which causes further damage to the intestines, including increased intestinal permeability and apoptosis.

E. coli O157:H7 is a concern for the food industry since the bacteria reside in bovine intestines without causing any pathogenic symptoms[3]. The bacteria are shed in the feces and pose a risk to people in contact with or consume contaminated food or drink. Additionally, E. coli can be transmitted between humans through occupational exposure. Water-borne outbreaks can occur in swimming pools contaminated with animal feces. Shedding of bacteria by beef cattle is higher in spring and summer than during winter months. Every outbreak of E. coli can be traced back to cattle and recalling contaminated products pose huge financial burdens to cow farmers. Several pre-harvest controls have been adopted in farms, including house cleaning, feed and water management, and vaccines against bacterial iron transport proteins such as siderophores[1-2]. Post-harvest control measures include thermal and chemical decontamination of infected carcasses.

In contrast to human colonization of E. coli O157:H7, binding of bacteria to cattle epithelium does not result in diarrhea. Cattle are known to lack Shiga toxin receptors and may be resistant to the toxic lesions in the gut, making them ideal reservoirs for the pathogen[4]. Earlier studies have shown bacterial binding to bovine epithelial cells but the exact bacterial OMPs involved in adherence and colonization has not been characterized. E. coli binding to human endothelial cells is mediated in part by OMPs and results in upregulation of several adhesion molecules, such as ICAM-1 and VCAM-1, leading to

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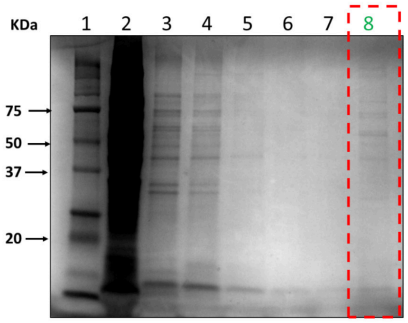
Enhanced leukocyte binding to endothelial cells5. It is unclear whether bacterial binding to bovine endothelial cells results in binding and activation of the immune system. As a first step, we sought to identify bacterial OMPs which are potentially utilized to bind the bovine endothelium.

The purpose of this study is to identify bacterial OMPs involved in the colonization of bovine endothelial cells. The hypothesis is that previously described E. coli O157:H7 OMPs which were involved in colonization of human cells are responsible in binding bovine endothelial cells. This would highlight common mechanisms in the colonization of human and bovine endothelial cells that lead to rapid transmission of infection from bovine to human hosts.

Results and Discussion Isolation of E. coli O15:H7 OMPs binding to bovine endothelial cells. Cell surface biotinylated bovine endothelial proteins were immobilized onto streptavidin beads. Unbound host proteins were washed away with mild detergent (0.1% Triton-X) followed by incubation with bacterial OMP preparation. Results are shown in Figure 1. The majority of the unbound host and bacterial proteins were detected in the flow-through fractions (Lanes 2 and 3). The efficiency of the washing procedure was monitored by a subsequent reduction of detectable proteins in the wash steps (Lanes 4-7). The acid elution procedure resulted in the isolation of a small subset of proteins from the total fraction, observed in lane 8. Stringent washing conditions with 5M NaCl did not yield any proteins.



12% SDS-PAGE
Coomassie blue stain

Lane	Samples
1	Molecular weight standard
2	Flow-through - host proteins
3	Flow-through- E. coli OMP
4.	Wash 1
5	Wash 2
6	Wash 3
7	Wash 4
8	**Elution output**

Figure 1. Proteins from the flow-through of the host and bacterial proteins were run under reducing and denaturing conditions using 12% SDS-PAGE and visualized with Coomassie blue stain.

Identification of bacterial OMPs. Partial sequence analysis of peptides revealed about 90 proteins including three OMPs, OmpA, OmpX and OmpSlp. The data is summarized in Table 1. A total of 8 peptides were identified for OMP A while fewer peptides were identified for OmpX and OmpSlp (Figure 2). Additionally, flagellin, a protein important for bacterial mobility, was identified as an interacting partner with bovine cell surface proteins. The putative roles of these proteins previously described for human cells are included in Table 1.

