



# *Escherichia coli* O157:H7 virulence factors differentially impact cattle and bison macrophage killing capacity

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## ABSTRACT

Enterohemorrhagic *Escherichia coli* O157:H7 colonizes the gastrointestinal tract of ruminants, including cattle and bison, which are reservoirs of these zoonotic disease-causing bacteria. Healthy animals colonized by *E. coli* O157:H7 do not experience clinical symptoms of the disease induced by *E. coli* O157:H7 infections in humans; however, a variety of host immunological factors may play a role in the amount and frequency of fecal shedding of *E. coli* O157:H7 by ruminant reservoirs. How gastrointestinal colonization by *E. coli* O157:H7 impacts these host animal immunological factors is unknown. Here, various isogenic mutant strains of a foodborne isolate of *E. coli* O157:H7 were used to evaluate bacterial killing capacity of macrophages of cattle and bison, the two ruminant species. Cattle macrophages demonstrated an enhanced ability to phagocytose and kill *E. coli* O157:H7 compared to bison macrophages, and killing ability was impacted by *E. coli* O157:H7 virulence gene expression. These findings suggest that the macrophage responses to *E. coli* O157:H7 might play a role in the variations observed in *E. coli* O157:H7 fecal shedding by ruminants in nature.

## 1. Introduction

Enterohemorrhagic *Escherichia coli* O157:H7 are gram-negative, flagellated bacteria, which are the causative agent of foodborne or waterborne illnesses in humans [1]. Some individuals, particularly the young and elderly, when infected with *E. coli* O157:H7 may experience bloody diarrhea and can develop Hemolytic Uremic Syndrome (HUS) [2]. HUS is characterized by kidney failure and may be fatal [3]. Conversely, within the healthy adult ruminant reservoir host, *E. coli* O157:H7 does not elicit clinical disease [4]. In ruminants, *E. coli* O157:H7 transiently colonizes the intestinal tract, including the recto-anal junction, which likely plays a role in shedding [5,6]. Bacterial shedding by ruminants can vary widely, with some animals becoming persistent, high-level shedders, often referred to as super-shedders [7]. This variation in shedding can be due to bacterial strain differences [8], gut microbiome composition [9] and genetic differences of the host breed or immune receptor repertoire [10,11].

Proteins produced by *E. coli* O157:H7 can have important adherence-promoting and immune-modulating effects on the host. One important protein is curli that produces adhesive fimbrial structures called curli fimbriae [12]. Curli fimbriae protrude from the bacterial surface and function to bind various host proteins, colonize animal tissues, activate the immune system, and promote production of biofilms [13]. Another important aspect of bacterial epithelial adherence is

the locus of enterocyte effacement (LEE)-encoded type III secretion system (T3SS). Previous studies have demonstrated that hemolysin expression-modulating protein Hha acts as a negative regulator of LEE expression and LEE-mediated adherence of *E. coli* O157:H7 to epithelial cells [14]. We have demonstrated previously that the strains of *E. coli* O157:H7 harboring deletion mutations within *hha* ( $\Delta hha$ ) over-expressed LEE-encoded proteins such as EspA, EspB, Tir, and Intimin [5]. The  $\Delta hha$  strains showed enhanced adherence to epithelial cells [15] as the proteins secreted through the T3SS (T3SP) are vital to the intimate contact between bacterial and epithelial cells. The T3SS projects on the bacterial cell surface as a needle-like structure which translocates bacterial proteins into the epithelial cell [16]. These translocated T3SP are implicated in modulation of cell signaling [17] and protein expression of the affected epithelial cell [18].

Immunologically, *E. coli* O157:H7 has been demonstrated to modify immune receptor signaling [19], cellular trafficking [20], and cytokine production [21] within the reservoir host to promote colonization. While *E. coli* O157:H7 doesn't induce observable lesions in healthy ruminants and generally colonizes transiently, cell types such as epithelial, dendritic cells, and macrophages may still interact with the bacteria to limit bacterial colonization and facilitate clearance. As highlighted above, there are numerous virulence mechanisms that contribute to epithelial cell attachment, and many of the same factors may contribute to attachment or infection of macrophages. The T3SS of

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*E. coli* O157:H7 can modulate macrophage phagocytic function, both in human and bovine cells [22]. Given the negative impact on bovine macrophage function, it is possible that the inability to clear *E. coli* O157:H7 contributes to bacterial burden and subsequent shedding of the organism by the host. *In vitro* studies using human macrophages have demonstrated it as a cell type supportive of *E. coli* O157:H7 replication [23] which suggests that macrophages may play a role in *E. coli* O157:H7 survival.

Predominantly, *E. coli* O157:H7 has been studied within the context of cattle because they are a primary meat source, and there are documented cases of contamination of beef, produce and water [24]. Similarly, bison represents an additional reservoir host to pathogenic bacteria as studies have demonstrated that the bison naturally shed enteropathogenic *E. coli* [25–27]. Given the increased economic growth of bison meat production [28] and intermingling of bison and cattle [29,30], there is a need to study *E. coli* O157:H7 interactions with the immune system of both species. Additionally, differences detected between ruminants may provide insight into differential interactions between host and bacterial organism for development of intervention strategies to limit colonization and shedding of *E. coli* O157:H7 in these animals.

Here, we utilized isogenic strains of *E. coli* O157:H7 expressing variable levels of curli fimbriae, and LEE-encoded T3SS and T3SP to assess the impact of virulence gene expression on the ability of macrophages to phagocytose and kill *E. coli* O157:H7. By using *E. coli* O157:H7 isogenic strains with mutations involving expression of adhesion virulence factors, we can identify virulence systems important for macrophage activation or killing. Importantly, bison macrophages had a reduced ability to kill the *E. coli* O157:H7 compared to cattle macrophages. Evasion of macrophage killing may play an important role in the shedding or persistence of *E. coli* O157:H7 in different ruminant species.

