ESCHERICHIA COLI O157: CATTLE COLONIZATION, THE NUTRITIONAL BASIS OF COLONIZATION, AND DIAGNOSTIC PROBE DEVELOPMENT

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This dissertation, culminating a six-year interlude between a career as a practicing veterinarian to a career as an academic veterinary pathologist, is dedicated to my wonderful family –

wife Julie,

daughter Aubrey,

daughter Lillie, and

son Samuel –

for their love and patience during this time of training and education.

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CHAPTER 1

Introduction

E. coli O157 is an intestinal pathogen of man that causes bloody and non-bloody diarrhea as well as potentially fatal systemic sequelae. It is causative of an emerging infectious disease first recognized in a foodborne disease outbreak in 1982. Since that time, additional outbreaks and sporadic cases have focused a great deal of research attention upon this pathogen and the diseases it causes.

Areas of active research for *E. coli* O157 and its diseases include genomics, epidemiology, pre-harvest and post-harvest food safety, pathogenesis, diagnostics, vaccination, and treatment.

Two of the studies described herein are important to the improved understanding of pre-harvest food safety related to food contamination by *E. coli* O157. In this regard, these studies demonstrate advances in reliably mimicking *E. coli* O157 colonization of adult cattle and also demonstrate a nutritional basis for its colonization. Reliably modeling natural infection allows hypotheses concerning *E. coli* O157 cattle colonization to be tested. As evidence, we demonstrate the testing of the hypothesis of a nutritional basis for colonization by utilizing a model that mimics natural colonization.

The final study described herein addresses the need for improved diagnostics by discovery-based investigations of the isolation of *in vitro* selected aptamers as recognition elements for *E. coli* O157. Application of novel recognition elements in diagnostic platforms could result in improved diagnostic tools utilized in settings that vary from cattle shedding surveillance to food contamination to bedside human diagnostics.

CHAPTER 2

Escherichia coli O157:

A Review of the

Literature

Abstract

E. coli O157 is a significant pathogen that causes foodborne illnesses in man without causing significant disease in cattle, the primary reservoir. This pathogen is the archetypal member of the enterohemorrhagic *E. coli* (EHEC) and is monitored and investigated closely as an emerging infectious disease pathogen. Herein, the pathogenesis, reservoir, epidemiology, and diagnostics of this pathogen and its disease are reviewed.

Introduction

E. coli O157 is a gram-negative, facultative anaerobic, enteric bacterium that is causative of significant human disease (67, 92, 105). First associated with human disease in 1982 in a foodborne illness outbreak (112), it is classified as an emerging infectious disease pathogen (94) and as such, has warranted a high level of attention recently as its disease pathogenesis and its epidemiology have become more fully understood.

E. coli is the most well-studied of all bacteria. Most *E. coli* are commensals of mammalian intestinal tracts (34) and typically colonize the intestine of human infants within a few hours of birth (67). However, a diversity of pathogenic *E. coli* strains have been characterized and are associated with illnesses ranging from diarrhea to urinary tract infections, meningitis, or septicemia (67). The diarrheagenic *E. coli* are a group of organisms that, upon infection, cause diarrhea in the host. The diarrheagenic *E. coli* are divided into a variety of pathotypes based upon their genotype and their mechanisms of pathogenesis. These pathotypes provide clinical diagnosticians, medical personnel, and

researchers a categorization schema allowing better understanding of the epidemiology, pathogenesis, diagnostics, and treatment of these significant enteric illnesses (92).

The three most fully understood and characterized diarrheagenic *E. coli* pathotypes are: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), and enterohemorrhagic *E. coli* (EHEC). Other pathotypes include enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAggEC), diffusely adherent *E. coli* (DAEC), and the recently characterized adherent and invasive *E. coli* (AIEC) (25, 67).

Enterotoxigenic *E. coli* (ETEC) are those *E. coli* strains that produce one or both of the enterotoxins known as labile-toxin (LT) and stable toxin (ST) (79). ETEC is largely a pathogen of developing countries, where it causes traveler's diarrhea (67). The labile-toxin (LT) is a classical AB_5 subunit toxin consisting of five identical B subunits that bind host cell gangliosides and an A subunit that mediates the toxin's ADP-ribosyl transferase activity. This activity transfers an ADP-ribosyl moiety from NAD to a G protein which regulates adenylate cyclase. The net result is persistent activation of adenylate cyclase, increased cAMP levels in affected cells, increased chloride secretion from activated crypt epithelial chloride channels, and inhibition of NaCl uptake by villus tip epithelium leading to an osmotic diarrhea (92). The stable toxin (ST) is a small peptide of two types: STa associated with human disease and STb associated with animal disease. STa binds the guanylate cyclase receptor on host cells and activates cGMP or cAMP-dependent kinases resulting in hypersecretion of ions. STb can mediate ion hypersecretion by increasing cytosolic calcium concentrations or by stimulating the release of serotonin or prostaglandin E_2 (67).

Enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) are both members of the attaching and effacing *E. coli* (AEEC) pathotype, a less descriptive pathotype designation that has fallen into disuse. EPEC and EHEC infection both result in an attaching and effacing histopathological lesion of enteric mucosa, the basis for their categorization in the AEEC pathotype (67). This similarity is based in the genomics of each pathotype, but they are dissimilar in other key features. These will be briefly summarized below, and a more comprehensive description of the pathogenesis and virulence of *E. coli* O157 will follow.

EPEC and EHEC both possess the locus of enterocyte effacement pathogenicity island, and encoded proteins from this island mediate attachment to host enterocytes similarly (34). The locus of enterocyte effacement has a similar complement of genes in both pathotypes, and the principal effectors include intimin, Tir (translocated intimin receptor), and a variety of secreted proteins (Esp's) (72). The principal change in host cells is intimate attachment of bacteria accompanied by effacement of the microvillus border as well as cytoskeletal rearrangement manifested as cellular pedestals composed of polymerized actin beneath the attached bacterium (34). With respect to issues concerning evolutionary divergence, it is interesting to note the sequence divergence of Tir and intimin within EPEC and EHEC (31, 139). Tir has been analyzed for functional interchangeability between EPEC and EHEC in knockout and complementation experiments. For EPEC, formation of the pedestals is dependent upon tyrosine phosphorylation of Tir; the phenotype is not complemented by Tir from EHEC. The reverse is not true as EHEC complemented with EPEC Tir was able to form pedestals

(31). Additionally, EPEC Tir's binding of host cell protein Nck is necessary to initiate actin polymerization, but is not required in EHEC (13).

In addition to differences in Tir and intimin between EPEC and EHEC, the type IV bundle-forming pilus is produced by EPEC, and not EHEC (45). The bundle-forming pilus (Bfp) is an early attachment factor for EPEC (45) and is encoded upon an EAF (EPEC adherence factor) plasmid (93). Conversely, Shiga toxins are produced by EHEC, and not EPEC (105). Shiga toxins are encoded on lysogenic lamboid phages integrated into the chromosome (105).

Virulence and Pathogenesis of *E. coli* O157

E. coli O157 infection in man results in asymptomatic infection to watery or bloody diarrhea (92). In a small subset of infections, especially in the elderly and in children under five years of age, an occasionally fatal sequela known as hemolytic-uremic syndrome (HUS) can develop. HUS is clinically defined and recognized by a triad of clinical symptoms including microangiopathic anemia, thrombocytopenia, and uremia (47).

E. coli O157 possesses several virulence factors, including: Tir and intimin within the locus of enterocyte effacement; the large pO157 virulence plasmid; and lipopolysaccharide; but the key virulence factor is the Shiga toxin (67), also known as verocytotoxin (VT). Greater than 200 serotypes of *E. coli* can produce Shiga toxins, and Shiga-positive and Shiga-negative strains exist in most serotypes (65). Additionally, many of these serotypes do not possess the locus of enterocyte effacement and do not cause disease (67). Because of this, additional nomenclature is utilized: STEC for Shiga-

toxin producing *E. coli* and VTEC for verotoxin-producing *E. coli* – which are both synonymous terms for any *E. coli* strain that produces Shiga toxins. The term EHEC is utilized to indicate the subset of *E. coli* strains that produce Shiga toxin and contain the locus of enterocyte effacement (67). For example, STEC strains associated with piglet edema disease do not possess the locus of enterocyte effacement and are not classified as EHEC.

E. coli O157 causes characteristic intestinal histopathology known as attaching and effacing histopathology (a term coined by Moon (87)) that is accompanied by hemorrhage and edema in the lamina propria (52). Attaching and effacing histopathology has been seen in animal and *in vitro* models including young calves (30), rabbits (102), lambs (132) intestinal explants (4), and cultured epithelium (76) infected with *E. coli* O157. However, this histopathology has not been seen in clinical human samples, a feature likely explained by the temporal delay between disease onset with high numbers of organisms and late disease, when attaching and effacing lesions are likely to be absent (92).

Shiga toxins are classical AB_5 toxins possessing five identical B subunits that mediate receptor binding and a catalytically active A subunit (105). Shiga B subunits bind the host glycolipid receptor globotriaosylceramide (Gb_3). This receptor is variably expressed across host species and across anatomic loci (86). In rabbits, Shiga toxin is thought to translocate via Gb_3 receptors in the ileum, but in humans the pathway is not known (105). Upon translocation and entry (126) into the bloodstream, the toxin targets tissues expressing the Gb_3 receptor, which in humans includes the renal cortex and vasculature. Because the kidney is a principal site of lesion formation in HUS (47, 105),

this focus of receptor expression is important for analysis. In a study of Shiga toxin binding to infant and adult human renal sections, it was shown that levels were highest in adult kidneys with most localization at the distal convoluted tubule. Although pediatric kidney had lower levels of localization in the kidney, pediatric expression occurred in the distal tubule as well as the glomerulus, a finding that is consistent with the increased incidence of HUS in young children (80).

Upon receptor binding, Shiga toxin is internalized via coated pits and is retrotranslocated to the endoplasmic reticulum via the Golgi apparatus. Here, the A subunit enters the cytosol and cleaves a single adenine residue from the 28S rRNA of the 60S ribosomal subunit via its N-glycosidase activity. This action terminates protein synthesis leading to the death of renal endothelium, renal tubular epithelium, and other loci where Gb_3 expression is present (105).

The contribution of Shiga toxins to intestinal disease is variable and differs according to animal model. Nataro's review concludes that the "ability of EHEC to produce the A/E (attaching and effacing) lesions is probably sufficient to cause nonbloody diarrhea but that Stx is essential for the development of bloody diarrhea and hemorrhagic colitis." (92) The contribution of Shiga toxins to HUS is more defined. Upon bloodborne translocation to the kidney, Shiga toxin damages renal endothelium and occludes the glomerular microvasculature. This is mediated through direct effects and through induction of cytokine and inflammatory pathways (3).

Although the linkage between HUS and the Shiga toxin is established (105), an *E. coli* O157 Shiga toxin-negative strain associated with a case of pediatric HUS has been reported (115). Schmidt and colleagues speculate that *E. coli* O157 factors other than

Shiga toxin may be capable of causing HUS (115). Consistent with that speculation is the observation that two other Shiga toxin-negative bacteria, *Streptococcus pneumoniae* (2) and *Neisseria meningitidis* (73), have been associated with cases of HUS.

The locus of enterocyte effacement (LEE) encodes a type III secretion system and effector proteins known as intimin, Tir (translocated intimin receptor), and Esps. Although differences of the LEE between EPEC and EHEC have already been highlighted, an additional difference is that the effects of LEE gene products of EPEC are seen in the small intestine, and in EHEC, in the large intestine (67).

The pO157 plasmid (12) encodes two additional virulence factors, whose importance have yet to be established. ToxB, a potential adhesin, is homologous to the EPEC LifA protein (75) and the non-O157 EHEC Efa-1 adhesin (96). Enterohemolysin is a second candidate virulence factor on the pO157 plasmid (114).

Human infection with *E. coli* O157 is usually by the fecal-oral route and the successful infectious dose is notoriously low, estimated from 1 to 100 to 100 to 200 microorganisms (92, 105). Upon ingestion, *E. coli* O157 can successfully traverse the acidic environment of the stomach because *E. coli* O157 has a complement of acid-resistance mechanisms (141). The human disease usually begins 24 hours after ingestion of contaminated food or water with abdominal cramping followed shortly by watery diarrhea. Bloody diarrhea, if it occurs, then follows 24-72 hours later. Therapeutics in man are generally supportive in nature (92), but treatment with antibiotics is controversial (62, 105) and one study recommended that antibiotics of choice should disrupt protein synthesis and not inhibit DNA repair (62).

In concluding this section on virulence and pathogenesis, inclusion of published and annotated genomes is appropriate as it provides a perspective on evolutionary relationships and highlights candidate genes for future investigations of pathogenesis. *E. coli* O157 has been sequenced twice, with both sequenced isolates originating from human outbreaks (57, 106). In comparing the two published *E. coli* O157 genomes to *E. coli* K12 MG1655, *E. coli* O157 and *E. coli* K 12 MG1655 share a largely collinear 4.1 Mb backbone (57), and the total genomic size of *E. coli* O157 is 5.5 Mb (57, 106). Perna, et al, predicted 1387 genes were novel to *E. coli* O157 (106) while Hayashi, et al, predicted 1632 novel *E. coli* O157 genes (57), the differences likely originating from differing annotation algorithms. Additionally, *E. coli* O157 EDL 933 and *E. coli* K12 MG1655 were compared to the genome of the closely related *Shigella flexneri* 2a, and it was found that the three bacteria share a 3.9 Mb, largely collinear, chromosomal backbone, and the key difference possessed by *S. flexneri* 2a was the plasmid pCP301, comprised of genes conferring the invasive phenotype upon it (64). As this genome information becomes more reliably annotated and as investigators utilize the information to confirm functional relationships, a more thorough understanding of pathogenesis and improved targets for epidemiologic surveillance will correlate with improved human health.

E. coli O157: Reservoir and Colonization Models

The primary and most frequently identified reservoir of *E. coli* O157 is the bovine gastrointestinal tract (95). Other animal reservoirs of *E. coli* O157 that have been identified include sheep, goats, pigs, horses, dogs, turkeys, wild birds, whitetail deer,

rabbits, rodents, and flies (1, 8, 9, 35, 44, 56, 60, 77, 91, 97, 110, 133). To date, a comprehensive investigation into sites of recovery of *E. coli* O157 from slaughtered cattle has not been performed. However, in an experimental colonization of young adult cattle, the predominant site of *E. coli* O157 colonization was the lower gastrointestinal tract with lesser magnitude recovery from the rumen and duodenum (48). The tropism for the lower intestine was further confirmed and restricted to the lymphoid follicle-rich mucosa of the terminal rectum (95). Additionally, Pohlenz demonstrated O157 colonization of the squamous mucosa of the recto-anal junction (107). Other important loci of O157 isolation in cattle include the oral cavity and hide surfaces. One prevalence distribution study showed that 74.8% of feedlot cattle were positive for *E. coli* O157 in the oral cavity and that 41.0 to 73.4% of these cattle were O157-positive at various places on the hide (70).

Quantitative assessment of shedding of *E. coli* O157 by cattle is important because of its impact on environmental contamination and its impact upon the bases for transmission and intermittent shedding. Investigations into numbers of *E. coli* O157 shed by cattle have yielded important, yet variable, insights. In one study, at the third day post-inoculation, adult cattle shed an average of 1.0×10^5 cfu/g feces, whereas similarly colonized young calves shed 6.3×10^6 cfu/g feces (23). This demonstrated the importance of O157-infected calves with respect to environmental contamination. In a field prevalence survey of dairy herds, shedding was documented to vary from 10^2 to 10^6 cfu/g feces (143).

Cattle have been intentionally colonized by *E. coli* O157 via a variety of procedures. Oral colonizations via dose syringe or stomach tube (22, 23, 26-30, 49, 61,

95, 117) , ruminal colonizations via rumen cannulas (48, 128), and rectal colonizations (117) have been documented. All methods have been utilized in adult cattle and each method has achieved similar colonization levels. With respect to ease of administration and persistence of colonization, the rectal administration model was superior to oral colonization in a direct comparison (117). Additionally, high infectious doses (10^{10} cfu/animal) consistently outperform lower infectious doses (10^{7} or lower) with respect to higher magnitude and longer persistence of colonization in many studies (23, 117).

While cattle are the primary reservoir of *E. coli* O157, they do not exhibit significant disease as adults (23) and only transient diarrhea as young calves (10, 23, 30). One of the key determinants of this immunotolerance may be the species differences in Shiga-toxin receptor expression. In man, the Shiga-toxin receptor, globotriaosylceramide (Gb3) is expressed in renal glomeruli, renal tubules, brain, and vasculature (105). Cattle lack vascular and intestinal epithelial receptors for Shiga toxin, but do express the Shiga-toxin receptor in renal tubules and in the brain (108). Although cattle appear resistant to the effects of Shiga toxin due to the lack of vascular receptors, they do exhibit attaching and effacing histopathology during colonization of calves (30), but this was not exhibited in adult cattle (23) until Naylor's recent report demonstrated the pathology in a yearling (95). Further, in a study of large intestinal explants from adult cattle, it was demonstrated that O157 exhibits attaching and effacing histopathology in adult tissues (4). The best explanation for the difficulty of demonstrating attaching and effacing histopathology in adult cattle is the fact that 10^{6} cfu/g of feces is a recognized threshold for recognition of adherent bacteria in histopathologic sections of intestine (6), and adult cattle typically do not maintain this level of colonization (23).

Some colonization models have been specifically directed at investigating attachment strategies of *E. coli* O157. Intimin was shown to be important in the colonization and enteropathogenicity in young calves by colonizing with intimin-positive and intimin-negative strains of *E. coli* O157 (30). Further, it was similarly shown to be important in young adult cattle and sheep (22). Although demonstrated only in sheep and pigs, long polar fimbriae knockout mutants of *E. coli* O157 also demonstrated a defect in colonization (66). Lipopolysaccharide O-antigen chains were investigated in an *in vitro* model of attachment and were found not to be required (21). As a whole, this summary of published results is reasonably consistent with a comprehensive determination of the genes essential for the colonization of the bovine intestinal tract (37) by use of signature-tagged mutagenesis. In that study by Dziva and colleagues (37), several genes involved in the type III secretion system were implicated; this is consistent with the colonization defects seen with absence of intimin, a type III secretion system effector protein. A fimbrial usher protein was implicated in Dziva's study; its association with the long polar fimbriae is not known. Finally, this study implicated at least five genes in lipopolysaccharide biosynthesis, and this appears to be at odds with the study on O-antigen contributions to adhesion (21).

Other models or field investigations have focused upon environmental influences, diet, and other factors. Intermittency of shedding has been documented by many (23, 109, 117). The impact of age and colonization bolus level has consistently shown that younger cattle are more reproducibly colonized with persistence and that high infectious doses provide more reliable colonizations (23, 29, 117). Dietary influences have been investigated, with more variability to the results. In a more comprehensive investigation

of dietary influence upon success of acid-resistant coliform colonization, Diez-Gonzalez (32) demonstrated higher colonizations with cattle on a grain diet. However, in a specific investigation of *E. coli* O157's acid resistance, Hovde (61) demonstrated the reverse to be true. A study authored by Van Baale (128) was in close agreement with Hovde's results. Seasonality influences have also been investigated in sheep and cattle, with generalization across studies inhibited by marked differences in design and sampling times. One bovine study analyzed culture swabs post-slaughter and demonstrated the highest culture positive incidence in the summer (18). In dairy cattle, peak culture positive incidence was seen in the spring and in November (85).

An *E. coli* O157 colonization model developed in mice (131) demonstrated stable and persistent colonizations with fecal shedding of 10^{7-8} cfu/g feces of the input *E. coli* O157. In addition to increased understanding of colonization, this murine model also assessed pathogenicity to some extent because the mice were not resistant to the effects of virulence factors. Young chickens have also been experimentally colonized. According to the authors, the benefit of this model was in the study of attaching and effacing lesions that were induced in the chickens (121). Rabbits have also served as a colonization model (102). Finally, although not directed at the problem of intestinal colonization, animal models of Shiga-toxin mediated disease have been developed in baboons (125) and mice (69).

Generalizing the results of these diverse studies is difficult. However, four things are clear: 1) the shedding of *E. coli* O157 by cattle is intermittent, 2) calves are consistently higher shedders than adults and are important only from an environmental contamination standpoint, 3) colonization success is impacted by multiple factors, and 4)

determination of environmental impacts such as diet and season is difficult and suffers from competing and uncontrolled variables.