Table 1. Summary of bacterial OMPs and their proposed functions

Protein	Gene	Coverage	#peptides	# amino acids	MW (kDa)	Predicted protein function
Outer membrane protein A	OMP A	40.17	8	346	37.2	Adhesin/invasin; binds GlcNAc1,4-GlcNAc epitopes on glycoprotein. Target for immune system
Outer membrane protein X	OMP X	13.45	2	171	18.6	Adhesion and invasion of lung epithelial cells
Outer membrane protein slp	OMP slp	9.57	1	188	20.9	Initial adhesion. Binds to human polymeric immunoglobulin receptor
Flagellin	Flagellin	56.75	21	585	59.9	Motility. Target for immune attack

Outer membrane protein A

MKKTAIAIAV ALAGFATVAQ AAPKDNTWYT GAKLGWSQYH DTGFINNNGP THENQLGAGA FGGYQVNPYV GFEMGYDWLG RMPYKGSVEN
GAYKAQGVQLTAKLGYPITD DLDIYTRLGG MVWRADTKSN VYGKNHDTGV SPVFAGGVEY AITPEIATRL EYQWTNNIGD AHTIGTRPDN
GMLSLGVSYR FGQGEAAPVVAPAPAPAPEV QTKHFTLKSD VLFNFNKATL KPEGQAALDQ LYSQLSNLDP KDGSVVVLGY TDRIGSDAYN
QGLSERRAQS VVDYLISKGI PADKISARGMGESNPVTGNT CDNVKGRAAL IDCLAPDRRV EIEVKGIKDV VTQPQA

Outer membrane protein X

MKKIACLSAL AAVLAFTAGT SVAATSTVTG GYAQSDAQGQ MNKMGGFNLK YRYEEDNSPL GVIGSFTYTE KSRTASSGDY NKNCIYGITA
GPAYRINDWASIYGVVGVGY GKFQTTEYPT YKHDTSDYGF SYGAGLQFNP MENVALDFSY EQSRIRSVDV GTWIAGVGYR F

Outer membrane protein slp

MNMTKGALIL SLSFLLAACS SIPQNIKGNN QPDIQKSFVA VHNQPGLYVG QQAIFGGKVI NVINGKTDTL LEIAVLPLDS YAKPDIEANY
QGRLLARQSGFLDPVNYRNH FVTILGTIQG EQPGFINKVP YNFLEVNMQG IQVVWHLREVV NTTYNLWDYG YGAFWPEPGW GAPYYTNAVS
QVTPELVK

Identified peptide sequences are shown in green

Figure 2. Full-length protein sequences of OMPs isolated from E. coli O157:H17 which bind bovine endothelial cell surface. Identified peptide sequences are highlighted.

In addition to these four well-characterized E. coli OMPs, several chaperone proteins were identified. The exact connection of these proteins to mediate bacterial adhesion to bovine endothelial cells is unclear since these proteins may represent a pool of non-specific proteins commonly detected during preparation of bacterial membranes. Other potential OMPs such as fimbrial biogenesis outer membrane protein, Phosphoporin PhoE, and murein lipoprotein OS were identified with lower peptide coverage and need further confirmation.

We have successfully developed a pull-down technique to isolate bacterial OMPs which bind to bovine endothelial cells. LC/MS/MS results showed several significant hits including OmpA, OmpX, OmpSlp, and flagellin. These adhesins should be further studied to to develop therapeutic interventions such as blocking antibodies or vaccine-based approaches.

OmpA is a highly expressed protein of E. coli and can aid in adhesion and invasion. The exposed surface of OmpA can trigger a host immune response6. OmpA has a Swiss army knife shape and forms a pore or barrel-like structure, although the exact function is unclear. OmpA and OmpX expression are tightly regulated by the bacteria under different growth conditions6.

Approaches to anchor host cell surface proteins to binding columns can potentially disrupt biologically relevant interactions between host cells and bacterial proteins. Alternative approaches, such as whole cell labeling with biotin followed by incubation of fixed bacteria and crosslinking prior to peptide sequencing, can reveal novel host-pathogen interactions which may have been missed during this study. Additional studies are needed to study the impact of bacterial adhesion to host cells using OMP-specific blocking antibodies to further understand the functional significance of our findings.