2. Materials and methods

2.1. Animals and sample collection

Four Holstein cattle and four American bison of approximately one-year of age were used as blood donors. All animals were clinically healthy at the time of blood collection, which was performed via jugular venipuncture into 50 mL syringes containing acid-citrate-dextrose (ACD). Animal procedures employed in these studies were approved by the USDA-National Animal Disease Center Institutional Animal Care and Use Committee.

2.2. Bacterial strains, culture medium and growth conditions

The bacterial strains used in this study are listed in Table 1. *E. coli* O157:H7 strains NADC 6564 and NADC 6565 did not produce (curli<sup>neg</sup>) or produced very low-levels of curli (curli<sup>lo</sup>) fimbriae, respectively, and were derived from a 1986 foodborne *E. coli* O157:H7 strain 86-24 [2,12,31]. *E. coli* strains NADC 6597 (curli<sup>neg</sup>  $\Delta$ hha) and NADC 6631

(curli<sup>hi</sup>  $\Delta$ hha) were constructed by deleting the *hha* or *stx2* gene in strains NADC 6564 and NADC 6565, respectively, as described in previously reported studies [32,33]. Bacterial strains were cultivated in Luria-Bertani broth (LB) or LB agar (Sigma-Aldrich, St. Louis, MO) supplemented with antibiotics (streptomycin, 100 mg L<sup>-1</sup>) (Sigma-Aldrich).

**Congo red binding and HEp-2 cell adherence assays.** The procedures for bacterial binding of Congo red and adherence to HEp-2 cells have been described previously [15,32]. For identification of amyloid fibrils (i.e. curli), Congo red staining was utilized. Briefly, cultures of bacterial strains were streaked on Congo red agar plates. After incubation for 48 h at 30 °C, plates were photographed. For adherence assays, 50  $\mu$ L of bacterial culture containing 1  $\times$  10<sup>5</sup> bacterial cells was added to the chamber portion of the tissue culture slide (Nalgene Nunc International, Naperville, IL) seeded with HEp-2 cells (ATCC, Manassas,VA). Slides were incubated at 37 °C for 1 h and then washed four times with phosphate-buffered saline. Slides were immersed in 0.4% crystal violet solution for 15 s, washed with distilled water, air dried, and examined for adherent bacteria at 400 $\times$  magnification.

2.3. Derivation of monocyte-derived macrophages

Macrophages were derived as previously described [34,35]. Briefly, peripheral blood mononuclear cells (PBMC) were isolated and treated with buffered ammonium chloride-potassium carbonate salt solution to lyse red blood cells (RBC). After RBC lysis, cells were washed with sterile phosphate-buffered saline (PBS). Cells were resuspended in complete RPMI 1640 (cRPMI) containing 10% fetal bovine serum, 2 mM L-glutamine, 1% streptomycin solution, and 5  $\mu$ g per ml of gentamicin sulfate (Life Technologies-Gibco, Carlsbad, CA). Monocytes were isolated from PBMC by plastic adherence in culture for 24 h. Non-adherent cells were removed, PBS was added to the plate, and adherent cells were gently removed with a cell scraper. Cells were centrifuged at 500  $\times$  g (Eppendorf, Hauppauge, NY) and resuspended in cRPMI, counted utilizing a Countess™ cell counter (Life Technologies) with trypan blue exclusion, and seeded at 10<sup>5</sup> live cells in 200  $\mu$ L medium per well in 96-well round bottom tissue culture plates. Monocytes were cultured for 7 days at 37 °C with a fresh media change every 2–3 days to derive macrophage cells.

2.4. Macrophage uptake and killing assay

Macrophage uptake and killing of *E. coli* O157:H7 was assessed with gentamicin protection assays as previously described [3]. Briefly, differentiated macrophages were washed twice and cultured in cRPMI without antibiotics. *E. coli* O157:H7 bacterial cell numbers per mL of culture were estimated from OD<sub>600</sub> readings (1 OD<sub>600</sub> = 2  $\times$  10<sup>8</sup> cells per mL), resuspended into cRPMI without antibiotics and inoculated into 96-well plates with macrophages at approximately 10<sup>6</sup> *E. coli* O157:H7 per well. Bacteria and cells were incubated for 60 min at 37 °C. Plates were centrifuged at 500  $\times$  g for 10 min, washed 3 times with cRPMI containing streptomycin and gentamicin (Life

Table 1  
Bacterial strains used in this study<sup>a</sup>.

<i>E. coli</i> O157:H7 strain	Genotype and description	Source or reference
NADC 6564	<i>stx2</i> <sup>+</sup> , curli-negative, and streptomycin-resistant isolate of strain 86-24	[2,12,31]
NADC 6565	Low-level curli-producing isolate of strain 86-24	[12]
NADC 6630	$\Delta$ stx2 NADC 6565	[32,33]
NADC 6631	$\Delta$ stx2 $\Delta$ hha derivative of NADC 6565 (produces higher amounts of curli fimbriae and LEE encoded T3SS and proteins secreted by the T3SS (T3SP))	[32,33]
NADC 6595	$\Delta$ stx2 NADC 6564	Manuscript Submitted (Vet Micro)
NADC 6597	$\Delta$ hha $\Delta$ stx2 derivative of NADC 6564 (produces no to very low levels of curli fimbriae and expresses LEE encoded T3SS and T3SP at high levels)	Manuscript Submitted (Vet Micro)

<sup>a</sup> Detailed description of bacterial strains listed in this table is provided in the manuscript.

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