Epidemiology of *E. coli* O157 Infection

Enterohemorrhagic *E. coli* O157 is found worldwide and has been reported in all continents except Antarctica (38, 74). Emergence of this pathogen has been extensively analyzed using evolutionary genetics techniques (134). It is thought that *E. coli* O157 arose from a locus of enterocyte effacement (LEE) and *Stx*2-expressing *E. coli* O55 progenitor via the stepwise acquisition of the O157 O-antigen *rfb-gnd* loci, loss of sorbitol fermentation, and loss of β-glucuronidase activity due to disruption of the *uidA* gene (39, 123, 135). Archetypal *E. coli* O157 is described as non-sorbitol fermenting and β-glucuronidase negative and is the most commonly isolated *E. coli* O157 strain in the U.S., U.K., Canada, and Japan (74). Based upon phylogenetic analyses, other subclones of *E. coli* O157 have diverged at various points of the proposed evolutionary pathway (39) and successfully populated geographically restricted areas (74). Of note, significant numbers of non-motile, sorbitol positive, β-glucuronidase-positive *E. coli* O157 are isolated in Europe (39) and non-motile, sorbitol-negative, β-glucuronidase-negative *E. coli* O157 are seen in Australia (113).

E. coli O157 was first associated with human disease in the U.S. in 1982 (112). Since that time, numerous outbreaks have been documented. Perhaps the most notorious outbreak occurred in 1993 in Washington where consumption of undercooked hamburger at a national fast-food chain sickened over 500 people and killed four children (5). Although large outbreaks dominate news headlines, it is estimated that the sporadic *E.*

coli O157 infections represent the major health burden associated with this pathogen (92). In the United States, it is estimated that *E. coli* O157 causes over 73,000 foodborne illness cases annually and that non-O157 STEC cause up to 36,740 more. It was further estimated that *E. coli* O157 causes 60 deaths per year in the U.S. (84).

Reports of infections with EHEC appear to follow a geographic trend with infection more common in the northern versus the southern United States and also more common in western than eastern Canada (92). Seasonality is also important with most sporadic cases documented in the summer (92).

Although most sporadic cases and outbreaks of hemorrhagic colitis are caused by *E. coli* O157 (92), incidence of disease due to other serotypes is rising (65). The most common serotypes other than O157 implicated in human disease are O26, O103, O111, and O113 (53). In fact, greater than 200 serotypes can express Shiga toxin, but Shiga-positive and Shiga-negative strains are seen in most of these serotypes (65). Such non-O157 serotypes represent a diagnostic dilemma, as most of them do ferment sorbitol, making selective media such as sorbitol-MacConkey (*vide infra)* largely useless. Even usage of Shiga toxin genetic markers is unreliable as Shiga toxin expression is not thought to solely confer full virulence upon EHEC (65).

The most important reservoir of *E. coli* O157 is the bovine intestinal tract (92, 95) and the fecal-oral route is the primary mode of infection in humans. The infectious dose for *E. coli* O157 infection has been estimated to be as low as 1 to 100 or 100 to 200 organisms (92, 105). Contaminated foods of bovine origin, in particular ground beef, are an important cause of human infection (53). In large packing houses, the preparation of ground beef involves the grinding of beef parts from thousands of cattle from different

farms. Beef patties are then formed from this mixture and widely distributed geographically. Therefore, *E. coli* O157 organisms from one infected animal can contaminate entire batches of ground beef (92). The economic costs to the beef packing industry from a ground beef recall are staggering and can negatively impact the reputation of meat packers (83).

Numerous other food vehicles have been implicated in the transmission of EHEC. These include mayonnaise (51), radish sprouts (122), alfalfa sprouts (15), lettuce (88), sausage (90), salami (14), apple cider (7), and milk (89). With the exception of milk, infection with these vehicles is thought to have occurred via water contaminated by cattle feces followed by consumption of these uncooked products (89, 92). Contamination of milk is documented in bulk tank (raw) milk samples (89) and presumptively implicates direct fecal contamination at the time of milking.

Waterborne sources of *E. coli* O157 are also significant to the understanding the epidemiology of O157. Because of its low infectious dose (92, 105), low level contamination of water could result in sporadic cases or outbreaks. Recreational lakes, municipal water supplies, and well water have been documented in waterborne *E. coli* O157 cases (17).

The epidemiology of *E. coli* O157 infection also includes some less common or less considered sources. Petting zoos have been documented to possess animals shedding *E. coli* O157 (59) and have been linked to human infections (59). Outbreaks have occurred at child day care centers (99, 116), and person-to-person spread was the suggested means of transmission (99). Additionally, an outbreak of *E. coli* O157 at a

county fair was epidemiologically concluded to originate from a contaminated building (129).

Finally, in addressing the epidemiology of *E. coli* O157, one can not ignore the potential of intentional usage of *E. coli* O157 as a biowarfare/bioterrorism agent. Although *E. coli* O157 has never been intentionally released, its low infectious dose and ease of environmental availability, coupled with its potential for morbidity or mortality, make it a candidate bioterrorism tool. The Centers for Disease Control have placed *E. coli* O157 as a category B bioterrorism agent (www.bt.cdc.gov/agent/agentlist-category.asp#b), and this placement has resulted in increased resources for surveillance and research in the recent past.

Diagnostics for *E. coli* O157

Detection of *E. coli* O157 in laboratory and field settings is a critical priority for *E. coli* O157 research and many exciting advances have been seen. Prompt laboratory diagnosis can translate into more efficient outbreak intervention and improved patient care (92). For example, the efficient diagnosis of initial cases in the 1993 Washington outbreak resulted in the recall of significant quantities of ground beef and prevented an estimated 800 additional primary infections (5).

Although several molecular-based diagnostic tests will be discussed in this section, the mainstay in clinical microbiology laboratories for the diagnosis of *E. coli* O157 continues to be routine culture of stool specimens. CDC recommendations provide for routine culture of bloody diarrhea specimens with the SMAC media (16). SMAC (Sorbitol-MacConkey) media is a selective media for the presumptive diagnosis of *E. coli*

O157 (81). This media preparation takes advantage of the fact that ≥ 80% of *E. coli* are sorbitol fermenters (50), whereas *E. coli* O157 is known to ferment sorbitol slowly or not at all (101). Colonies not fermenting sorbitol (presumptive *E. coli* O157) appear colorless on this medium (81). However, this test lacks sensitivity as there is no known direct genetic linkage coupling Shiga toxin production and the inability to ferment sorbitol (92). Also, sorbitol-fermenting *E. coli* O157 strains have been documented (54, 74), and these isolates would be incorrectly diagnosed on SMAC media.

Modification of SMAC media to improve selectivity has been accomplished. A common modification is the inclusion of cefixime and/or tellurite (potassium salt) to yield media known as C-SMAC, T-SMAC, or CT-SMAC (41, 92, 142). Cefixime is a cephalosporin antibiotic advantageous to selectivity as it is more inhibitory to *Proteus sp.* than *E. coli* organisms (19). Potassium tellurite is advantageous for selection as most *E. coli* O157 strains possess one or multiple copies of a tellurite resistance genetic island (124). Another alternative to improve selectivity is to use the fluorogenic substrate 4-methylumbelliferyl-B-D-glucuronide (MUG) incorporated into agar media. Most strains of *E. coli* O157 and other enterohemorrhagic strains do not produce β-glucuronidase and cannot cleave MUG. Most other *E. coli* do produce the enzyme, cleave the MUG substrate, and are fluorescent with 366nm UV light (50, 127).

An extensive body of literature has been published on utilization of genetic sequences for detection. These methods usually utilize the polymerase chain reaction and are highly sensitive and specific (92). Because of their importance in pathogenesis, detection of one or both Shiga toxin genes (*stx*1 and *stx*2) has received much focus. An early technique utilized a single degenerate primer pair to amplify both *stx*1 and *stx*2

genes (68), but now two primer sets are usually utilized and analyzed in tandem (92). Detection of O-antigen biosynthesis genes specific to *E. coli* O157 is utilized with success. Study authors advocated utilizing PCR coupled with primary enrichment protocols to rapidly and specifically identify *E. coli* O157 (82). Genes composing the locus of enterocyte effacement (LEE; *eae* genes) are additional attractive genetic amplification targets. Here, detection is of the intimin gene product that, problematically, is common to EHEC and EPEC. However, the 3' end of the *eae* is highly divergent among EHEC and EPEC strains (139), thereby allowing *eae* PCR primers specific for *E. coli* O157 to be designed and used to detect EHEC specifically (43). Finally, genetic content of the large virulence plasmid is unique and restricted to *E. coli* O157 and other EHEC (92). Specific primers and a PCR protocol were published to take advantage of this diagnostically-specific genetic restriction (40).

Although much of the PCR-based diagnostic testing is directed toward rapid human diagnostics, the technique is utilized in pre-harvest and post-harvest food safety surveillance. PCR-based testing has been specifically adapted to bovine *E. coli* O157 shedding surveillance (100) and to monitor for contamination of ground beef (42).

Many immunoassays are currently widely available for the detection of *E. coli* O157. Most developed assays are directed at the detection of H antigens, O antigens, or Shiga toxins (92) and the latter two are discussed here.

O antigen detection for EHEC in immunoassay format is almost exclusively restricted to detection of O157. This is a limitation because such testing can not detect representatives of the numerous, presumptively minor, other EHEC serotypes. *E. coli* O157 antisera have been incorporated into ELISA kits, latex agglutination kits, and other

kits (92). ELISA testing is utilized to directly detect O157 antigen in stool samples. In one study, the ELISA kit utilized was more sensitive (91.2% vs.82.4%) than and similarly specific to SMAC plating coupled to immunofluorescence microscopy (104). Latex agglutination kits for confirmation of *E. coli* O157 are available from numerous suppliers, and three of them were evaluated in one study. In this study, 159 strains of *E. coli* were analyzed and sensitivity and specificity were both 100% when compared to internal reference sera (119). Any immunoassay developed with monoclonal antibodies to the O157 O antigen could suffer from cross-reactivity issues because *E. coli* O157 has identical or cross-reactive epitopes with *E. coli* O44 and O55 (118), *Brucella abortus*, *Salmonella* O30 strains, (33) *Yersinia enterocolitica*, *Citrobacter freundii*, *Citrobacter sedlakii* (130), *Escherichia hermannii* (111), and some non-O1 strains of *Vibrio cholera* (137).

Shiga toxin immunoassays are available to detect either Shiga toxin-1 or Shiga toxin-2. Two commercially available tests evaluated in the literature are the Premier EHEC Test (Meridian Diagnostics) and the Duopath Verotoxin Test (Merck, Germany) (71, 103). The Premier EHEC test is an ELISA format and performed well in one study comparing sensitivity and specificity to SMAC agar (71). The Duopath test is also in ELISA format, and when compared to Premier EHEC as a gold standard, it exhibited 100% specificity and sensitivity (103).

Immunomagnetic separation is a technique based upon commercially available paramagnetic particles (Dynal) which are derivatized with O157 monoclonal antibodies. Immunomagnetic separation with these particles has been successfully used to detect *E. coli* O157 in food (136, 138), water (138), and cattle feces (20). In the detection of *E.*

coli O157 from cattle feces, Chapman, et al showed an approximately 100-fold increase in sensitivity over conventional culture methods (20). It has also been shown to be highly sensitive in the detection of *E. coli* O157 in human feces (24).

As an alternative to antibody-based techniques, aptamers have been proposed to be developed and utilized in diagnostic testing for infectious agents (63), including *E. coli* O157. Aptamers are DNA or RNA oligonucleotides that bind a target of interest on a physicochemical and electrostatic basis (58), in contrast to traditional usage of oligonucleotides as hybridization probes. Here, in addition to a genotype (which is only meaningful to the accurate reproduction of the probe), the aptamer possesses a phenotype in its exhibition of affinity and selectivity for targets. An aptamer selected to bind thrombin is perhaps the most successful and certainly most-studied in this emerging class of biomolecules (55). Aptamers are selected from a complex, synthetic, oligonucleotide pool by an iterative process known as SELEX (systematic evolution of ligands by exponential enrichment) (46). Each iteration (divided into "rounds") results in affinity partitioning of winning ligands, which are then amplified via PCR and subjected to subsequent rounds. To date, little has been published in the area of aptamer selection for bacterial targets except for the preliminary reports on aptamers that bound the anthrax toxin (11) and *E. coli* O111 (36). However, if aptamer selection for bacterial targets is increasingly successful, their binding properties and other advantages will make them a useful alternative to antibodies in the future.

Other testing modalities not addressed include serology, which is not frequently used to diagnose infection of diarrheagenic *E. coli* strains other than EHEC. Serology would suffer from the potential cross-reactivity of O antigen epitopes described above.

Still others include quantum dots coupled to immunomagnetic separation (120), time-resolved fluorescence(140), fluorescence polarization (98), flow cytometry (78), and others.

Conclusions

Although *E. coli* O157 is conventionally regarded as an emerging infectious disease agent, it is perhaps more appropriately considered to be an established infectious disease as the pathogen is routinely recognized in surveillance studies, is routinely screened for in human fecal analyses, and is the subject of comprehensive research endeavors. Areas of active investigation in the near future will focus on interventional strategies in cattle to address pre-harvest food safety and non-antimicrobial strategies to prevent or reduce disease in man.

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Chapter 3

Streptomycin-treated Adult Cattle Model for *E. coli* O157 Colonization

Abstract

The gram-negative bacterium *Escherichia coli* O157 causes bloody diarrhea and potentially fatal sequelae in man. Cattle, the recognized primary reservoir, are resistant to the disease, but shed the pathogen in their feces which contaminate food products. Modeling the colonization of cattle with *E. coli* O157 is important to understand the host-pathogen relationship, but many current approaches are only moderately reproducible, especially in adult cattle, from which bovine-derived food originates. Therefore, a highly reproducible *E. coli* O157 colonization model effective in adult cattle was developed in this study. Five yearling cattle were surgically cannulated in the duodenum, were treated daily with streptomycin via the duodenal cannula prior to and during experimental colonizations, and were colonized with 10^{10} cfu/animal of a streptomycin-resistant *E. coli* O157 via the duodenal cannula. Colonization of rectal mucus and shedding in feces was monitored. Streptomycin treatment benefitted the experimental colonization when compared to untreated controls during initiation (days 0-6) and early maintenance (days 7-12) of colonization. Chemotherapeutic elimination of the colonizing strain followed by five repeated colonizations in the same animals was successful. Culture positivity at 7 days and 12 days was 100% and 84%, respectively, across all animals and trials. Animals were euthanized following the final colonization trial and quantitative localization of *E. coli* O157 was assessed temporally, with highest magnitude recovery occurring within terminal rectal mucus. The described model is highly reproducible and is novel with respect to streptomycin treatment, usage of duodenal cannulas, and repeated colonizations of the same animals.

Introduction

The gram-negative bacterium *Escherichia coli* O157 is an intestinal pathogen of man that causes hemorrhagic colitis and the occasionally fatal sequela known as hemolytic-uremic syndrome (21, 25). Since 1982, when *E. coli* O157 was first associated with human disease following consumption of undercooked hamburger (29), the epidemiological link to food contamination, especially ground beef, has become established. Contamination of ground beef and other food products, including leafy vegetables, processed meats, and milk, usually occurs via exposure to bovine feces (reviewed in (21)). Indeed, the bovine intestinal tract is recognized as the primary reservoir of this pathogen, and yet, a comprehensive understanding of this host-pathogen interaction is still lacking. Prior studies demonstrated variability with respect to occurrence, magnitude, and duration of *E. coli* O157 shedding by cattle in field and experimental situations. Because such variability suggests that unknown host and/or environmental factors are at play, modeling *E. coli* O157 colonization in cattle is valuable to the improved understanding of colonization and in the evaluation of pre-harvest food safety interventional strategies.

Cattle are recognized as the primary reservoir of *E. coli* O157. In the natural setting, factors impacting initial colonization of the naïve animal are unknown, but horizontal transmission is presumed and has been observed experimentally (10, 30). Sources implicated in potential inter-animal transmission include feces (10, 30), as well as water sources (10), hides, and the oral cavity (19). Upon infection, age-related differences in pathogenicity are observed experimentally. Neonatal calves (≤ 3 weeks) exhibit diarrhea and attaching/effacing histopathology that is most severe in the youngest

(6). *E. coli* O157 pathogenicity is usually not observed in adult cattle (4, 6, 14, 30), but conflicting studies report the occurrence (7) or the absence (4) of pathogenicity of *E. coli* O157 following experimental colonization in young calves. Establishment of experimental colonizations routinely requires inocula $>10^7$ cfu/animal (4), and many studies utilize $\geq 10^{10}$ cfu/animal to colonize cattle (4, 14, 30). Higher colonization doses result in more consistent and persistent colonizations (30). One group has speculated that, if experimental shedding data mimics the field observations, then heavy fecal contamination (≥ 10g) during high shedding periods is required for propagation of colonization within a herd (4). Another group has hypothesized that, among *E. coli* O157-positive herds, a small number of "supershedders" enhance transmission within a herd (23).

Prevalence of *E. coli* O157 colonization within herds and individual animals has been extensively studied. Within herds, *E. coli* O157 is widespread in dairy and beef operations (27). Recent herd studies have reported a point-in-time prevalence ranging from 63% (5) to 100% (31). Individual animal fecal prevalence in recent studies has ranged from 7% in a range calf survey (20) to 28% in a feedlot survey (9). Yet, when hides and the oral cavity were sampled in addition to feces in a feedlot survey, prevalence increased to 96%, indicating that epidemiologic surveys in which only feces were sampled may have underestimated prevalence (19). Other factors under investigation that impact prevalence include seasonality, with summer months being the recognized period with higher shedding (reviewed in (27)), and diet, with hay (16, 35) or grain (8) feeding favoring the colonization of *E. coli* O157 or generic, acid-resistant *E. coli*, respectively. Therefore, *E. coli* O157 shedding in cattle appears to be intermittent within a small

proportion of cattle within a herd, and factors impacting recrudescence of infection are not well understood.

Contaminated ground beef is recognized as the primary vehicle for *E. coli* O157 infection in man (21). In the U.S., per capita retail beef consumption in 2002 was 67.5 pounds (34), a significant portion of which is ground beef originating from adult cattle. Between January and November 2004, greater than 30 million adult cattle were slaughtered in the U.S., while 768,000 (39-fold fewer) calves were slaughtered. (22) Because adult cattle represent the large majority of cattle slaughtered and most ground beef is derived from adult cattle, adult cattle are likely the primary source of contaminated ground beef. Further, both beef and dairy cattle spend the majority of their lifespan as post-weaning adults instead of the neonatal or calfhood stage. Therefore, special attention to modeling adult colonization by *E. coli* O157 is favored over modeling colonization in young calves.

Young calves and adult cattle have been successfully utilized in *E. coli* O157 colonization models; however, key differences exist. In a study in which both young calves and adults were colonized and assessed by fecal shedding, the adult colonizations were more short-lived and of lower magnitude when compared to the young calves (4). Low-level, short duration shedding has also been observed by others colonizing adult cattle (14, 30) and is also consistent with what is seen in the field (38). The reasons for differing shedding patterns between adults and young calves are thought to be due to diet, age-related differences in rumen function, or an immune response. However, a study of serological responses of cattle to *E. coli* O157 cast some doubt on the impact of the immune response upon *E. coli* O157 carriage in cattle. In that study, there was no

correlation with the serological titer and elimination of the pathogen nor with protection from re-infection (18).

The primary objective of this study, the development of a colonization model in adult cattle to assess lower gastrointestinal factors influencing initiation and persistence of *E. coli* O157 colonization, was framed by several conceptual issues. First, establishment of such colonization was explored and proposed in the context of Freter's nutrient-niche hypothesis. Next, persistence of the colonization and consistency of results were identified as significant problems. Finally, factors impacting the cost of experimentation with adult cattle were deemed significant.

The large intestinal microflora is composed of hundreds of bacterial species, the majority of which are anaerobic. This microflora is characterized by stable coexistence, with each population controlled by competition for limiting nutrients. By implication, this microflora is resistant to "invading" species, unless the microflora is perturbed by an exogenous factor or the "invader" can more successfully compete for a given nutrient (11). This, the essence of Freter's nutrient-niche hypothesis, has not previously been addressed in established bovine *E. coli* O157 colonization models. However, evidence supporting the hypothesis was demonstrated with a murine *E. coli* O157 colonization model. In this model, mice were colonized with streptomycin-resistant *E. coli* O157 strains and were treated continuously with oral streptomycin (36). Streptomycin, an aminoglycoside antibiotic with poor oral absorption, is satisfactory for the treatment of susceptible, gram-negative enteric flora (17). In the murine model, streptomycin treatment effected a perturbation of the native microflora by decreasing the facultative anaerobic bacteria from 10^8 cfu/g feces to 10^2 cfu/g feces, allowing the "invading" *E. coli*

O157 strains an opportunity to occupy a niche (36). On this basis, we proposed a similar strategy of streptomycin treatment in the development of the model described herein. However, streptomycin could adversely affect rumen microflora if administered per os. The rumen microflora is composed of anaerobic and facultative anaerobic flora (as well as protozoa), and complex and interacting factors of this polymicrobial community control normal digestive and fermentation processes (13). To the extent streptomycin would affect rumen microflora and function, adverse clinical side effects such as indigestion and anorexia (13) could affect the study. Therefore, administration of streptomycin via a surgically-placed duodenal cannula as an alternative to oral administration was investigated.