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Conclusion. Our study has revealed remarkable similarities between the adhesion molecules involved in binding E. coli to human and bovine endothelial cells. The lack of expression of immunoreactive receptors for Shiga toxin by bovine endothelial cells in contrast to the expression in other tissues such as convoluted kidney tubules4 may provide an explanation for the lack of hemorrhagic diarrhea in cattle in spite of conserved colonization mechanisms in human and bovine hosts. Further studies are needed to profile additional bacterial OMPs which may interact with bovine endothelial cells by culturing bacteria under different stress conditions including nutrient deprivation. Additionally, bacterial invasion results in the release of inflammatory mediators by host cells. Such inflammatory mediators can upregulate expression of additional cell surface host proteins which interact with E. coli OMPs. Thus, manipulation of both host and bacterial culture conditions prior to membrane preparations may reveal additional binding partner of bacterial OMPs. In summary, this study is the first to demonstrate the role of these OMPs for attachment of E. coli to bovine endothelial cells.

Methods. Bacterial OMP extraction. Extraction of cell surface proteins from bovine endothelial cells (EJG cells). Confluent EJG cells were quickly washed twice with ice-cold phosphate buffered saline (PBS) to prevent rounding and detachment. Cell surface biotinylation with sulfo-NHS-SS-biotin was performed according to the manufacturer's protocol (Pierce Biotechnology). Cells were incubated with 10 ml biotin solution in an orbital shaker for 30 minutes at 4 ºC. The reaction was stopped by the addition of a quenching solution. Cells were gently scraped and centrifuged at 500 g for 3 minutes. Cell pellets were resuspended in lysis buffer and disrupted by sonication. Cell lysates were centrifuged at 10,000 g for 2 minutes and the clarified supernatant was collected in a fresh tube and stored at -20 ºC. A stepwise protocol is shown in Figure 3.

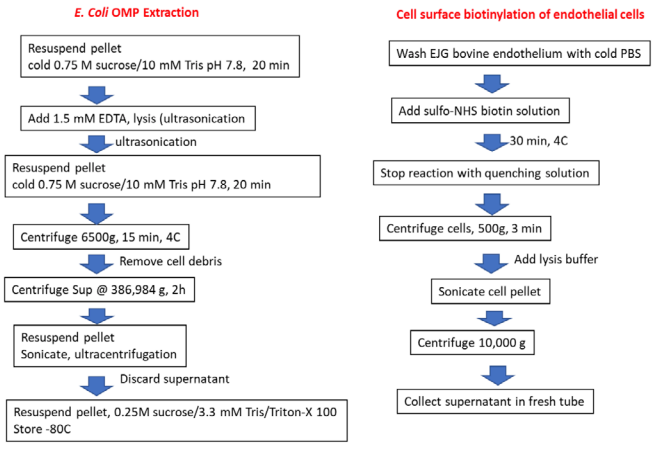

Figure 3. Stepwise protocol for extraction of bacterial OMPs (Left panel) and biotinylation and membrane preparation of bovine endothelial cells (Right panel) is described.

Pull-down Assay and peptide analysis. All procedures were conducted using the Pierce cell surface protein isolation kit. Briefly, bovine EJG endothelial lysate was incubated with streptavidin beads for 60 minutes at room temperature in a rotating mixer. Unbound proteins were washed three times with 0.5% Triton-X Tris buffer followed by incubation with the bacterial OMP preparation for an additional 60 minutes at room temperature. Free OMPs were washed three times with 0.5% Triton-X Tris buffer followed by elution under acidic conditions.

Peptide Analysis Eluted proteins were digested with trypsin, concentrated and desalted according to standard procedures by an external contract research organization (Poochon Scientific, Frederick, MD). Samples were analyzed using LC/MS/MS analysis and bacterial OMP proteins were identified based on peptide sequence analysis. Briefly, LC/MS/MS analysis was carried out using a Thermoscientific Q-Extractive hybrid mass spectrometer. Peptide mixtures were loaded on reverse phase PicoFrit column and trapped peptides were eluted using 3-36% linear gradient of acetonitrile in 0.1% formic acid. Eluted peptides were ionized and sprayed into the mass spectrometer. Raw data was searched against E. coli O15:H7 strain protein sequence database downloaded from UniportKB using the Proteome Discoverer 1.4 software. The maximum false peptide rate was specified as 0.01 (confidence scores were not provided by the external CRO).

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