Duodenal cannulas permit diversified approaches to placement of analytes and/or procurement of samples based upon a physical segregation of the complex and voluminous ruminant gastrointestinal tract. Such cannulas are routinely placed and stably maintained for up to two years (15). A duodenal cannula has been used in *E. coli* O157 bovine colonizations, but in that study, it was used only for sampling (14). The employment of a duodenal cannula in a streptomycin-treated adult cattle model could provide two key advantages. First, it could provide a means for post-gastric administration of streptomycin, and second, it could provide the mode of administration of the experimental bacterial bolus. The possibility of streptomycin side effects would be minimized or eliminated because normal ruminant digestive processes limit duodenal-abomasal and abomasal-omasal reflux with sphincters (3).

An accurate assessment of colonization consistency and persistency should incorporate not only numerical data, but also the phases of colonization, which can be

divided into three functional stages: 1) initiation (days 0-6), 2) early persistence or maintenance (days 7-14), and 3) long term persistence (>14 days). Support for this concept in this field is documented by Hovde and colleagues (28, 30), in which culture positivity for one week or less indicates the lack of a stable association between the host and bacterium. Colonization consistency, especially in the early maintenance phase, appears to be a significant problem in bovine *E. coli* O157 colonization models. In a selective review of three studies in which cattle were orally or ruminally colonized with 10^{10} cfu/animal, there were 6 out of 10 (30), 8 out of 12 (14), and 9 out of 9 (4) culture positive animals at days 12, 12-14, and 14, respectively. Furthermore, in the former two studies (14, 30) in which animal-specific shedding levels were reported, culture-positive animals at 25 days post-infection and beyond were the same animals positive at days 12-14 the majority of the time. Although this meta-analysis is not statistically validated, visual inspection of these data sets indicates that culture positivity at days 12-14 is a strong predictor of long term persistence. Improved consistency and persistence was observed in a novel rectal administration model (30). In this study, 8 of 8 animals inoculated rectally with 10^9 cfu were positive at day 14 and most were persistently culture positive through 6 weeks (30).

Although prioritizing adult cattle *E. coli* O157 colonization models over young calf models has been justified, it does not come without concomitant problems, the most significant of which is cost. The purchase price and per diem maintenance costs of adult cattle are significantly higher than analogous costs with young calves, and such expense usually has the effect of sample size restriction. Because sample sizes are generally smaller, this places greater emphasis upon consistency to achieve appropriate statistical

power. It also focuses greater interest on the possibility of repeated colonization of adult cattle for use in multiple colonization trials. This is particularly important with respect to the use of duodenally cannulated cattle as described herein. Single repeat colonizations of cattle with *E. coli* O157 have been demonstrated in two studies (4, 14). However, these repeat colonizations relied upon natural elimination of the pathogen (4) and/or a lengthy interval between colonizations (4, 14). Adapting a colonization protocol to the chemotherapeutic elimination of the organism and decreasing the time interval between colonizations minimizes experimental costs for use of adult cattle for colonization studies.

The terminal rectal mucosa of cattle has recently been identified as a site of tropism for *E. coli* O157 colonization (23). This known tropism provides an important sampling site for colonization or surveillance studies. In a more recent study, researchers found that a rectoanal mucosal swab from this site exhibited superior sensitivity over other samples when monitoring colonization (28). In the model described herein, acquired samples included feces as well as rectal mucus. This latter sample is analogous to the rectoanal mucosal swab (30). Sampling of mucus was further based upon prior research that demonstrates that *E. coli* and other microflora grow preferentially in mucus, rather than feces (11, 37).

In this study, we present an *E. coli* O157 colonization model effective in adult cattle. The advantages of novel features of this model are discussed. In the development of this model, we investigated the following: 1) the success of colonization via a duodenal cannula, 2) the effect of streptomycin treatment upon colonization success, 3)

the difference between fecal shedding and rectal mucus recovery, and 4) the possibility of using the same animals for repeated colonization trials.

Materials and Methods

Research Animals/Environment. Five mixed-breed beef cattle (4 steers, 1 heifer) were purchased at approximately 227kg body weight and allowed to acclimatize to their initial environment, the Oklahoma State University Veterinary Teaching Hospital. Duodenal cannulas were placed 6cm caudad to the pyloric junction and anchored and exteriorized between the last two ribs following a published technique (33). Cattle were maintained on injectable broad-spectrum antibiotics for one week and daily cannula site Betadine rinses for two weeks. During experimental colonizations, animals were transferred to and maintained in a BSL-2 large animal facility; each animal was placed in a pen alone. Researchers wore BSL-2 personal protective equipment; additional animal care personnel wore face shields and masks during high pressure spray cleanings. Pens were scraped daily; feces were collected and treated with household bleach (1 part bleach to 9 parts fecal matter) for a minimum of four hours prior to disposal in the sewage system. Pens were spray-washed once daily with an antimicrobial mixture. Cattle were fed twice daily a pelleted total mixed ration composed of corn, ground alfalfa hay, and mineral supplements. Water was provided free choice. At termination of experiments, cattle negative by enrichment culture for *E. coli* O157 were temporarily transferred to an outdoor facility and maintained similarly while the BSL-2 facility was cleaned.

Bacterial strain and inoculation preparation. *E. coli* O157 EDL 933 used was streptomycin-resistant and nalidixic acid-resistant and was provided by Dr. Paul Cohen of the University of Rhode Island. Bacteria were grown overnight in 250mL of Luria broth (Sigma) to an OD_{600} of 1.5-1.6. For inoculation, 100mL of cell suspension was pelleted and washed three times with PBS and finally resuspended in 50mL of PBS. Ten-fold

dilutions of the final inocula were prepared and plated with spreading on selective SMAC media (described below) to accurately assess the inoculation level.

Inoculation, Streptomycin treatment. For inoculation, an EMT endotracheal tube (Bound Tree Medical, Dublin, OH) was placed in the opened cannula followed by cuff inflation for sealing. Each animal received 10mL of the prepared inoculum via the medicinal access port of the EMT tube followed by 5mL flush of PBS via the same port followed by a 40-120mL flush of PBS via the main lumen of the EMT tube. No loss of the inoculating bolus was ever observed. For cattle on daily streptomycin treatment, a 1 g/mL solution of streptomycin sulfate (Sigma) in water was prepared and dosed via the cannula at 15mg per pound of body weight. On the morning of colonization initiation, streptomycin treatment was delayed approximately 12 hours until the evening sampling. Cattle receiving daily streptomycin began the treatment regimen three days prior to colonization and continued until the colonization was terminated.

Sampling/Media. Samples of feces and rectal mucus were collected at 0, 0.5, 1, 2, 3, 5, 7, 9, 12, 15, and 18 days. Fecal samples ranged from 5-20g and were collected either as free-catch samples or directly from the terminal rectum. Feces was weighed and suspended at a 1:10 ratio in 1% tryptone (Becton-Dickinson) and placed on ice for one hour prior to plating. Mucus samples were acquired from the rectum via palpation; briefly, following manual evacuation of the feces, mucus was collected from the ventral half of the most caudal 30 cm of the rectum by scraping with the lid from a sterile syringe casing. Mucus was scraped until 200-500mg was acquired; it was suspended at a 1:2 ratio in 1% tryptone, vortexed vigorously, and placed on ice for one hour prior to plating. Feces and mucus samples were processed into 10-fold dilution series by diluting 100μL

of initial sample into 900µL of sterile PBS, and 100µL of each dilution was placed onto appropriate agar media and evenly spread on the plate with an alcohol-flamed spreading tool. Bacterial plates were incubated 12-18 hours at 37°C prior to assessment and counting. Diluted feces and mucus samples were plated onto two types of media:1) Sorbitol-MacConkey (SMAC) agar (Remel) and 2) SMAC agar with the following antibiotics added: streptomycin at 40µg/mL and nalidixic acid (Sigma) at 50µg/mL. Sorbitol-negative (white) colonies were enumerated daily and, where colonial morphology was unusual, subjected to confirmatory testing with a commercially available immunoassay (O157 Immunocard STAT; Meridian Biosciences). Enrichment cultures accompanied all daily samplings. Briefly, 100-150µL of primary samples were placed in TSI broth (Becton-Dickinson) with 40µg/mL streptomycin and grown at 37°C for 16 hours with shaking (210 rpm). If primary samples were negative, the enrichment samples were subjected to dilution series and plated similarly. Enrichment samples yielded qualitative data of presence (enrichment positive) or absence (enrichment negative). For purposes of computation of means, enrichment data were transformed to a semi-quantitative estimate of 50 cfu/g feces or 10 cfu/g mucus, exactly 50% of the lowest limit of detection for each of the sampling regimens. Rectal biopsies were taken at days 0, 3, 9, and 15 of each colonization trial. Biopsies were procured in the terminal 3cm of rectum via an equine uterine biopsy instrument and fixed in 10% buffered neutral formalin. Tissues were routinely processed, paraffin embedded, sectioned, and stained with hematoxylin and eosin, and examined by light microscopy.

Colonization termination. At the conclusion of an experiment, cattle were treated with two antibiotics to which the colonizing strain was determined to be susceptible.

Neomycin (Biosol; Upjohn) was administered daily for three days via the cannula at 10 mg/kg. Ceftiofur sodium (Naxcel; Pfizer) was administered subcutaneously daily for three days at 0.5mg/kg. Enrichment cultures of the last day of such treatment and two subsequent days were grown, plated, and assessed to determine reliable elimination of the colonizing strain.

Terminal Study/Euthanasia and Necropsy Methods. In the final colonization trial, 3 groups of animals were colonized and then euthanized: Group I, euthanized at one-day post-colonization (n=1), Group II, euthanized at three days post-colonization (n=2), and Group III, euthanized at seven days post-colonization (n=2). To prevent unintentional early colonization, animals were penned according to their planned date of euthanasia and separated by a minimum of 20 feet. During this terminal study, steer #3 (assigned to group II) experienced sudden, severe, free gas bloat on the third post-colonization day that resulted in death before treatment could be administered. This was presumed to be due to, in part, its predisposition to bloat and its dominance during feeding with the co-penned smaller steer. The other four cattle were sedated with xylazine (Rompun, Bayer) and killed with a captive-bolt gun. Necropsy followed a standard protocol and was performed by an American College of Veterinary Pathologists board-certified pathologist. Contents from the rumen, abomasum, duodenum, ileum, cecum, proximal colon, and feces were diluted and plated identically for that described for feces above. Gall bladder contents and mucus from duodenum, ileum, cecum, proximal colon, and terminal rectum were diluted and plated identically to that described above for rectal mucus. Tissues examined by light microscopy were fixed in 10% buffered neutral

formalin, processed through graded alcohols, sectioned at 5µm, stained with hematoxylin and eosin, and coverslipped.

Statistical Methods. Two groups were compared for the effect of daily streptomycin treatment. A fair toss of the coin determined the unequal initial assignments of animal numbers per group (streptomycin treated n=3; no treatment n=2). Animals assigned to each group were determined by chance. In the second phase of the experiment, the groups and assignments were reversed, creating a balanced, paired experiment. The Wilcoxon-signed ranks test, a non-parametric statistical test, was utilized in a one-tailed fashion to test for significant differences, with $P<0.05$ deemed significant.

Results

Animal health. All animals remained healthy throughout the course of the five repeated colonizations. Occasional animals would experience transient, moderate bloat during the termination of colonization trials. This was presumed to be secondary to the effects of the two antibiotics given. The cannula of one steer became broken and displaced during the course of one experiment and was replaced. Sporadic diarrhea was noted in variable animals but did not correlate with any colonization event. All cattle experienced moderate average daily gains of 0.71 +/- 0.05kg over the 221 days of these studies. No attaching and effacing histopathology was recognized in any cow during the course of the colonizations or the necropsy study.

Streptomycin treatment benefited colonization success. To test the effect of streptomycin upon experimental colonization, five study animals were randomly assigned to a streptomycin-treated group (n=3) and a non-treated control (n=2) and colonized per cannula identically. After 18 days of sampling, the colonization was terminated with antibiotics, a "washout" period ensued, and the group assignments were reversed, creating a paired experiment. Figures 1A and 1B summarize the recovery of the input strain in a time-relative fashion for feces and mucus, respectively. For analysis of the effect of streptomycin, colonization phases were defined as initiation (days 2, 3, and 5), early maintenance (days 7, 9, and 12), and late maintenance (days 15 and 18). Streptomycin treatment favored the magnitude of shedding in mucus during initiation ($p<0.01$) and early maintenance ($p=0.02$), but not late maintenance ($p>0.10$). Streptomycin treatment favored the magnitude of shedding in feces only during initiation

($p<0.01$) with levels in early maintenance and late maintenance deemed insignificant ($p>0.05$ and $p>0.15$, respectively).

Greater persistence of recovery and increased magnitude of shedding was seen in mucus samples instead of feces samples. Feces and rectal mucus samples were procured and subjected to dilution and plating at every sampling day. Greater persistence of recovery was seen in mucus than feces during the two-phased trial that tested the effect of streptomycin (Figures 1A and 1B). In determination of persistence, days of persistence was defined as the last sample day positive unless a minimum of two intervening samples were negative between two positive samples. The latter situation was negated as it possibly implied a re-infection from another animal. When cattle were streptomycin-treated, *E. coli* O157 persistence was 16.8 +/- 2.7 days in mucus and 14.0 +/- 4.6 days in feces. Likewise, when not streptomycin-treated, *E. coli* O157 persistence was 15.6 +/-2.5 days in mucus and 9.8 +/- 5.4 days in feces. In analyses of the other three trials in which streptomycin was in continuous use (Tables 1 and 2), the input strain was recovered in mucus for 13.13 +/- 3.25 days and in feces for 10.93 +/- 3.97 days (Wilcoxon-signed rank test: $p\leq0.02$). Higher magnitude of shedding was seen in mucus over feces at day 2 ($p<0.025$), day 3 ($p<0.005$), and day 5 ($p<0.05$), but magnitude of shedding was not significantly different between feces and mucus at days 7, 9, 12, and 15.

Colonization was reproducible and amenable to repetition within animals. Five full-length trials of 15 or 18 days were conducted on the same five animals. Upon examining bacterial recovery from mucus across all five trials, 92% of all samples were positive at day 9, 84% of all samples were positive at day 12, and 72% of all samples were positive

at day 15. A period of time ranging from 6 days to 4 weeks intervened between the termination of one colonization trial and the beginning of a subsequent trial. No diminished colonization magnitude or persistence was seen in any subsequent trial. Indeed, the latter two full-length trials conducted exhibited 100% positive shedding in mucus at day 15.

The lower intestinal tract quickly became the primary site of localization. A sixth and final colonization trial was conducted on these cattle to spatially define colonization sites in a temporal fashion within this model. Cattle were colonized via the cannula and euthanized at 1 day (n=1), 3 days (n=2, 1 euthanized, 1 died), and 7 days (n=2) post-colonization. Following euthanasia (or death, steer #3), gross necropsy was performed, and thirteen samples were procured for primary and enrichment cultures. Significantly, no retrotranslocation of the input strain was detected in the abomasum or the rumen (Table 3). The input strain was recovered from the gall bladder in three of the five cattle. Overall, the highest magnitude of recovery was seen in the rectal mucus samples. However, this was not true for steer #5, which exhibited enrichment positivity in many samples even though it was terminated at only 24 hours post-colonization. This animal received a one-log order of magnitude lower colonization bolus which could explain the reduced recovery. Overall, it was evident that within seven days, the terminal rectum became the primary site of localization with ileal mucus being a secondary site of recovery. It is also evident that the colonizing bolus was cleared from the duodenal cannulation site very quickly, being only detected in the animal sacrificed at 1-day post-colonization. Histopathologic samples of various gastrointestinal and gall bladder sites showed no evidence of attaching and effacing histopathology.

Discussion

A primary goal of developing an improved bovine colonization model for the study of *E. coli* O157 is a model that consistently yields reliable and reproducible establishment of colonization. The streptomycin-treated cow model described here exhibits several positive attributes consistent with this goal. Additionally, compared to other bovine *E. coli* O157 colonization models, the model described here is novel in three respects: 1) colonization initiation via the duodenal cannula, 2) facilitation of niche occupation by the continuous streptomycin treatment, and 3) repeated use of the same cattle for repeated colonization experiments.

The most significant positive attribute of the streptomycin-treated cow *E. coli* O157 colonization model is its reliability and reproducibility. Adult cattle are more difficult to experimentally colonize than calves, and this difficulty is observed in the areas of shedding levels and proportions of positive study animals after one week post-inoculation (1, 4, 14, 24, 30). The shedding levels of these streptomycin-treated cattle at 7 and 15 days post-inoculation (10^1 to 10^4 and 10^1 to 10^3, respectively) were similar to those found in other published adult cattle colonization models. Further, the streptomycin-treated cow model appears comparable or slightly superior to other adult models with respect to proportions of positive study animals after one week of colonization. In four of five trials, 100% of the cattle in this model were actively shedding at day 12. In the remaining trial, 100% were actively shedding through 7 days. Also, colonization persisted in all five animals for 15 days in at least one of the trials. For comparison, Cray and colleagues showed that nine of nine orally colonized adults were actively shedding at 14 days (4). Experiments performed by Sheng and colleagues

showed that six of ten orally colonized cattle were positive on day 12, but eight of eight rectally colonized cattle were positive at day 14 (30). Additionally, twelve of eighteen orally dosed adults were positive at day twelve to fourteen in a study by Grauke and colleagues (14). Further difficulty in adult colonization was documented by Ohya (24) and Buchko (1) in which 4 of 8 calves and 9 of 18 yearling steers, respectively, were fecal culture negative by 7 and 9 days, respectively.

Repetitive colonizations of the same individual animals were demonstrated here. We successfully colonized the same five animals five (six, including the terminal experiment) times without significant variations of shedding levels and proportions colonized. Repetitively colonizing the same animals had been demonstrated before (4, 14), and in those studies, cattle were reinoculated once instead of the five times demonstrated here. In one study (4), there was a significant drop in the persistence of colonization upon re-inoculation, but the other study demonstrated no differences in shedding patterns following re-inoculation (14). Furthermore, re-inoculation in those studies (4, 14) followed a lengthy interval to allow for the natural elimination of *E. coli* O157. In contrast, the strain and methods employed herein allowed for reliable chemotherapeutic elimination of *E. coli* O157 followed by re-colonization without diminished success. The measured lengths of persistence in the other studies (4, 14) extend beyond the 15 to 18 days measured here, so a direct comparison is not possible. Regarding the immune response of cattle to *E. coli* O157, Johnson and colleagues demonstrated that the serological responses of cattle were not correlated with elimination or reinfection with *E. coli* O157 (18). Their findings implied that persistence and reinfection in the face of an immune response contributed to herd persistence and that

vaccine-based interventions could be of questionable efficacy (18). The repetitive colonizations demonstrated here seem to add additional weight to that argument.

Administration of the colonizing bolus via the duodenal cannula conferred the principal advantage of avoiding ruminal dilution of the bolus. Whether or not the traversing of ruminal contents (estimated to be 100-120L in these animals) impacts colonization success is not known and was not tested here. However, the data acquired from the streptomycin-treated cows are more similar to the rectal administration model (30) than to the oral and/or ruminal colonization models (4, 14, 30). The results of this study and the rectal colonization model seem to indicate that experimental colonization success is negatively impacted by transit of the upper gastrointestinal tract. To further support this idea, conditions simulating rumen fluid of well-fed animals have been shown to negatively affect growth of *E. coli* O157 (26). Also relevant to the discussion is the bypass of the acidic abomasal environment in the streptomycin-treated cow model. In *Salmonella typhimurium*, adaptation to acidity influences the expression of full virulence (reviewed in (12)). A similar adaptive response may occur in *E. coli* O157 in the bovine gastrointestinal tract, and this model would provide the means to test such a hypothesis.

Streptomycin effected a dramatic reduction of susceptible facultative anaerobic bacteria by six log-orders of magnitude in the murine *E. coli* O157 colonization model (36). However, although the results indicated a beneficial effect of streptomycin upon *E. coli* O157 colonization success, such a magnitude of reduction was not appreciated in the streptomycin-treated cattle, where two log-orders of magnitude (10^6 to 10^4 cfu/g) reduction was usually observed (data not shown). The reasons for this difference could be: 1) a higher proportion of naturally streptomycin-resistant microflora in cattle than in

mice, 2) the cumulative acquisition of resistance by facultative anaerobes in the present study, 3) the much larger volume of the bovine intestine and its subsequent dilution of the antibiotic, or 4) the acidity of the bovine rectum diminishing the efficacy of the drug (17).

In the terminal colonization trial, site-specific localization and magnitude of *E. coli* O157 colonization was assessed. Most significantly, the greatest magnitude of bacterial recovery, on average, was in the terminal rectal mucus, lending additional support to the findings of Naylor and colleagues that the terminal rectal mucosa is a site of tropism for *E. coli* O157 (23). However, the consistent recovery of *E. coli* O157 from ileal mucus or feces was partially at odds with the Naylor study (23) although animals in that study were younger and sacrificed at 3-4 weeks post-colonization. Ileal recovery parameters for these streptomycin-treated cows are most similar to the results in sheep reported by Grauke (14). Both the terminal rectal mucosa and ileum are characterized as being rich in lymphoid tissue, and this feature may be important for the confirmed and suspected areas of tropism for *E. coli* O157. Gall bladder recovery of the input strain was demonstrated in three of this study's animals, a finding that corroborates previous work by Stoffregen and colleagues (32).

In sampling feces as well as mucus scraped from the terminal rectum, it was demonstrated that rectal mucus samples yielded higher magnitudes of shedding during early colonization and also resulted in longer term recovery of the organism. This method of sampling and improved recovery of *E. coli* O157 is broadly similar to the rectoanal mucosal swab culture method described by Rice and colleagues (28). However, the basis for mucus sampling in the present study was the body of evidence indicating that generic *E. coli* resides in the mucus layer of the intestine and metabolizes mucin-

derived sugars (2, 37). Further, in the development of the murine *E. coli* O157 colonization model, Wadolkowski and colleagues demonstrated that murine cecal and colonic mucus supported growth of *E. coli* O157 EDL 933 (36), the strain used in the present study.

The novel methods employed by this *E. coli* O157 colonization model should not be construed as specific for *E. coli* O157 studies. The streptomycin-mediated reduction of competing flora, coupled with bacterial colonization via a duodenal cannula could be beneficial and should be amenable to other bacterial colonization studies in cattle, provided that the input strains are streptomycin-resistant.

Thorough discussion of the model would be incomplete without a discussion of disadvantages. The expense of this model is the chief disadvantage. A principal contributor to the expense – usage of adult animals – is inferred to the goals of the model development and cannot be altered. Other factors contributing to the expense include the cost of cannulation surgery and the antibiotics used to facilitate or eliminate colonizations. Another disadvantage is its labor intensity. The frequency of sample acquisition, the frequency of streptomycin treatment, and the maintenance and protection of the cannulas are chief contributors to the labor intensity of the model. The chief compensation for these disadvantages is the reproducible and repetitive colonizations of animals, with a fully realized goal of decreased animal usage and expense.

This study demonstrated an advance in the understanding of *E. coli* O157 colonization of adult cattle. It demonstrated the reproducibility conferred by novel methods, the effect of reducing competitive flora, and the repetitive colonization of the

same animals. The model should serve as a useful alternative when other investigators seek to advance the understanding of *E. coli* O157 and its bovine reservoir.

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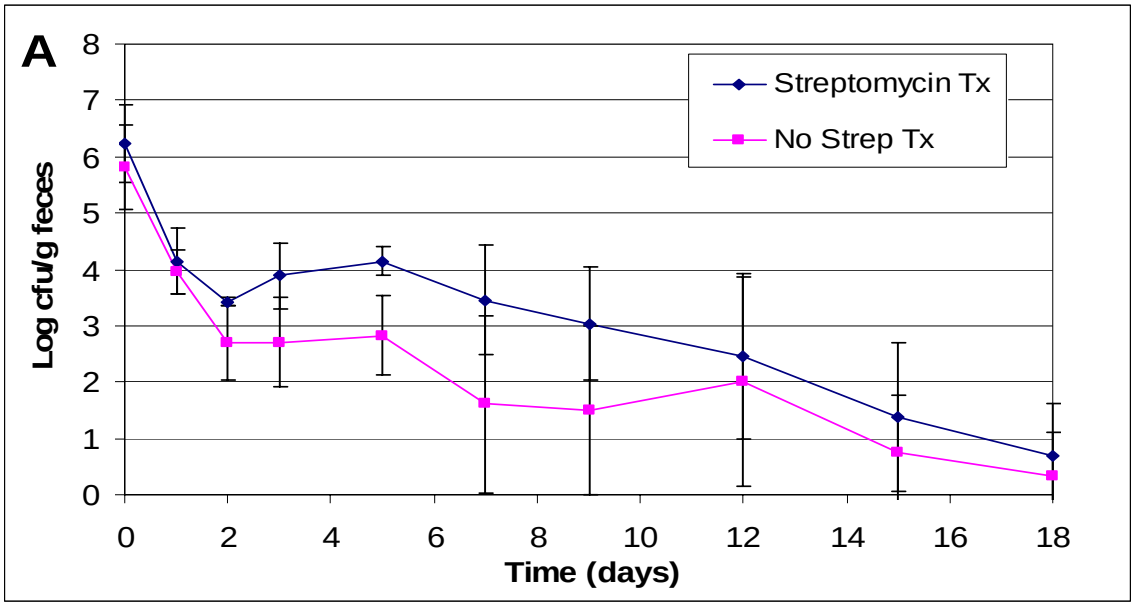
Figures 1A and 1B:



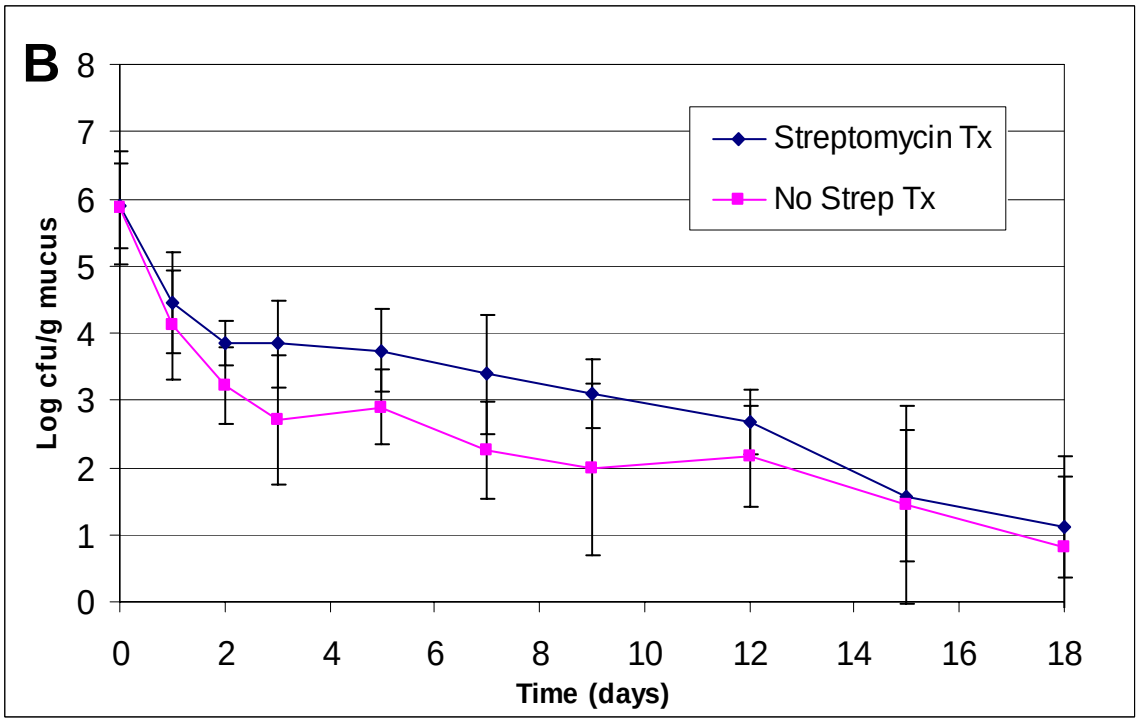


Figure 1. Streptomycin-treated and non-treated animals were colonized with 10^{10} cfu of *E. coli* O157 and samples were taken at the indicated times, homogenized, and plated as described. Enumerated counts were transformed to $\log_{10}$ levels. A) Monocolonization in feces. B) Monocolonization in mucus. Bars represent the standard error of the $\log_{10}$ (cfu/g sample). Value at day 0 reflects samples acquired at 12 hours post-colonization.

Table 1.

TABLE 1. *E. coli* O157 concentrations within feces of streptomycin-treated adult cattle

	Animal	*E. coli* O157 concentration[a] (CFU/g feces) on post-inoculation[b] day:								
	#/Sex	0.5 (12 hrs)	1	2	3	5	7	9	12	15
Trial 1	1/M	5.9×10^7	7.0×10^4	9.0×10^2	2.0×10^2	E+	0	E+	5.0×10^2	0
	2/F	5.8×10^5	1.1×10^4	1.0×10^3	4.0×10^2	E+	E+	0	0	0
	3/M	6.4×10^7	9.4×10^4	2.3×10^4	1.0×10^3	4.0×10^2	0	E+	2.0×10^2	0
	4/M	1.1×10^7	5.0×10^4	3.0×10^2	0	0	0	0	0	0
	5/M	3.9×10^5	7.0×10^4	2.5×10^3	1.3×10^3	7.0×10^2	0	E+	0	0
Trial 2	1/M	2.0×10^5	1.8×10^4	2.4×10^3	1.8×10^3	1.0×10^3	5.0×10^2	E+	1.2×10^3	6.0×10^2
	2/F	1.3×10^6	1.5×10^3	1.1×10^3	6.0×10^2	1.0×10^2	2.0×10^2	2.1×10^3	9.5×10^2	2.5×10^2
	3/M	8.9×10^6	8.8×10^4	1.0×10^3	1.0×10^2	2.3×10^3	E+	0	E+	0
	4/M	5.1×10^5	4.0×10^3	1.0×10^3	1.0×10^2	2.8×10^3	9.0×10^2	2.0×10^2	1.5×10^2	4.0×10^2
	5/M	8.4×10^5	2.2×10^4	2.6×10^3	2.5×10^2	4.5×10^3	3.0×10^2	1.3×10^3	3.5×10^2	9.0×10^2
Trial 3	1/M	3.4×10^6	6.0×10^2	2.0×10^2	2.5×10^2	2.0×10^2	4.0×10^2	3.0×10^2	0	0
	2/F	1.6×10^5	3.6×10^3	1.3×10^3	5.0×10^2	E+	4.5×10^2	E+	1.2×10^3	E+
	3/M	3.9×10^5	5.3×10^3	4.0×10^2	1.0×10^2	E+	2.0×10^2	0	0	0
	4/M	2.4×10^6	1.1×10^3	1.3×10^3	1.0×10^2	E+	E+	0	0	0
	5/M	1.6×10^6	4.0×10^3	1.6×10^3	5.5×10^2	3.5×10^2	1.8×10^3	1.0×10^2	1.1×10^3	0

[a] Values rounded to two significant figures and expressed in scientific notation; E+: positive by enrichment culture only; 0: not detected by primary or enrichment cultures.

[b] Cattle were dosed with 10^{10} cfu of *E. coli* O157 via the duodenal cannula.

Table 2.

TABLE 2. *E. coli* O157 concentrations within rectal mucus of streptomycin-treated adult cattle

	Animal	*E. coli* O157 concentration[a] (CFU/g rectal mucus) on post-inoculation[b] day:								
	#/Sex	12 hrs	1	2	3	5	7	9	12	15
Trial 1	1/M	9.0×10^5	1.0×10^5	5.8×10^4	9.6×10^2	1.0×10^2	1.4×10^2	E+	0	0
	2/F	1.1×10^6	5.6×10^3	4.8×10^2	6.0×10^1	1.8×10^2	E+	0	0	0
	3/M	1.4×10^7	8.2×10^4	9.2×10^3	1.1×10^3	5.8×10^2	5.2×10^2	2.4×10^2	3.6×10^2	5.0×10^2
	4/M	2.6×10^6	3.2×10^4	1.4×10^4	1.5×10^3	2.2×10^2	E+	0	0	0
	5/M	2.3×10^5	3.2×10^4	1.7×10^3	1.4×10^3	1.0×10^2	1.5×10^3	E+	0	0
Trial 2	1/M	4.8×10^5	5.6×10^3	1.1×10^4	2.7×10^3	5.8×10^2	1.4×10^2	2.2×10^2	6.0×10^2	E+
	2/F	2.7×10^6	1.5×10^5	7.6×10^2	1.1×10^3	4.1×10^4	4.2×10^3	4.4×10^2	2.8×10^2	3.8×10^2
	3/M	6.4×10^6	7.8×10^4	3.5×10^4	6.9×10^4	2.9×10^4	8.0×10^1	8.0×10^1	2.3×10^2	3.2×10^2
	4/M	5.8×10^5	4.8×10^3	5.1×10^3	4.8×10^2	3.1×10^3	3.4×10^2	2.0×10^2	5.0×10^1	8.0×10^1
	5/M	3.9×10^5	1.1×10^4	6.8×10^3	1.5×10^3	1.2×10^3	1.0×10^2	3.0×10^2	5.0×10^1	5.6×10^2
Trial 3	1/M	1.3×10^6	4.4×10^3	4.5×10^3	1.5×10^3	1.6×10^3	1.2×10^3	2.6×10^3	3.4×10^2	2.0×10^1
	2/F	3.2×10^5	1.4×10^4	9.6×10^2	1.2×10^3	5.2×10^2	3.8×10^2	E+	1.8×10^2	8.0×10^1
	3/M	1.8×10^5	6.2×10^3	8.4×10^2	7.8×10^2	2.3×10^3	4.8×10^2	3.0×10^1	1.4×10^3	1.2×10^2
	4/M	1.6×10^6	4.2×10^3	8.2×10^3	2.2×10^3	1.7×10^3	6.6×10^2	2.0×10^1	8.0×10^1	E+
	5/M	4.9×10^5	4.6×10^4	3.4×10^3	7.8×10^2	9.2×10^2	7.4×10^2	1.0×10^2	2.0×10^2	8.0×10^1

[a] Values rounded to two significant figures and expressed in scientific notation; E+: positive by enrichment culture only; 0: not detected by primary or enrichment cultures.

[b] Cattle were dosed with 10^{10} cfu of *E. coli* O157 via the duodenal cannula.

Table 3.

TABLE 3. *E. coli* O157 localization by time in the streptomycin-treated cow model

E. coli O157 concentration[a] (CFU/g sample)						
Days Post-Inoculation:		1[b]	3[c]		7[c]	
Sample Site	ID	5/Steer	2/Heifer	3/Steer	1/Steer	4/Steer
Rumen, Abomasum		0, 0	0, 0	0, 0	0, 0	0, 0
Gall bladder		E+	E+	0	0	E+
Duodenum Contents		1.0×10^2	0	0	0	0
Duodenum Mucus		E+	0	0	0	0
Ileum Contents		4.0×10^2	3.0×10^2	2.1×10^3	7.0×10^2	8.0×10^2
Ileum Mucus		E+	2.0×10^1	1.2×10^3	1.0×10^2	1.1×10^3
Cecum Contents		1.0×10^2	0	0	7.0×10^2	E+
Cecum Mucus		E+	E+	4.0×10^1	8.0×10^1	E+
Prox. Colon Contents		E+	1.0×10^2	0	1.0×10^2	2.0×10^2
Prox. Colon Mucus		E+	E+	2.0×10^1	4.0×10^1	6.0×10^2
Feces		E+	2.0×10^2	1.4×10^3	2.0×10^2	1.0×10^2
Rectal Mucus		E+	8.2×10^2	6.0×10^1	4.7×10^3	6.2×10^2

[a] Values rounded to two significant figures and expressed in scientific notation; E+: positive by enrichment culture only; 0: not detected by primary or enrichment cultures.
[b] Steer #5 was dosed with 10^9 cfu of *E. coli* O157 via the duodenal cannula.
[c] All other cattle were dosed with 10^{10} cfu of *E. coli* O157 via the duodenal cannula.

Chapter 4

Role for Sugar Catabolism in the Colonization of the Adult Bovine Rectum by *E. coli* O157

Abstract

The gram-negative bacterium *E. coli* O157 causes human intestinal illness with occasional fatal systemic sequelae. The most important reservoir of *E. coli* O157 is the bovine intestinal tract, and fecal shedding is the usual source of *E. coli* O157 contamination of food. However, comparatively little is known about factors important for its colonization of the bovine intestine. Prior animal colonization models have established a tropism for the terminal bovine rectum and the modest importance of adhesins such as intimin and long polar fimbriae. In this study, we investigate whether a nutritional basis exists for colonization of the bovine rectum by *E. coli* O157. This was investigated by competitively co-colonizing streptomycin-treated adult cows with a wild-type strain of *E. coli* O157 and an isogenic mutant in a catabolic pathway for either ribose, *N*-acetylgalactosamine, or fucose. Strains were streptomycin-resistant and had additional antibiotic selectable markers for the differential enumeration of input strains sampled from terminal rectal mucus. All three catabolic knockout strains exhibited modest to marked colonization defects during initiation (days 0-6). The fucose catabolic knockout exhibited the greatest colonization defect during maintenance (days 7-15), the ribose catabolic knockout exhibited a modest colonization defect during late maintenance (days 12-15), and a colonization defect was not appreciated during maintenance for *N*-acetylgalactosamine. These results support the idea that growth and colonization of *E. coli* O157 in the bovine rectum has a nutritional basis, with a nutrient preference for fucose of highest importance of the three tested.

Introduction

The gram-negative bacterium *E. coli* O157, an intestinal pathogen of man that causes diarrhea, hemorrhagic colitis, and the occasionally fatal hemolytic-uremic syndrome (21), was first associated with human illness in 1982 when an outbreak of diarrhea was associated with the consumption of undercooked, contaminated ground beef (26). Outbreak investigations and research performed since have established bovine-origin food products as the primary vehicle of infection, cattle as the principal reservoir, and bovine fecal shedding as the primary mode of food contamination. Because of the importance of the bovine reservoir and fecal shedding to the disease epidemiology, the colonization of cattle by *E. coli* O157 has been extensively studied (6, 7, 9, 14, 22, 29). Recently, the large intestine (14) and the rectum (22) were identified as sites of high magnitude recovery following experimental *E. coli* O157 colonization of adult cattle. However, the underlying factors impacting bovine colonization by *E. coli* O157 are not known. The steps involved in the colonization of the intestinal mucosa are proposed to include chemotaxis and motility, penetration of the mucus layer, adhesion to mucin-derived oligosaccharides within the mucus layer, adhesion to epithelial surfaces, and growth and catabolism (reviewed in (5, 17)). These factors frame some of the recent research on adhesion and the study on sugar catabolism described herein.

Adhesion of *E. coli* O157 to epithelium has been recently reviewed (32). The outer membrane protein intimin and its translocated intimin receptor Tir, both encoded within the locus of enterocyte effacement (LEE) pathogenicity island, are important mediators of attaching and effacing (A/E) histopathology (6, 9, 10, 21, 34). Intimin is

important in the colonization of neonatal calves (9), young adult cattle (6), sheep (6, 34), and neonatal pigs (10). The importance of LEE was reaffirmed in a recent examination of bovine *E. coli* O157 colonization factors studied by colonizing a library of signature-tagged mutants (11). In conventional pigs, *E. coli* O157 with long polar fimbriae mutations (*lpf*A1 and *lpf*A2) exhibited modest colonization defects and fewer A/E lesions than the wild-type parent (15). The O-polysaccharide side chain of *E. coli* O157 may play a role in the colonization process (23), but is not required for attachment (4). Non-fimbrial adhesins encoded by *tox*B and *efa-1*, potentially important in *E. coli* O157 adherence, remain to be fully functionally characterized (reviewed in (32)).

A role for growth and nutrient catabolism in successful colonization is best explained by Freter's hypothesis, which holds that the 500-1000 different species of the gut microbiota can stably coexist as long as each constituent successfully competes for one or a few limited nutrients, and that their growth rate is at least equal to their intestinal washout rate (3, 5, 13). Nutrient sources include ingested food, shed epithelia, and intestinal mucins (3). Intestinal mucins, macromolecular glycoproteins that comprise the major protein component of intestinal mucus (27, 28), are produced mainly by goblet cells, provide nutrient sources (3, 5) and attachment sites (16) for bacteria, and have gel-forming properties (28) that protect intestinal mucosa (27, 28). Mucins consist of a protein backbone extensively glycosylated by *O*-linked oligosaccharides of marked structural and compositional diversity (27, 28). These oligosaccharides contain five major sugars: L-fucose, D-galactose, sialic acids, *N*-acetylgalactosamine, and *N*-acetylglucosamine (5). Other sugars detected in mucus, originating from sloughed epithelium, diet, or bacterial metabolism, include arabinose, ribose, gluconate (5), and

mannose (5, 19). Evidence from recent studies indicates that commensal *E. coli* strains occupy the intestinal mucus layer (18, 25) and catabolize sugars present in mucus (3, 5). Similarly, Wadolkowski and colleagues demonstrated *in vitro* growth of *E. coli* O157 EDL 933 in murine colonic mucus (33). Therefore, *E. coli* O157 catabolism of sugars present in rectal mucus may be important to its colonization of the bovine rectum.

In this study, we examined the nutritional basis of *E. coli* O157 bovine rectal colonization by hypothesizing that *E. coli* O157 catabolizes mucin-derived sugars during its colonization of that site. We tested this hypothesis by competitively co-colonizing wild-type *E. coli* O157 with isogenic mutants deficient in catabolic pathways for two mucin-derived sugars (L-fucose and *N*-acetylgalactosamine) or another sugar present in mucus (ribose) and monitored for colonization defects by differentially enumerating the *E. coli* O157 strains in rectal mucus. Herein, we report the results of these three trials.

Materials and Methods

Research animals and environment. Five mixed-breed beef cattle (4 steers, 1 heifer) were purchased at approximately 227kg body weight and allowed to acclimatize to their initial environment, the Oklahoma State University Veterinary Teaching Hospital. Duodenal cannulas were placed 6cm caudad to the pyloric junction and anchored and exteriorized between the last two ribs following a published technique (30). Cattle were maintained on injectable broad-spectrum antibiotics for one week and daily cannula site Betadine rinses for two weeks. During experimental colonizations, animals were transferred to and maintained in a BSL-2 large animal facility; each animal was placed in a pen alone. Research personnel wore BSL-2 personal protective equipment; additional animal care personnel wore face shields and masks during high pressure spray cleanings. Pens were scraped daily; feces were collected and treated with household bleach (1 part bleach to 9 parts fecal matter) for a minimum of four hours prior to disposal in the sewage system. Pens were spray-washed once daily with an antimicrobial mixture. Cattle were fed twice daily a pelleted total mixed ration composed of corn, ground alfalfa hay, and mineral supplements. Water was provided free choice. At termination of experimental trials, cattle experienced mild, transient bloat secondary to antibiotics. Further, at trial termination, cattle were temporarily transferred to an outdoor facility and maintained similarly while the BSL-2 facility was cleaned.

Bacterial strain and inoculation preparation. *E. coli* O157 EDL 933 (wild-type) used was streptomycin-resistant and nalidixic acid-resistant. Isogenic mutants in catabolic pathways for ribose, *N*-acetyl-galactosamine, and L-fucose were constructed in the streptomycin-resistant *E. coli* O157 EDL 933 with a chloramphenicol resistance cassette

inserted into the gene of interest following a published method (8). A summary of strains used and their source is in Table 1. Polymerase chain reaction verified the correct insertion, and growth on appropriate minimal media confirmed the phenotype. For colonization, all strains were grown for 16 hours in 200mL of Luria broth (LB, Sigma) to an OD_{600} of 1.5-1.6. Growth curves for the wild-type and isogenic mutant strains in LB broth were identical and reproducible and allowed for reasonably accurate predictions of inoculation levels for both strains. An input ratio of 3-4:1 favoring the mutant was chosen to more conclusively demonstrate the colonization defects of the mutants. For inoculation, 60mL of wild-type cell culture and 200-240mL of mutant cell culture was pelleted and washed three times with PBS and finally resuspended in 50mL of PBS. Ten-fold dilutions of the final bolus were prepared and plated with spreading on selective SMAC media (described below) to accurately assess the inoculation level.

Inoculation and streptomycin treatment. For inoculation, an emergency medical technician (EMT) endotracheal tube (Bound Tree Medical) was placed in the opened cannula followed by cuff inflation for sealing. Each animal received 10mL of the prepared inoculum via the medicinal access port of the EMT tube followed by 5mL flush of PBS via the same port followed by a 40-120mL flush of PBS via the main lumen of the endotracheal tube. No loss of the inoculating bolus was ever observed. Cattle were treated daily via the cannula with streptomycin prepared as a 1 g/mL aqueous solution of streptomycin sulfate (Sigma). Cattle were dosed 15 mg/lb of body weight beginning three days prior to initiation of colonization and continued until termination of a trial. On the morning of colonization initiation, streptomycin treatment was delayed approximately 12 hours until the evening sampling.

Sampling and media. Samples of feces and rectal mucus were collected at 0, 0.5, 1, 2, 3, 5, 7, 9, 12, and 15 days. Fecal samples ranged from 5-20g and were collected either as free-catch samples or directly from the terminal rectum. Feces was weighed and suspended at a 1:10 ratio in 1% tryptone (Becton-Dickinson) and placed on ice for one hour prior to plating. Mucus samples were acquired from the rectum via palpation; briefly, following manual evacuation of the feces, mucus was collected from the ventral half of the most caudal 30 cm of the rectum by scraping with the lid from a sterile syringe casing. Mucus was scraped until 200-500mg was acquired; it was suspended at a 1:2 ratio in 1% tryptone, vortexed vigorously, and placed on ice for one hour prior to plating. Feces and mucus samples were processed into 10-fold dilution series by diluting 100μL of initial sample into 900μL of sterile PBS and so forth. 100μL of each dilution was placed onto appropriate agar media and evenly spread on the plate with an alcohol-flamed spreading tool. Bacterial plates were incubated 12-18 hours at 37C prior to assessment and counting. Diluted feces and mucus samples were plated onto three types of media: 1) Sorbitol-MacConkey (SMAC) agar (Remel), 2) SMAC agar with streptomycin at 40ug/mL and nalidixic acid (Sigma) at 50 ug/mL (NS-SMAC), and 3) SMAC agar with streptomycin at 40ug/mL and chloramphenicol (Sigma) at 34 ug/mL (CS-SMAC). Colonies enumerated on SMAC media reflected total flora supported by growth on MacConkey before and during streptomycin treatment and also monitored for introduction of non-input *E. coli* O157 strains (which was not detected). NS-SMAC was used for the differential enumeration of wild-type *E. coli* O157; CS-SMAC was used for differential enumeration of mutant strains of *E. coli* O157. Sorbitol-negative (white) colonies were enumerated daily and, where colonial morphology was unusual, subjected

to confirmatory testing with a commercially available immunoassay (O157 Immunocard STAT; Meridian Biosciences). Enrichment cultures accompanied all daily samplings. Briefly, 100-150μL of primary samples were placed in trypticase soy infusion (TSI) broth (Becton-Dickinson) with streptomycin (40 μg/mL) and grown at 37C for 16 hours with shaking (210 rpm). If primary samples were negative, the enrichment samples were subjected to dilution series and plated similarly. Enrichment samples yielded qualitative data of presence (enrichment positive) or absence (enrichment negative). For purposes of computation of means, enrichment data was transformed to a semi-quantitative estimate of 50 cfu/g feces or 10 cfu/g mucus, exactly 50% of the lowest limit of detection for each of the respective samples.

Colonization termination. At the conclusion of an experiment, cattle were treated with two antibiotics to which the colonizing strains were determined to be susceptible. Neomycin (Biosol; Upjohn) was administered daily for three days via the cannula at 10 mg/kg. Ceftiofur sodium (Naxcel; Pfizer) was administered subcutaneously daily for three days at 0.5mg/kg. Enrichment cultures of the last day of such therapy and two subsequent days were grown, plated, and assessed to determine reliable elimination of the colonizing strain.

Statistical Methods. In the three competitive co-colonization trials described, colonies were counted on differential media, converted to a $\log_{10}$ scale, means for the five cows computed, and a standard error of the $\log_{10}$ data computed. The initiation phase of colonization was defined as days 2, 3, and 5 of sampling, excluding the 12 and 24 hour samples because transient intestinal passage and equilibration of the inoculum was occurring. The maintenance phase of colonization was defined as days 7, 9, 12, and 15.

The Wilcoxon-signed ranks test, a non-parametric statistical test, was utilized in a one-tailed fashion to test for significant differences during colonization phases, with $p<0.05$ deemed significant. The null hypothesis was that co-colonizations of wild-type and mutant strains would be equal. The alternative hypothesis tested was that the mutant would exhibit a colonization defect during competition.

Results

Animal Health. All animals remained healthy and experienced moderate average daily gains of 0.74 +/- 0.07kg over the 98 days of these studies. No attaching and effacing histopathology was observed in any of the cattle during the course of the colonizations.

Ribose is important in the late maintenance phase of colonization and may be important during initiation. Wild-type *E. coli* O157 was co-colonized with *E. coli* O157 Δ*rbs*K, a mutant constructed with a chloramphenicol resistance cassette disrupting the ribokinase (*rbs*K) gene. The mutant:wild-type input ratio was 3.96:1, and both strains were differentially enumerated in feces and rectal mucus over 15 days (Figure 1). We artificially favorably biased all mutants at the point of colonization to ensure a minimum of a 1:1 ratio of input strains and to allow the possible development of a ratio reversal to be demonstrated. The ratio reversed to 0.5:1 by day 3 (Figure 2). In addition to the ratio reversal, the wild-type was recovered in significantly higher numbers than the mutant in the aggregate analysis of days 2, 3, and 5. During the entire maintenance phase the average number of wild-type organisms recovered was higher than the mutant ($p<0.05$). However, upon examining days 7 and 9 ($p=0.065$) and 12 and 15 ($p<0.01$) in separate analyses, it appeared that the later maintenance phase was more important than early maintenance. All five animals were positive for both strains within mucus through day 12, but on day 15, all five were positive for the wild-type while only two were positive for the mutant. Results for the feces samples were similar through day 9. Fecal persistence for both strains beyond day 9 was sporadic (data not shown).

***N*-acetyl-galactosamine may be important in initiation of colonization.** Wild-type *E. coli* O157 was co-colonized with *E. coli* O157 Δ*aga*WEFA, a mutant constructed with a

chloramphenicol resistance cassette replacing a portion of the phosphotransferase (PTS)-dependent import system (*aga*WEF) and the de-acetylase (*aga*A) gene for *N*-acetylgalactosamine. The mutant:wild-type input ratio was 4:1, and strains were differentially enumerated in feces and rectal mucus over 15 days (Figure 3). The 4:1 ratio reversed to 0.69:1 by day 3 (Figure 4). Consistent with this observation, the wild-type was recovered in higher average numbers than the mutant on days 2, 3, and 5 ($p<0.05$). In aggregate analysis of sample days within the maintenance phase, there was no significant difference in recovery of bacteria between the two strains ($p>0.2$). By day 15, one animal each was negative for one of the strains within rectal mucus. Therefore, there was no significant difference in persistence observed between the wild-type and mutant strain. The fecal shedding data was similar in the progressive, day-to-day, decrease in recovery and in the lack of a significant difference during maintenance. However, the day-to-day differences between the strains during maintenance fluctuated more widely (data not shown).

Fucose is important in initiation and maintenance phases of colonization. Wild-type *E. coli* O157 was co-colonized with *E. coli* O157 Δ*fuc*AO, a mutant constructed with a chloramphenicol resistance cassette disrupting the fucose aldolase (*fuc*A) and oxido-reductase (*fuc*O) gene for fucose catabolism. The mutant:wild-type input ratio was 3.8:1, and both strains were differentially enumerated in feces and rectal mucus over 15 days (Figure 5). The input ratio of 3.8:1 reversed markedly to 0.03:1 on the third day of this trial (Figure 6). In aggregate analysis of samples collected during the initiation phase, the wild-type was recovered in mucus in significantly higher numbers than the fucose mutant ($p<0.01$). Similarly, in aggregate analysis of maintenance phase samples, the wild-type

was recovered in mucus in significantly higher numbers than the fucose mutant ($p<0.01$). Persistence of the wild-type strain was 9.4 +/- 3.3 days, and persistence of the mutant was 4.8 +/- 2.3 days ($p<0.05$). Overall, fecal shedding of both strains was only somewhat similar to the mucus recovery, being of generally lower magnitude and shorter duration (data not shown).

Discussion

Cattle were successfully co-colonized with parent and isogenic catabolic mutant strains of *E. coli* O157 EDL933 in this study. Mutants were constructed in key carbohydrate source catabolic pathways and, with the aid of antibiotic selectable markers, were differentially enumerated with the parent strain in feces and rectal mucus with a frequent sampling regimen. This system permitted the *in vivo* observation of colonization defects in the complex and dynamic large intestinal environment. The rational selection of appropriate catabolic mutants was guided by results of similar competitive colonizations in CD-1 mice (Cohen, P.S. and Conway, T., unpublished) and by selected literature (11, 19).

The contribution of ribose to the nutritional basis of *E. coli* O157 cattle colonization was shown to be important during initiation and of modest importance during late maintenance. By comparison, similar experiments in CD-1 mice showed that the *rbs*K mutant exhibited a three-log order of magnitude colonization defect in competition with its wild-type parent at day 11 (Cohen, P.S. and Conway, T., unpublished), displaying the importance of *E. coli* O157 ribose catabolism to a higher degree than that observed in the adult cattle model. Such an effect is likely not generic to all *E. coli* as ribose was the least preferred nutrient for commensal *E. coli* MG 1655 (3) in similar experiments in CD-1 mice. Ribose does not appear to be mucin-derived; instead, it is detected in mucus (5) and is likely derived from nucleic acids of sloughed epithelium and dead microorganisms. Its modest importance in adult cattle *E. coli* O157 colonization could possibly be reflective of limited compositional availability in the

mucus layer. Unfortunately, the contribution of ribose to the available pool of sugars within animal mucus has not been analyzed in many previous studies (19, 27, 28, 31).

N-acetylgalactosamine (Aga) was demonstrated to be of modest importance during initiation, but not during maintenance. Overall, this was broadly similar to the finding that Aga was of minor importance to *E. coli* O157 in the CD-1 mice (Cohen, P.S. and Conway, T., unpublished). A preliminary conclusion from these results in cattle is that Aga is not essential for growth during the maintenance phase. Other alternative mechanisms for not observing a maintenance defect could have been: 1) limited sugar availability, or 2) redundancy in catabolic pathways. The compositional proportion of Aga in bovine intestinal mucin was ~24% in a study of young calf ileal mucin (19). Similar data is not available for other sites or other ages of cattle. Therefore, proportional availability of Aga in the adult bovine rectum is not known. Interestingly, Dziva and colleagues colonized young calves with signature-tagged mutants of *E. coli* O157 and found a colonization defective mutant with a transposon insertion in *aga*B, annotated to be a portion of the phosphotransferase (PTS) - dependent import pathway for Aga. (Disparate gene annotation exists as Brinkkotter and colleagues indicate *aga*B to be part of the PTS import pathway for galactosamine, not Aga (2).) Additional commentary on the construction of the mutant, *E. coli* O157 Δ*aga*WEFA, provides a relevant background to discuss catabolic pathway redundancy. Disruption of *aga*WEF eliminated genes of the Aga-specific PTS import pathway (2), and disruption of *aga*A eliminated the gene for the deacetylase step, the first degradative step following import (2). Therefore, catabolic pathway redundancy both in Aga importation and deacetylation would need to exist to fulfill this mechanistic possibility.

The competitive co-colonization involving the fucose mutant demonstrated a marked colonization defect in initiation and maintenance. The underlying mechanism for this defect may reflect: 1) a critical importance of fucose as a carbon source for *E. coli* O157 , 2) that fucose may be the dominant glycan in the region sampled, or 3) both. Support for the former mechanism comes from the competitive co-colonization of the same strains in CD-1 mice. In that study, competitive co-colonization of wild-type *E. coli* O157 with *E. coli* O157 Δ*fuc*AO demonstrated the most dramatic colonization defect in the fucose mutant when compared to several other competitive co-colonizations (Cohen, P.S. and Conway, T., unpublished). These results are highly similar to the adult cattle colonization results contained herein. This is likely not a generic *E. coli* phenomenon as a similar catabolic knockout constructed in *E. coli* MG 1655 was competitively co-colonized in CD-1 mice, and the fucose knockout demonstrated a less dramatic colonization defect. Furthermore, fucose was ranked as the sixth (of seven) most preferred nutrient for *E. coli* MG1655 in *in vitro* growth experiments (3). In consideration of the second possible mechanism, whether fucose is the dominant glycan of bovine rectal mucin, there is no published analysis of bovine rectal mucin. However, fucose is a minor constituent of calf ileal mucin (19).

Returning to Freter's hypothesis, that successful colonization is predicated upon successfully competing for one or a very few limited nutrients while having a growth rate that exceeds intestinal washout (3, 5, 13), it appears that fucose may be a preferred nutrient for *E. coli* O157 colonization of the bovine rectum. A corollary of this idea is that the transient occurrence of *E. coli* O157 shedding documented in field settings could be partially explained by initial colonization by heavy fecal contamination (advanced by

Cray and Moon (7)) followed later by other microflora occupying a fucose nutrient-niche displacing *E. coli* O157 by virtue of more efficient utilization of fucose or a higher growth rate. Furthermore, probiotic strategies aimed at limiting fucose bioavailability could possibly limit the successful colonization of *E. coli* O157 in the bovine rectum and may be a rational application of these results .

The composition of bovine rectal mucin could be a determinant of unknown significance upon these results. In the young calf, Aga and fucose were major and minor, respectively, constituents of ileal mucin (19), yet if sugar availability dictates nutrient preferences, one could hypothesize that these levels of Aga and fucose would be reversed in adult cattle rectal mucin. Indeed, one cannot generalize mucin oligosaccharide composition from the calf ileum to the adult bovine rectum because differences likely exist. In comparative studies, mucin has been shown to exhibit regionally specific patterns of glycosylation in rodents (12, 31) and man (27, 28). Further, expression of glycosyltransferase genes is regionally specific in rodents, as well (20). Robbe and colleagues suggest that such regionally specific mucin glycosylation could be explanatory for colonization of specific intestinal regions by bacteria (tropism) by virtue of a diversity of attachment sites (27). In addition and as an alternative, we speculate that such regionally specific glycosylation could possibly be a factor in defining nutrient-niches and even regional tropisms for bacteria.

In a recent study, the lymphoid-dense, terminal rectal mucosa was identified as a site of tropism for *E. coli* O157 colonization of cattle (22), and Naylor and colleagues speculated that the tropism was likely based on a bacterial adhesin-epithelium receptor interaction occurring at this site (22). Attaching and effacing histopathologic lesions

have been demonstrated in rectal and colonic mucosal explants (1) and ileal explants (24) in adult cattle. *In vivo* attachment in an adult cow was first demonstrated by Naylor and colleagues in the rectum (22). The wide-ranging spatial occurrence of *E. coli* O157 attachment to ileum (24), colon (1), and rectum (1, 22) calls into question the likelihood that adhesin-mediated attachment is the principal defining factor in the terminal rectal tropism. Certainly, the present study does not conclusively present an alternative hypothesis. However, a nutritional basis for colonization of *E. coli* O157 may be spatially variable and could be a factor defining colonization tropism. Future studies using additional competitive co-colonizations, as well as biochemical definition of bovine mucin glycosylation by age and by intestinal region, will be needed to further test these ideas.

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Figure 1. Competitive Co-colonization in Mucus: *E. coli* O157 WT and *E. coli* O157 Δ*rbs*K.

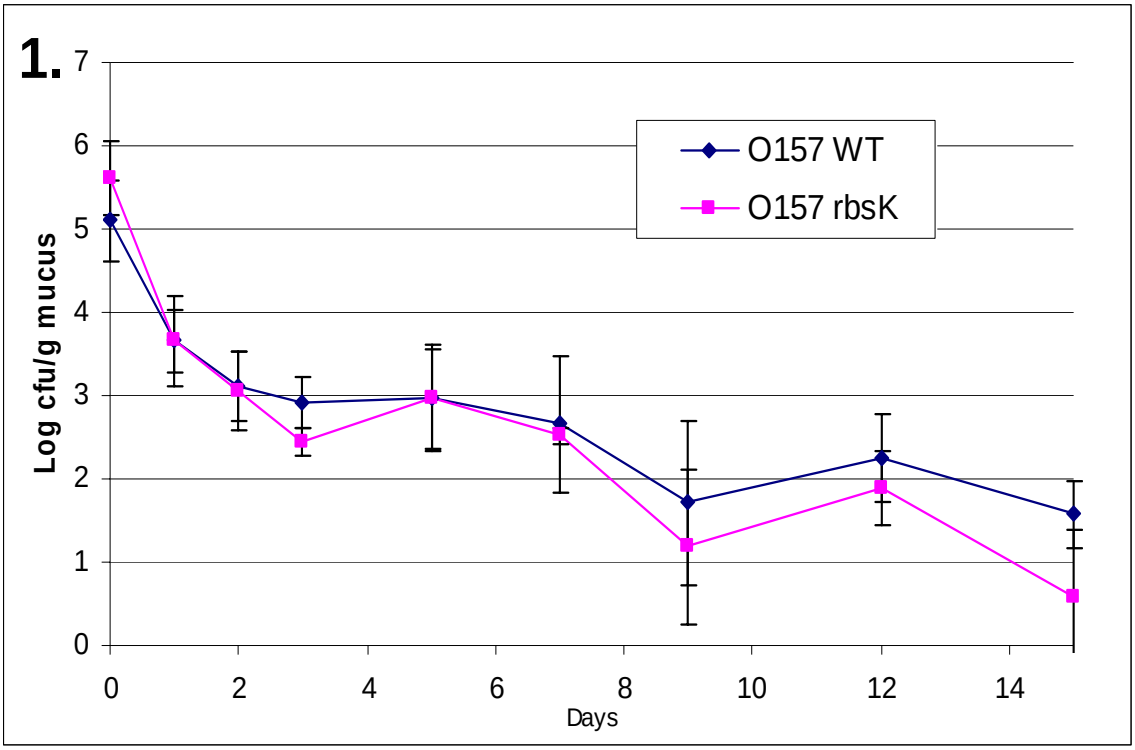

Figure 1. Streptomycin-treated cattle were colonized with 10^{10} cfu of wild-type *E. coli* O157 and *E. coli* O157 Δ*rbs*K and mucus samples were taken at the indicated times, homogenized, and plated as described. Enumerated counts were transformed to $\log_{10}$ levels. Bars represent the standard error of the $\log_{10}$ (cfu/g sample). Value at day 0 reflects samples acquired at 12 hours post-colonization.

Figure 2. Absolute ratio of input strains in mucus: *E. coli* O157 WT and *E. coli* O157 Δ*rbs*K

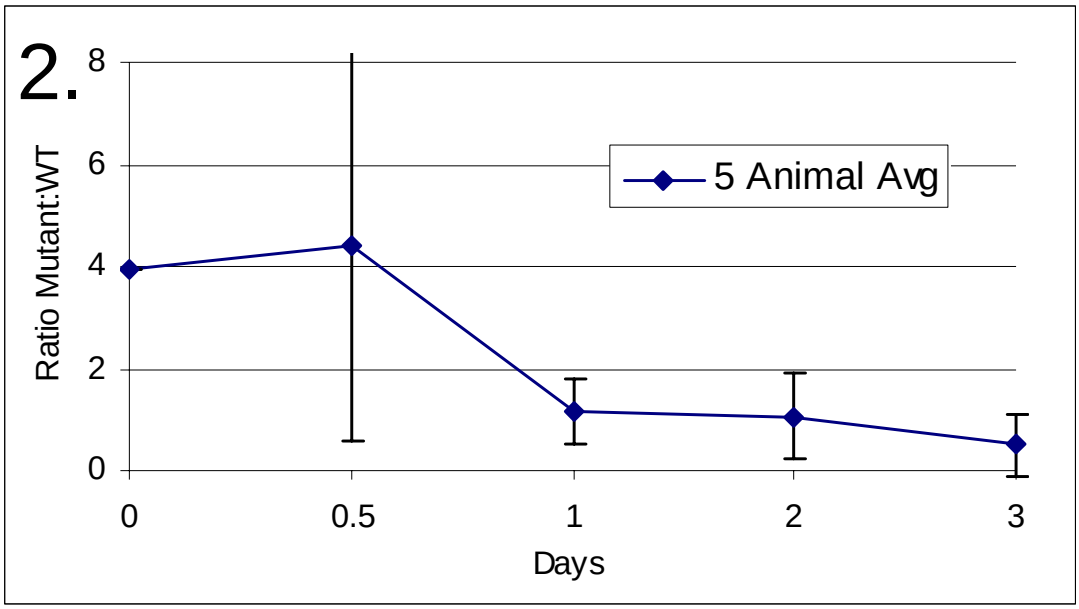

Figure 2. The absolute numerical ratio of input strains *E. coli* O157 WT and *E coli* O157 Δ*rbs*K recovered in mucus over time during initiation. Plot is the average of ratio of strain by input or recovery for five adult cows.

Figure 3. Competitive Co-colonization in Mucus: *E. coli* O157 WT and *E. coli* O157 Δ*aga*WEFA

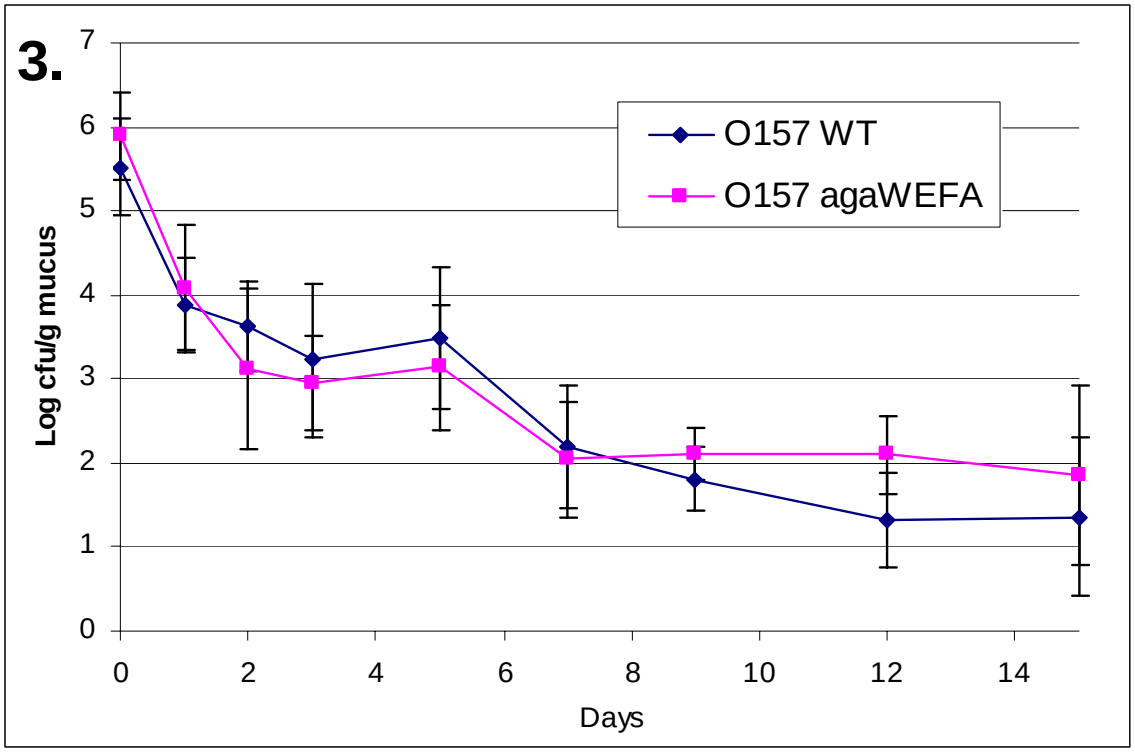

Figure 3. Streptomycin-treated cattle were colonized with 10^{10} cfu of wild-type *E. coli* O157 and *E. coli* O157 Δ*aga*WEFA and mucus samples were taken at the indicated times, homogenized, and plated as described. Enumerated counts were transformed to $\log_{10}$ levels. Bars represent the standard error of the $\log_{10}$ (cfu/g sample). Value at day 0 reflects samples acquired at 12 hours post-colonization.

Figure 4. Absolute ratio of input strains in mucus: *E. coli* O157 WT and *E coli.* O157 Δ*aga*WEFA

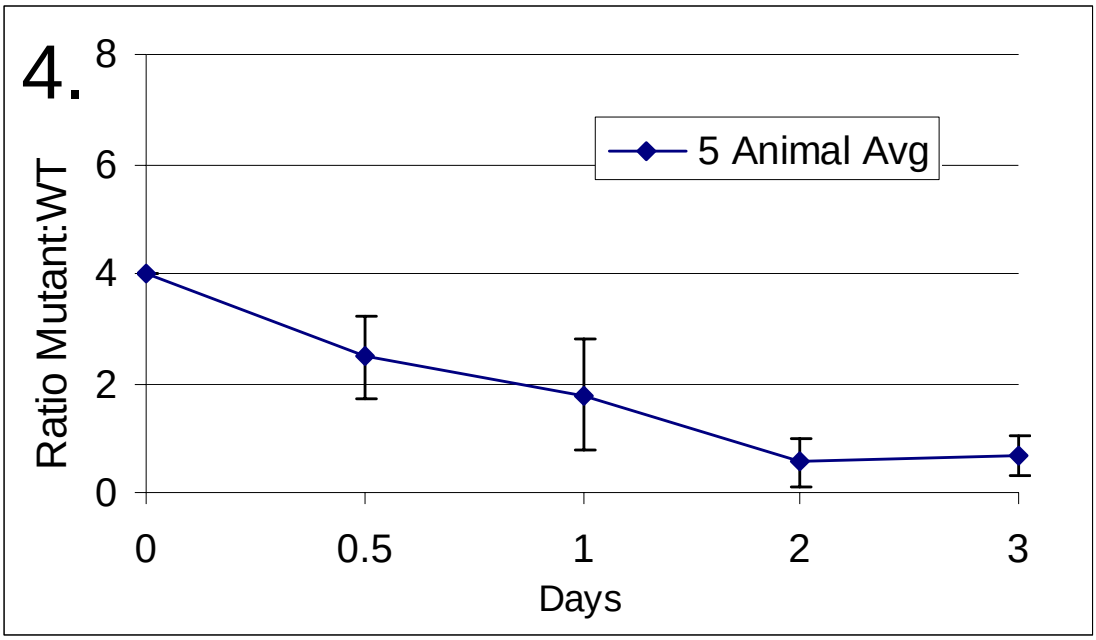

Figure 4. The absolute numerical ratio of input strains *E. coli* O157 WT and *E coli.* O157 Δ*aga*WEFA recovered in mucus over time during initiation. Plot is the average of ratio of strain by input or recovery for five adult cows.

Figure 5. Competitive Co-colonization in Mucus: *E. coli* O157 WT and *E. coli* O157 Δ*fuc*AO

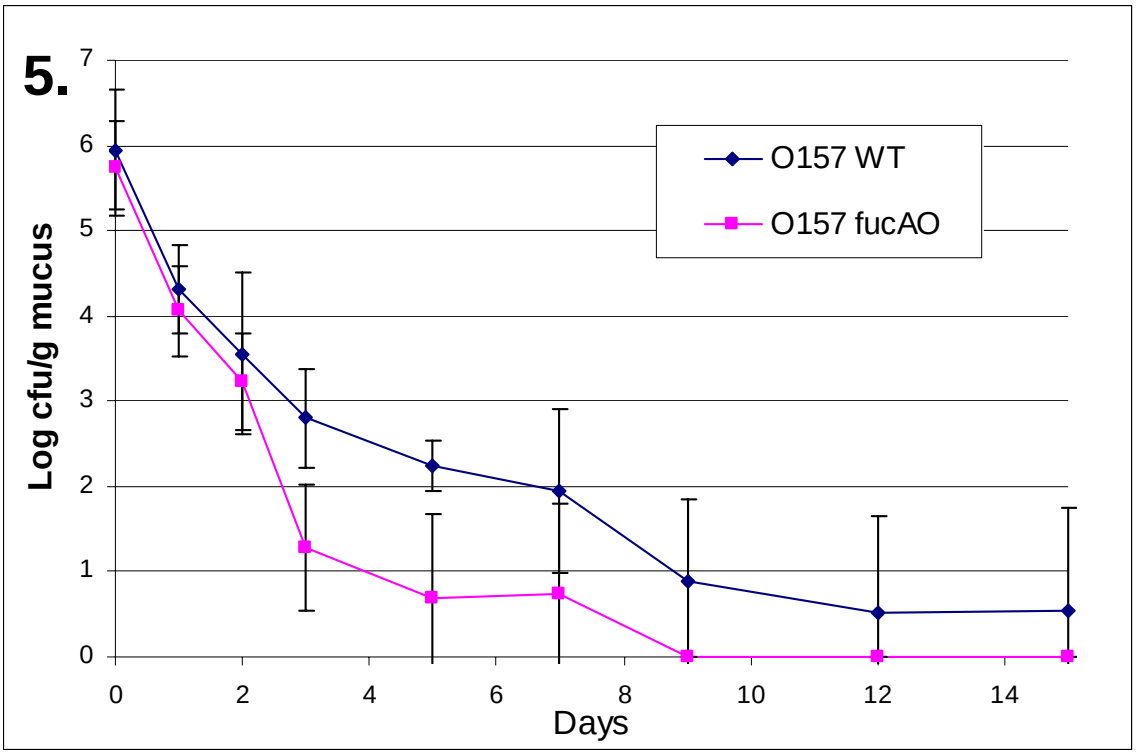

Figure 5. Streptomycin-treated cattle were colonized with 10^{10} cfu of wild-type *E. coli* O157 and *E. coli* O157 Δ*fuc*AO and samples were taken at the indicated times, homogenized, and plated as described. Enumerated counts were transformed to $\log_{10}$ levels. Bars represent the standard error of the $\log_{10}$ (cfu/g sample). Value at day 0 reflects samples acquired at 12 hours post-colonization.

Figure 6. Absolute ratio of input strains in mucus: *E. coli* O157 WT and *E. coli* O157 Δ*fuc*AO

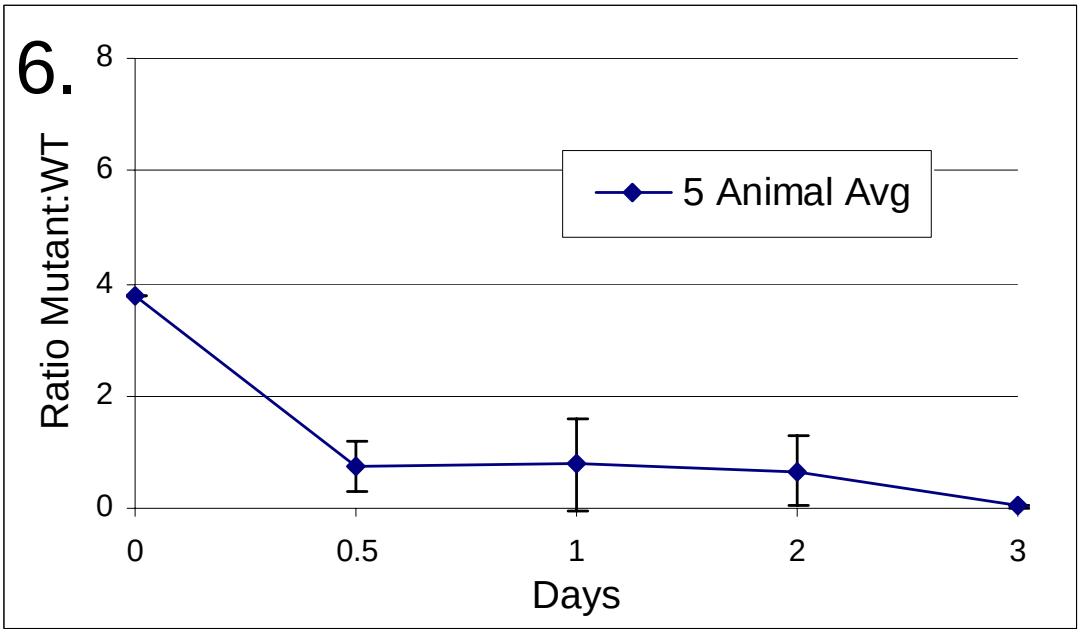

Figure 6. The absolute numerical ratio of input strains *E. coli* O157 WT and *E coli* O157 Δ*fuc*AO recovered in mucus over time during initiation. Plot is the average of ratio of strain by input or recovery for five adult cows.

Table 1. Strains utilized in this study.

Strain	Antibiotic Resistance	Source
E. coli O157 EDL933 WT[a]	Str[b], Nal[c]	
E. coli O157 Δ*fuc*AO	Str, Cam[d]	P. Cohen, Univ. of
E. coli O157 Δ*aga*WEFA	Str, Cam	Rhode Island
E. coli O157 Δ*rbs*K	Str, Cam	

[a] Wild-Type [b]Streptomycin [c]Nalidixic acid [d]Chloramphenicol

Chapter 5

Aptamer Selection for

E. coli O157

Abstract

Background/Objective: *E. coli* O157 is an intestinal pathogen of man that causes diarrhea and the hemolytic-uremic syndrome. It is usually transmitted in food contaminated by bovine feces. Because of its public health importance, novel diagnostic tools with advantages in speed, sensitivity, cost, and field adaptability are in demand. Aptamers, oligonucleotides selected from random pools for desired affinities, have the potential to satisfy these demands. Aptamers can bind targets in a manner similar to antigen-antibody interactions, with similar to superior specificity, dissociation constants, and repertoire. We have performed aptamer selection experiments (SELEX) against *E. coli* O157 targets with the goal of identifying useful binding species. **Methods:** In one targeting strategy, *E. coli* O157 lipopolysaccharide was purified, biotinylated, and immobilized on streptavidin-coated paramagnetic particles. Multiple rounds of SELEX were performed on this target with DNA and RNA aptamer pools. Nitrocellulose immobilization of purified lipopolysaccharide was also attempted for target presentation. In a second targeting strategy, whole *E. coli* O157 cells were used as a target, and *E. coli* K-12 and other Enterobacteriaceae were used as negative selection targets. Partitioning of binding ligands was performed by centrifugation. Radiolabeled aptamer binding assays and cloning and sequencing enriched pools were strategies utilized to monitor progress. **Results:** Selective enrichment of the aptamer pool was not observed during the targeting of immobilized lipopolysaccharide. However, selective enrichment of the pool was observed through 19 rounds of SELEX against *E. coli* O157 whole cells. Additionally, a binding assay indicated 5:1 preferential binding for O157 over K-12, and the enriched

aptamer bound O157 in a significantly greater proportion than the round 0 background.

Conclusions: Candidate aptamers for detection of *E. coli* O157 have been selected.

Introduction

E. coli O157 is an intestinal pathogen of man that causes bloody diarrhea and the occasionally fatal hemolytic-uremic syndrome. It is currently estimated that *E. coli* O157 causes more than 70,000 illnesses with 60 deaths annually in the U.S. [1] It is also prioritized by the U.S. Centers for Disease Control (CDC) as a category B bioterrorism agent (http://www.bt.cdc.gov/agent/agentlist.asp). Because of the public health importance of *E. coli* O157, robust diagnostic tools that exhibit superior performance with respect to speed, cost, and field adaptability without sacrificing specificity and sensitivity are especially needed. This introduction will briefly review the current state of diagnostic testing for *E. coli* O157 (for more detail, see [2-4]) and illustrate the unique properties and potential applications of aptamers for the detection and surveillance of *E. coli* O157.

Basic clinical microbiological detection of *E. coli* O157 is achieved by using the sorbitol-MacConkey media (SMAC), recommended by the CDC to be routinely used in the culture of bloody diarrhea specimens [5]. SMAC, selective for the presumptive diagnosis of *E. coli* O157 [6], differentially identifies *E. coli* O157 because its inability to ferment sorbitol confers a colorless colonial morphology [6], whereas ≥ 80% of *E. coli* are sorbitol fermenters and appear pink [7]. The specificity of this technique is problematic as sorbitol-fermenting, *E. coli* O157 strains have been documented [8, 9].

Diagnostics based on chromosomal and/or plasmid DNA content of *E. coli* O157 have been reviewed [3] and utilize unique genetic signatures in either probe-based assays or the polymerase chain reaction. Recently, specific primers for the O157 O-antigen biosynthesis genes were designed resulting in a PCR technique specific for the detection

of *E. coli* O157 [10]. Perhaps more clinically relevant are PCR techniques targeting Shiga toxin genes or the *eae* gene from the locus of enterocyte effacement (reviewed in [3], because these genes are specific for the larger group of enterohemorrhagic *E. coli*, instead of restricted to *E. coli* O157. The higher expense and increased technical expertise required for PCR somewhat restricts its application in primary diagnostics.

Immunomagnetic separation and latex agglutination testing are diagnostic tools that exhibit high specificity and require less technical expertise. Immunomagnetic separation is a technique based upon commercially available paramagnetic particles (Dynal) that are derivatized with O157 monoclonal antibodies. Immunomagnetic separation with these particles has been successfully used to detect *E. coli* O157 in food [11, 12], water [12], cattle feces [13], and human feces [14], with markedly greater sensitivity of detection in cattle feces [13] and human feces [14]. Latex agglutination testing is accomplished via several end-user, marketed kits. In one study, three such kits were evaluated by testing 159 strains of *E. coli* (O157 and other serotypes). The sensitivity and specificity were both 100% when compared to internal reference sera [15]. The primary pitfall for both immunomagnetic separation and latex agglutination (as well as other immunoassay techniques) is the serologic cross-reactivity of *E. coli* O157. Indeed, *E. coli* O157 O antigen has identical or cross-reactive epitopes with *E. coli* O44 and O55 [16], *Brucella abortus*, *Salmonella* O30 strains[17], *Yersinia enterocolitica*, *Citrobacter freundii*, *Citrobacter sedlakii* [18], *Escherichia hermannii* [19], and some non-O1 strains of *Vibrio cholerae* [20].

As an alternative to antibody-based techniques, aptamers have been proposed to be developed and utilized in diagnostic testing for infectious agents [21], including *E.*

coli O157. Aptamers are DNA or RNA oligonucleotides that bind a target of interest on a physicochemical and electrostatic basis [22], in contrast to traditional usage of oligonucleotides as hybridization probes. Here, in addition to a genotype, the aptamer possesses a phenotype in its exhibition of affinity and selectivity for targets. An aptamer selected to bind thrombin is perhaps the most successful and certainly most-studied in this emerging class of biomolecules [23], but aptamers have also been selected against polysaccharides, small molecules, and whole cells (reviewed in [22]). Aptamers are selected from complex, synthetic, oligonucleotide pools by an iterative process known as SELEX (systematic evolution of ligands by exponential enrichment) [24]. Each iteration ("round") results in affinity partitioning of winning ligands, which are then amplified via PCR and subjected to subsequent rounds. To date, little has been published in the area of aptamer selection for bacterial targets except for two reports on aptamers for anthrax toxin [25] and *E. coli* O111 LPS [26]. Thus, if aptamer selection for bacterial targets is increasingly successful, their binding properties and other advantages could make them a useful alternative to antibodies in the future.

To select an aptamer specific for *E. coli* O157, we chose to target the bacterial outer membrane because it is predominantly comprised of the immunodominant lipopolysaccharide [27, 28] and because any selected aptamer could be applied in a diagnostic platform without a need for cellular disruption. Two targeting strategies were employed. In the first, the purified lipopolysaccharide (LPS) from *E. coli* O157 was utilized as a target and, in the second, whole *E. coli* O157 cells were utilized as the target, presenting an array of potential, externally-presented targets. Herein, we report the progress made on aptamer selection for *E. coli* O157.

Materials and Methods

Bacterial strains. *E. coli* O157 ATCC 43888 is a Shiga-toxin negative strain isolated from human feces. It produces a fully substituted O antigen. *E. coli* O157 MA6, a gift of Choong Park, VA Fairfax Hospital, Falls Church, VA, is a naturally occurring, rough mutant of *E. coli* O157 [29]. It was isolated from Malaysian beef. It does not produce the O antigen, as detected by serologic tests and lipopolysaccharide analyses. It does group with *E. coli* O157 strains on various genotypic analyses. *E. coli* TOP10F' is a K-12 derivative (Invitrogen). *Salmonella typhimurium* used was a bovine fecal isolate from the archives of the Oklahoma Animal Disease Diagnostic Laboratory. *Yersinia pestis* KIM5 D1 was a gift of Dr. Robert Brubaker, Michigan State University. The former two bacteria were used in lipopolysaccharide purification and targeting. *E. coli* O157 43888 and the latter three isolates were used for whole cell selections. Luria/glycerol stocks of both strains were prepared and archived at -80°C. Standard dilutions were used to present known quantities of cells for whole cell selection experiments.

Lipopolysaccharide Extraction. Lipopolysaccharide (LPS) was extracted from bacterial cells using a protocol supplied by Andrew Preston, Centre for Veterinary Science, University of Cambridge. This protocol is a modification of the hot phenol:water extraction process described by Johnson and Perry [30]. Bacteria were inoculated into 2L of Luria broth (Sigma) for 16 hours of growth. Cells were harvested with centrifugation at 7,500 rpm for 15 min and washed three times with ddH_20. Water sufficient to make a thick slurry was added, and the suspension was sonicated with a Branson Sonifier 450 with the following parameters: Output-8; Duty cycle-constant; pulse-constant; 30 second pulses for a total of 90 seconds. Lysozyme (Sigma) was added

to 0.2% w:v and the suspension incubated at room temperature overnight followed by a 24 hour incubation at 4°C. Next, the suspension was sonicated for an additional 30-second pulse using the above conditions. Micrococcal nuclease (30U, Sigma) and ribonuclease A (100µg/mL, Sigma) was added, and the suspension was incubated at 4°C an additional 48 hours. Phenol (Sigma) was melted and equilibrated with the bacterial digest at 65°C. It was sonicated for 30 sec and incubated an additional 30 minutes. Next, the suspension was placed on ice for 30 minutes and then centrifuged at 6000 rpm at 4°C. The aqueous phase was removed, an equal volume of water was added to the phenol phase, and the heat incubation, ice, and centrifugation were repeated. Both aqueous phases were pooled, the phenol phase was recovered, and both phases were extensively dialyzed against ddH20 for 3-7 days using dialysis tubing (15,000 MWCO, cellulose acetate, SpectraPor). Dialysis proceeded until the phenol odor was non-detectable. Volumes from both dialyzed phases were then ultracentrifuged at 100,000xg in a 55.2Ti rotor and Beckman ultracentrifuge for 16 hours at 4°C. The pellet was collected, resuspended in ddH_20 and subjected to an 8 hour proteinase K (Sigma) digestion (25 mg/mL PK prepared and added to LPS solution at 100µL per 500µL) at 60°C. The final preparation was lyophilized to result in a dry product utilized in future analyses and modifications.

Other LPS Sources: LPS from *E. coli* O55 (Sigma) was utilized as supplied.

LPS Analysis by SDS-PAGE. Lipopolysaccharide products purified from *E. coli* O157 43888 and *E. coli* O157 MA6 were analyzed with SDS-polyacrylamide gel electrophoresis. Minigels were cast at 12.5% using Bio-Rad or Hoefer apparatus. Acrylamide mixtures, ammonium persulphate, TEMED, and buffering solutions used

chemicals from Sigma. LPS samples were diluted into Laemmli sample buffer (Sigma) and boiled for 3 minutes prior to loading. Gels were run at constant voltage (100V; 80-110 minutes). Gels were stained by a silver stain kit (Owl) following manufacturer's instructions. Principal of the silver stain kit was based upon Tsai and Frasch's described methods [31].

LPS Biotinylation. Bacterial lipopolysaccharides were biotinylated following a method described by Luk and colleagues [32]. One milligram of LPS was dissolved in 0.9mL of fresh, 0.1M sodium acetate (pH 5.0) followed by addition of 0.1mL of 100mM sodium m-periodate (Sigma) and the overall mixture was incubated on ice for 30 minutes in the dark. The reaction was stopped by addition of a drop of glycerol (~2µL). The mixture was dialyzed (15,000 MWCO, SpectraPor) overnight against sterile PBS. Next, biotin-LC-hydrazide cross-linker (Pierce) was prepared in DMSO to a final concentration of 50mM, and 0.1mL of this solution was added to the dialyzed, oxidized LPS sample. This mixture was incubated at room temperature for 2 hours with end to end rocking and then dialyzed again overnight and stored at 4°C. Successful biotinylation was confirmed by dot-blotting and electrotransfer (Bio-Rad transfer case) of SDS-PAGE gels onto nitrocellulose (0.45µm, Bio-Rad) followed by probing with a streptavidin-alkaline phosphatase conjugate and colorimetric detection with a BCIP/NBT substrate (Sigma).

Streptavidin-coated paramagnetic particles. M-270 Dynabeads (Dynal) are uniform, spherical, superparamagnetic, polystyrene beads with streptavidin covalently attached to the surface. These beads were utilized in Dynabead-immobilized lipopolysaccharide target presentations and also were utilized during strand separations performed as part of the SELEX protocol. Prior to utilizing beads for strand separation or for target

presentation, they were captured and washed two times with 1X binding buffer, described below.

DNA Aptamer Library. The DNA aptamer library utilized in most experiments was composed of a 23-base upstream constant primer binding site and a 20-base downstream constant primer binding site flanking a central, randomized region thirty nucleotides in length (IDT, Coralville, IA). The sequence of the DNA library was 5'-TTGAGCGTTTATTCTGAGCTCCC- $(N)_{30}$ – TTCGACATGAGGCCCGGATC – 3', the forward primer sequence was 5'-TTGAGCGTTTATTCTGAGCTCCC, and the reverse primer sequence was 5'-GATCCGGGCCTCATGTCGAA. Biotinylated reverse primer was utilized for polymerase chain reaction in all post-SELEX amplification except when it preceded planned cloning and sequencing of the pool. Prior to cloning, the enriched aptamer pool was amplified utilizing the forward primer and a non-biotinylated reverse primer.

DNA Aptamer SELEX for Bead-Presented LPS. The DNA SELEX protocol is summarized in Figure 1A. Biotinylated, purified LPS was loaded at a minimum of 2X saturating load as determined in an ELISA-like format. LPS was loaded fresh onto Dynabeads prior to each round of SELEX in the presence of 1X binding buffer and washed three times with 1X binding buffer prior to incubation with pool. Following incubation with pool, unbound ligands were partitioned via magnetic capture of bead-bound target and bound ligands and washed twice with 1X binding buffer. Bound ligands were PCR-amplified for the next round of selection in the presence of bead-bound target and amplified pool was prepared for subsequent rounds by methods described below. Thirty total rounds were performed designated 1-12, 13A-21A, and

13B-21B. Starting pool for subsequent rounds originated from the round number/letter preceding in all instances except the starting pool for rounds 13A and 13B was from round 12 pool. Alterations in stringency, including altered target concentrations, incubation times, and negative selections, occurred round by round and are summarized in Table 1.

DNA Aptamer SELEX for Nitrocellulose-Presented LPS. The DNA SELEX protocol is summarized in Figure 1A. LPS was immobilized onto nitrocellulose (0.45µm nitrocellulose, Bio-Rad, cut into 8mm disks with a hole-punch) filter disks by immersion into native LPS solutions (1mg/mL) for 2-6 hours followed by three washes with 1X binding buffer. Bound aptamers were eluted by heating the membrane in 300-400µL 7M urea at 95-100°C for 10 minutes. Eluted aptamer DNA was ethanol precipitated and amplified by PCR and prepared for the next round of selection by methods described below. Alterations in stringency, including altered target concentrations, incubation times, and negative selections, occurred round by round and are summarized in Table 2.

DNA Aptamer SELEX for Whole Cell Targeting. DNA SELEX protocol is summarized in Figure 1A. Ultracentrifugation was selected as the means of partitioning the whole cells/bound ligands from unbound ligands. The rationale for the selection of this partitioning method was twofold: 1. sample tubes (8x35mm snap cap, polypropylene, Fisher 02-681-230) and adaptor tubes (1mL polycarbonate, Beckman 355657) used in the Beckman type 25 rotor under ultracentrifugation conditions (90,000 x g, 20-150 min) resulted in compact bacterial pellets that were not easily disturbed, and 2. ultracentrifugation conditions utilized were considered sufficient to co-pellet derived outer membrane vesicles (T. Beveridge, University of Guelph, personal communication),

thought to be an additionally useful target likely to be present in diagnostic samples. Alterations in stringency, including altered target concentrations, incubation times, and negative selections, occurred round by round and are summarized in Table 3.

Binding Buffer. The binding buffer utilized in DNA aptamer selection experiments and in aptamer binding assays was composed as follows: 20mM Trizma base (Sigma), 5mM $MgCl_2$, and 100mM NaCl. The pH was adjusted to 7.5.

PCR Methods. PCR reagents (Invitrogen) included Platinum Hot-start polymerase. Unless otherwise indicated, PCR cycling was performed on a Peltier-PCR engine (MJ Research) with the following cycling conditions: 95°C (5 minutes), 30 cycles of 95°C (30 sec), 55°C (30 sec) and 72°C (1 min), with a final extension at 72°C for 8 min. $MgCl_2$ concentration was optimized to 3.5mM. Scale of PCR reaction was usually at 500µL (10 tubes holding 50µL reaction volumes.)

Amplicon Analyses and Preparation for Subsequent Rounds. Following PCR, sample tubes were pooled and 500µL volume was subjected to concentration with a Microcon YM-30 filter (Millipore) following the manufacturer's directions. Concentrated amplicon was mixed with standard 6X sample buffer and electrophoresed on 3.6% low-melting agarose (NuSieve, Cambrex) slab gels at 100V with Tris-acetate-EDTA buffer**.** Post-electrophoresis staining was by SYBR-gold (Molecular Probes) following manufacturer's directions, and gels were documented by a gel documentation system. The 73-bp amplicon was carefully excised with a clean razor blade and gel purification was accomplished by using a commercial kit (QIAEX II, Qiagen). Manufacturer's instructions for the kit were followed except for the amount of Qiaex II particles were increased to 40-50µL. Following elution of dsDNA from the Qiaex II

particles, 200μL of M-270 Dynabeads were prepared (see below) and concurrently, the dsDNA aliquot was heated to 95-100°C for 5 minutes and flash-cooled in an ice-ethanol bath and carefully transferred to the Dynabeads. Aptamer pool was incubated in the presence of Dynabeads for 5-10 min at 4°C, followed by magnetic capture of beads and recovery of the single-stranded, positive-sense, supernatant. This pool was utilized in the subsequent round of SELEX.

End-Labeling. For aptamer binding assays, oligonucleotide pools or oligonucleotides of interest were 5' end-labeled with γ^{33}P-ATP (EasyTides, Perkin-Elmer, NEG612H) using T4 polynucleotide kinase (OptiKinase, USB) following a protocol adapted from [33]. Briefly, 4-10 pmoles of oligonucleotide were incubated with T4 kinase, radiolabeled ATP, kinase buffer, and ddH_20 in a total volume of 50μL at 37°C for 30-45 minutes. Purification of end-labeled oligonucleotides was by Sephadex G-25 columns (Amersham) following the manufacturer's directions. Incorporation efficiency was calculated using a standard method.

DNA Aptamer Binding Assays. Binding assays were performed at periodic intervals for all DNA based selections. Without exception, the methods and environment of the binding assay conditions were similar to the selection conditions. All aptamers were end-labeled as described above. For Dynabead-bound lipopolysaccharide binding assays, standard quantities and loads of Dynabead-bound lipopolysaccharide were incubated with enriched round aptamers and beginning round 0 aptamers. Following a period of binding, Dynabeads were pelleted with brief centrifugation and captured with a magnet. Unbound supernatant and three washes with binding buffer, representing the nonbound fraction, were collected and analyzed on a scintillation counter. Radiolabeled aptamers

bound to target were counted by analyzing the entire mixture of beads, target, and bound aptamers. The percent bound represented bound counts divided by the sum of bound and unbound counts, multiplied by 100. For nitrocellulose-based selections, a similar binding assay occurred upon nitrocellulose with bound aptamers detected by direct counting of the nitrocellulose disk. Other parameters such as washing and calculations were similar to the Dynabead-based binding assay. For whole cell selections, the binding assay was ultracentrifuge-based and differed from the two prior assays in that five washing steps with binding buffer occurred instead of three. Bound aptamers were detected by direct counting of the bacterial pellet. For all types of assays, negative controls consisted of analyzing the round 0 background and false targets.

Cloning and Sequencing. A TOPO-TA Cloning Kit (Invitrogen) with supplied chemically competent *E. coli* K12 TOP10F' cells was utilized to clone enriched aptamer pools. The manufacturer's protocol was followed and modifications supportive of improved cloning for libraries were followed (prolonged cloning step). Transformed cells were grown on Luria agar supplemented with 80μg/mL ampicillin (Sigma). White colonies were grown overnight in Luria broth in deep-well plates or tubes and plasmid DNA was extracted with a FastPlasmid kit (Eppendorf). Plasmid DNA was analyzed on a 1% agarose gel and routinely stained with SYBR-Gold (Molecular Probes) and documented. Low yield or low quality plasmids were excluded from sequencing. DNA sequencing was performed by the Oklahoma State University Recombinant DNA/Protein Resource Facility or the Oklahoma Medical Research Foundation.

Sequence Analysis. Following retrieval of aptamer pool sequences from a sequencing facility, sequences were visually inspected for quality, strand sense, vector location,

primer location, homology to non-input sequence data (BLAST), and length. All sequences were converted to the positive sense. Sequences with a high degree of poor quality sequence were excluded; those which had 1-5 uncalled nucleotides were further inspected by examining their electropherograms and manually corrected. Other sequences excluded included those with a <21 nucleotide length of the random aptamer section and those with inserts from non-input genetic sources.

Utilized sequences were converted to FASTA format and imported to the SeqWeb v2.0 accelrys (GCG, Inc., Madison, Wisconsin) software suite for analyses. Analyses carried out included "Pretty" (calculated consensus sequence), "PileUp" (multiple sequence alignments), and "GrowTree" (evolutionary relationships).

RNA Aptamer SELEX Methods.

RNA Aptamer Library. The RNA aptamer library used (RNA 30N) is composed of $10^{13\text{-}14}$ species and is synthesized at the University of Texas Ellington Lab. Its sequence is GGGAAUGGAUCCACAUCUACGAAUUC$(N)_{30}$ UUCACUGCAGACUUGACG AAGCUU where N is A, G, C, or U. Primers used for reverse transcriptase PCR are 41.30 (5'GATAATACGACTCACTATAGGGAATGGATCCACATCTACGA) and 24.30 (5' AAGCTTCGTCAAGTCTGCAGTGAA).

Selection Strategies. The RNA SELEX protocol is summarized in Figure 1B. Performed in collaboration with and at the University of Texas Institute for Molecular Biology (Ellington Lab), Dynabead-immobilized lipopolysaccharide from *E. coli* O157 43888 was selected against with RNA aptamer pools. The pool in use was designated "RNA 30N", and SELEX was performed in an automated fashion six rounds at a time on a Beckman Biomek robotics station following published methods [34]. Nitrocellulose

binding assays used a standard technique that is more fully described in [34]. Two major selection experiments, encompassing 18 rounds, were performed.

Results and Discussion

Lipopolysaccharide purification and analyses. Lipopolysaccharide was extracted and purified using a modified hot phenol:water method. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver staining revealed, as expected, a fully substituted O antigen for the *E. coli* O157 ATCC 43888 as evidenced by the characteristic ladder-like banding pattern (Figure 2). The *E. coli* O157 MA6 rough mutant LPS was analyzed on the same gel and revealed a similar darkly-staining, lipid A region, and a single band equivalent in size to the lowest ladder "rung" of the smooth 43888 strain, and an absent ladder-like structure. The identity of this single band is likely the LPS species comprised of lipid A plus inner and outer core. The presence or absence of portions of the first repeat of the O antigen subunit is unknown. The differential structure of the two LPS species served as the rationale for negative and positive selections of streptavidin-bead immobilized and nitrocellulose-immobilized LPS. The *E. coli* O157 ATCC 43888 would serve as the positive target, and the *E. coli* O157 MA6 would be the negative target, with the subtractive selection process hypothesized to enrich for the O antigen region.

Selections against Dynabead-immobilized lipopolysaccharide with DNA aptamer pool. Thirty rounds of SELEX were performed utilizing biotinylated *E. coli* O157 43888 and MA6 LPS targets to select an aptamer specific for the fully-substituted LPS of *E. coli* O157. Altering target concentrations, incubation parameters, and positive and negative targets were primary means of increasing stringency and are summarized in Table 1. A binding assay was performed at round 12 and exhibited a 3.2% pool affinity for the true target which exceeded a negative control (bead immobilized *E. coli* O55 LPS) by a 2:1

ratio. Further stringency alteration occurred from rounds 13A to 21A (Table 1), and most significantly, six SELEX rounds were performed with target-pool incubation times of 4-10 minutes. The round 21A binding assay showed that both *E. coli* O157 positive target and the negative control *E. coli* O55 bound <1% of the pool, while the unloaded Dynabeads bound 7.5% of the pool (Figure 3). We speculated that the six rounds of 4-10 minute incubations with the true target may have been disadvantageous at this stage of selection for the enriched round 12 pool was putatively comprised of aptamer species that bound best following 30-45 minute incubations, which were used during rounds 1-12. Conversely, the round 12 pool putatively possessed few species capable of binding so rapidly. We therefore selected anew for nine additional rounds, designated rounds 13B-21B, by using an archived round 12 pool template. During these nine rounds, incubation times were lengthened back to 45-60 min, and only mild alterations in stringency were introduced by washing steps. Similar to the round 21A binding assay, again the enriched pool (round 21B) favored unloaded Dynabead (27.3% of pool) by a 6:1 ratio over true target, *E. coli* O157 LPS. However, assay of this pool exhibited 5.6:1 favorable binding for *E. coli* O157 LPS (4.6% of pool) over negative control, *E. coli* O55 LPS (0.8%).

The reasons for favorable binding of the unloaded bead surface in latter rounds of this selection are not known. At round 21A, it was proposed that markedly shortened incubation times during SELEX rounds of a pool enriched under longer incubation and binding conditions possibly eliminated slower binders. Although the subsequent rounds did not prove or disprove that speculation, certainly returning to the longer incubation time did not correct the problem of binding the unloaded bead.

A second mechanism that could have led to the favorable binding of the bead surface was the methods employed to negatively control binding to the bead surface. The method was simply to use the same bead for target presentation as was used for preparation of ssDNA (strand separation). The operation of preparing ssDNA was to melt dsDNA PCR product and flash cool it as the pool was applied to the Dynabeads. While the Dynabeads captured the (-) sense, biotinylated ssDNA strand, the (+) sense, non-biotinylated ssDNA strand would be exposed to the Dynabead surface. If the (+) sense ssDNA bound, then it would have been removed from future rounds of SELEX. If this was ineffective, enrichment for the bead surface could occur. Operationally, different temperature conditions employed for target presentation (25°C) and ssDNA preparation (4°C) had been utilized throughout and was speculated to be a factor that could have led to favored binding of the bead surface - the implication being that exposure of the (+) ssDNA to the beads at 4°C did not effectively control against their possible binding at 25°C. The methodological problem with the negative control could have allowed some aptamer species to bind the bead surface during target presentation, and therefore undergo enrichment. One piece of evidence that mitigates against this speculation is that such enrichment for the unloaded Dynabead did not occur through the first 12 rounds, even though identical methods were employed.

Selections against Dynabead-immobilized lipopolysaccharide with RNA aptamer pool. Concurrent with SELEX experiments using a DNA-based pool, collaborators at the University of Texas utilized the Dynabead-immobilized LPS from *E. coli* O157 ATCC 43888 as a target for RNA selections. Negative control targets (ie biotinylated LPS from *E. coli* O55 or *E. coli* O157 MA6) were not used. Eighteen rounds of SELEX were

performed on an automated robotics system, and a binding assay showed 0.4% and 0.3% of enriched pool bound the bead-immobilized and the non-immobilized LPS, respectively (Figure 4). This slightly exceeded the round 0 background for the cognate groups, but the levels did not exceed the background level of binding nitrocellulose by the round 18 RNA aptamers (0.44%) (Figure 4). An additional eighteen rounds were repeated at a later time with no appreciable change in the results (data not shown).

Selections against nitrocellulose-immobilized lipopolysaccharide with DNA aptamer pool. Because selections against Dynabead-immobilized LPS failed with DNA and RNA-based aptamer pools, and because enrichment for the Dynabead surface was deemed to be favored because its target properties were deemed superior to LPS, we sought to eliminate the introduced biotin linkage and Dynabead. We therefore chose to present purified LPS species immobilized on nitrocellulose membrane disks.

The targets presented and selection parameters through the completed 13 rounds are summarized in Table 2. Through 13 rounds of SELEX, no affinity for *E. coli* O157 LPS developed when presented on nitrocellulose. Indeed, the non-enriched round 0 background binding level was 0.6%, and round 13 enriched pool bound *E. coli* O157 LPS, *E. coli* O55 LPS, and *E. coli* O157 MA6 LPS at levels of 0.3%, 0.6%, and 0.4%, respectively (Figure 5). These disappointing results were somewhat at odds with unpublished results of SELEX experiments targeting the LPS of *Francisella tularensis*. In these experiments, a specific aptamer for *Francisella tularensis* was selected by targeting the purified O-antigen only (N. Thirumalapura, personal communication). This was performed similarly by immobilization of O-antigen upon nitrocellulose disks. A key difference between these disparate results is the different nature of the whole *E. coli*

O157 LPS and the *Francisella tularensis* O antigen. The nature of this difference is detailed further (*vide infra)*.

To summarize selections against purified LPS, three major selection efforts were completed. These efforts varied across two classical presentation strategies and varied across both DNA and RNA aptamers. All selection efforts resulted in no affinity enrichment for the purified LPS target.

Although some details differed across selection platforms, the consensus of results indicated to us that the LPS target was more likely to be problematic than the methods employed. Not unlike antibodies which bind to large and complex targets, aptamers exhibit some preferences with respect to target properties. Favorable target moieties include: hydrophilicity, cationicity, and presence of sites for hydrogen-bonding [21]. Unfortunately, purified lipopolysaccharide is a polyanion exhibiting numerous phosphate groups and also possesses a large, hydrophobic lipid A moiety [27]. This polyanionic and partially hydrophobic nature could have made aptamer interaction highly disfavored thermodynamically. Comparative data from the experiments of Thirumalapura and colleagues indicate that this idea may be correct. Indeed, by Thirumalapura's presentation of O antigen only, he removed the problematic moieties of lipid A and core that possess the disfavorable properties, hydrophobicity and polyanionicity.

Although optional strategies to compensate for disadvantageous properties of purified LPS existed, we instead chose to terminate the LPS selections and pursue a different target presentation strategy.

Selections against *E. coli* O157 and other whole bacterial cells with DNA aptamer pool. With observations of low binding and other problems with LPS targeting, we chose to reverse our targeting approach. The former LPS targeting strategy is best described as a "target in search of an aptamer." In contrast, we chose a strategy summarized as "an aptamer in search of a target" by presenting whole bacterial cells to the aptamer pool. Whole bacterial cells present an array of potential targets in their undisrupted form, including polysaccharides and protein-comprised structures such as flagella, fimbriae, outer membrane proteins, and porins.

Nineteen rounds of positive and negative selection were performed, and the selection strategy and alterations of stringency are summarized in Table 3. Ultracentrifugation was employed as the partitioning method. The round 13 binding assay revealed favorable binding to both *E. coli* O157 and *E. coli* K-12 (Figure 6), with 14.6% and 24.0% of the enriched pool binding these targets, respectively. The round 0 background level of binding was 4.2-6.0%, and the enriched pool showed no affinity for *Salmonella typhimurium* cells. The higher affinity for *E. coli* K-12 was interesting and somewhat surprising in the face of negative selection against it during the first 3 rounds (Table 3). We speculate that negative selection against this target in early rounds could have been insufficient to eliminate enriched binding for *E. coli* K-12. Additionally, the outer membrane structure is likely very similar between *E. coli* O157 and *E. coli* K-12.

The round 13 pool was cloned and sequenced at this point of experimentation and yielded 70 clones for analysis. Raw sequences were analyzed by DNA sequence analysis software and an evolutionary relationship was calculated for the sequences (Figure 7).

Visual inspection of this phylogenetic dendrogram revealed six broad families of sequences, indicative of significant pool enrichment occurring.

The strategy employed over the next 6 rounds (14-19) to decrease the binding for *E. coli* K-12 was to perform a two-stage selection at each round. This was performed by incubating the enriched pool with *E. coli* K-12 cells first, followed by incubation of the non-binding supernatant with *E. coli* O157 cells, without intervening amplification via PCR. Aptamer species that bound *E. coli* O157 after not binding *E. coli* K-12 were amplified via PCR and recycled into subsequent SELEX rounds.

The round 19 pool was cloned and sequenced and yielded 26 clones for analysis. Sequence analyses of raw sequence data resulted in a phylogenetic dendrogram that was dominanted by one large family with a minority of sequences in a second family. (Figure 8) Sequence alignment of all round 19 sequences revealed that 7 were identical and 2 additional clones had only a single mismatch or 5' deletion (Figure 9). Additionally, the clone repeated 7 times in this analysis was also present as repeats within the round 13 sequence analysis. This clone, designated 2A5, was used in the final binding assay (Figure 10). Finally, a conserved 11-mer motif was observed in 18/26 of the round 19 clones. The sequence of this 11-mer motif was CACCACMMMCM, where M is an abbreviation for the two nucleotides, cytosine and adenine, with an aMino group.

The round 19 binding assay (Figure 10) utilized the highly conserved 28-mer, 2A5-short, as well as the 71-mer, 2A5-long (including constant primer binding sites), as consensus representatives of round 19. Aptamer 2A5-long bound *E. coli* O157 whole cells at a higher level (2.5%) than 2A5-short (2.1%) and the round 0 background (1.3%), of which the difference between 2A5-long and background was statistically significant

($p<0.05$). Additionally, 2A5-long binding to *E. coli* O157 cells exceeded its binding to *E. coli* K-12 (0.6%) and *Salmonella typhimurium* (1.6%) in a statistically significant fashion ($p<0.05$). Furthermore, the interaction of 2A5-long or 2A5-short with *E. coli* K-12 or *Salmonella typhimurium* was nearly identical to the round 0 background control.

Based upon the favorable round 13 binding for K-12 cells and the dramatically reduced binding affinity for K-12 at round 19, it is apparent that aggressive negative selection achieved its purpose. The round 13 pool affinity for *E. coli* K-12 was 24% and exceeded background by a ratio of 4:1. Following the six rounds of negative selection, the round 19 (2A5 as representative) pool affinity for *E. coli* K-12 was <0.5%, and was less than the background. *Salmonella typhimurium* never exceeded its cognate negative control in binding assays.

In the round 19 binding assay, aptamer 2A5-long preferentially bound *E. coli* O157 over K-12 and *Salmonella*, as well as its negative control. 2A5-long also bound *E. coli* O157 at higher levels than 2A5-short. This is interpreted as meaning that a portion of the upstream, downstream, or both constant primer binding sites confers upon the aptamer an improved binding ability. Additional assays utilizing different segments of the 2A5 aptamer are planned to possibly further increase the binding affinity.

Sequence analyses performed at rounds 13 and 19 demonstrate the pool reduction accomplished by the SELEX protocol. However, only binding assays can demonstrate the binding properties of the reduced pool. Partially disappointing in the 2A5-binding assay is the overall level of aptamer binding for the *E. coli* O157 target. However, this binding data must be interpreted cautiously. In addition to overall low binding, it also illustrates a glaring deficiency in this area of aptamer research: relevant positive controls

for microbial surface:nucleic acid interactions do not exist. Therefore, we cannot make firm conclusions regarding its binding properties, nor can we predict the ligand's future utility.

The overall low binding of the 2A5 species for *E. coli* O157 cells, though apparently specific, is likely of low avidity. This is partially supported by the methods employed in the centrifugation-based binding assay, in which five washes of the *E. coli* O157 cellular target pellet were employed. A weakly avid aptamer species could have the potential to exhibit higher overall binding levels with fewer washing steps. However, in our hands, we have demonstrated that five washes are necessary during this binding assay to eliminate a leaching effect of non-specific (possibly enmeshed) aptamers from the target pellet (data not shown). Techniques to improve the binding of 2A5 to *E. coli* O157 cells are possible and are proposed to include testing different regions and fragment lengths of 2A5-long for improved binding and/or to possibly demonstrate higher binding to an isolated bacterial cellular component.

In summary, *in vitro* selection of DNA and RNA aptamers for *E. coli* O157 purified LPS was not accomplished. Though problematic target properties have been emphasized as possibly causative, brief commentary on general SELEX methods is important in summation. The number of rounds of SELEX to isolate a binding aptamer in published studies ranges from a low of two rounds [35] to a high number around fourteen rounds [36], with many of the reports achieving aptamer isolation within 7-12 rounds. That the three aptamer selections against LPS took 13-21 rounds, without success, again points to the possibly problematic moieties of the target, but could also indicate methodological problems with stringency of binding and partitioning.

Additionally, with acquired expertise, it became apparent that simplified selection strategies were more appropriate than complicated iterations of positive and negative selections, altering target concentrations and incubation times, and the like. The more simplified selection strategy developed for *E. coli* O157 whole cells, both in target presentation and a more nearly constant set of target incubation conditions, could have played a role in the more promising results obtained.

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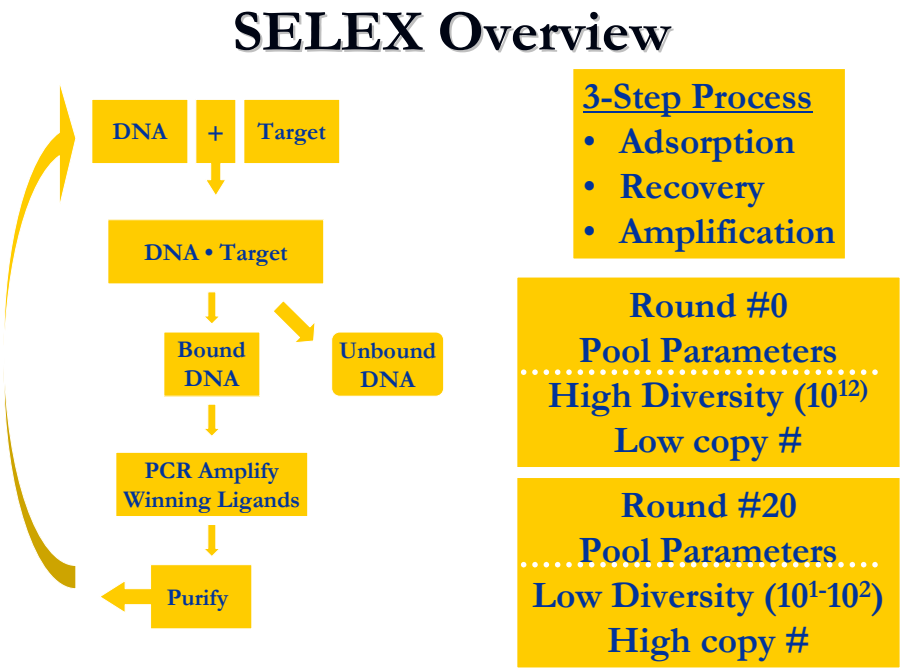
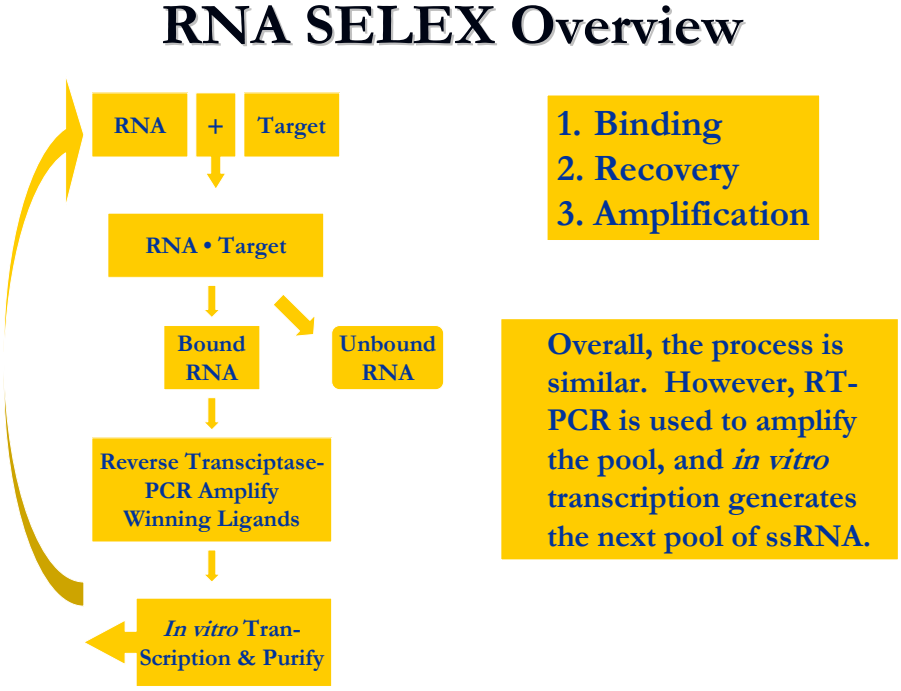
Figure 1. The SELEX Protocol

A. DNA



B. RNA



Legend for Figure 1. A) DNA SELEX. SELEX is the three-step process of adsorption of random pool to target, partitioning and recovery of bound ligands, and amplification of bound ligands via polymerase chain reaction. At the beginning, the pool complexity is high. With successful enrichment, the ending pool complexity is low, though remaining species are high copy number. B) RNA SELEX as specifically performed by the Ellington group in collaboration. Overall, the process is similar to DNA SELEX. However, two key differences are: 1) reverse transcriptase (RT) PCR (RT-PCR) is used for amplification, and 2) amplified sequences are *in vitro* transcribed to RNA species prior to the next SELEX round.

Figure 2. Lipopolysaccharide SDS-PAGE analysis.

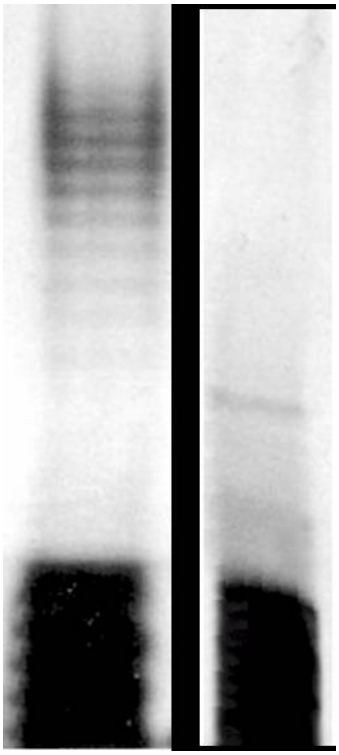


Figure 2. Left lane. Fully-substituted O-antigen ladder-like structure apparent in *E. coli* O157 ATCC 43888 LPS. Right lane. Single band at base of region where O-antigen ladder should be present in rough mutant strain *E. coli* O157 MA6.

Figure 3. DNA SELEX with Dynabead-immobilized LPS.

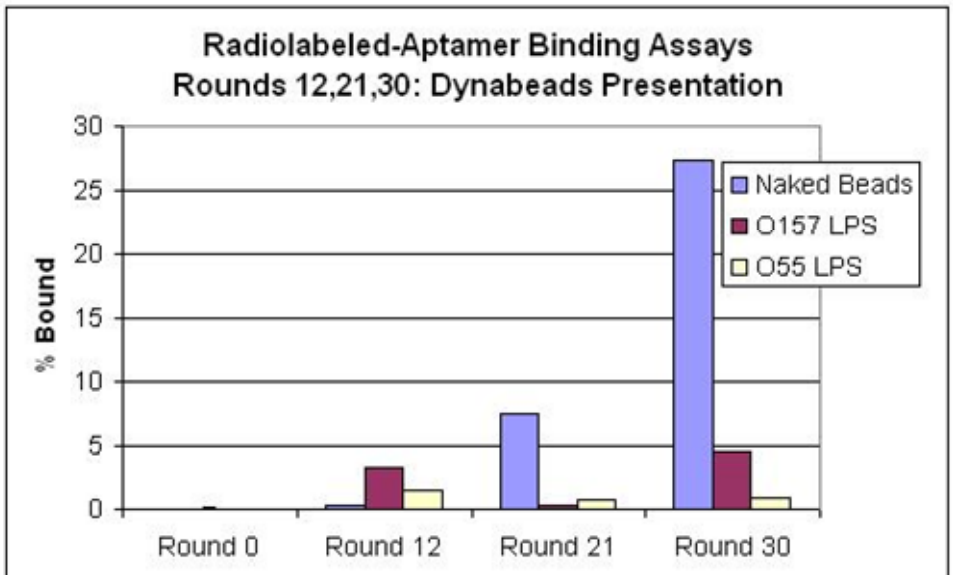


Figure 3. Binding assays performed at rounds 12, 21 (21A), and 30 (21B).

Percent bound indicates the percent of a radiolabeled starting pool (round 0 or enriched pool) bound target in a bead presentation assay.

Figure 4. RNA SELEX with Dynabead-immobilized LPS.

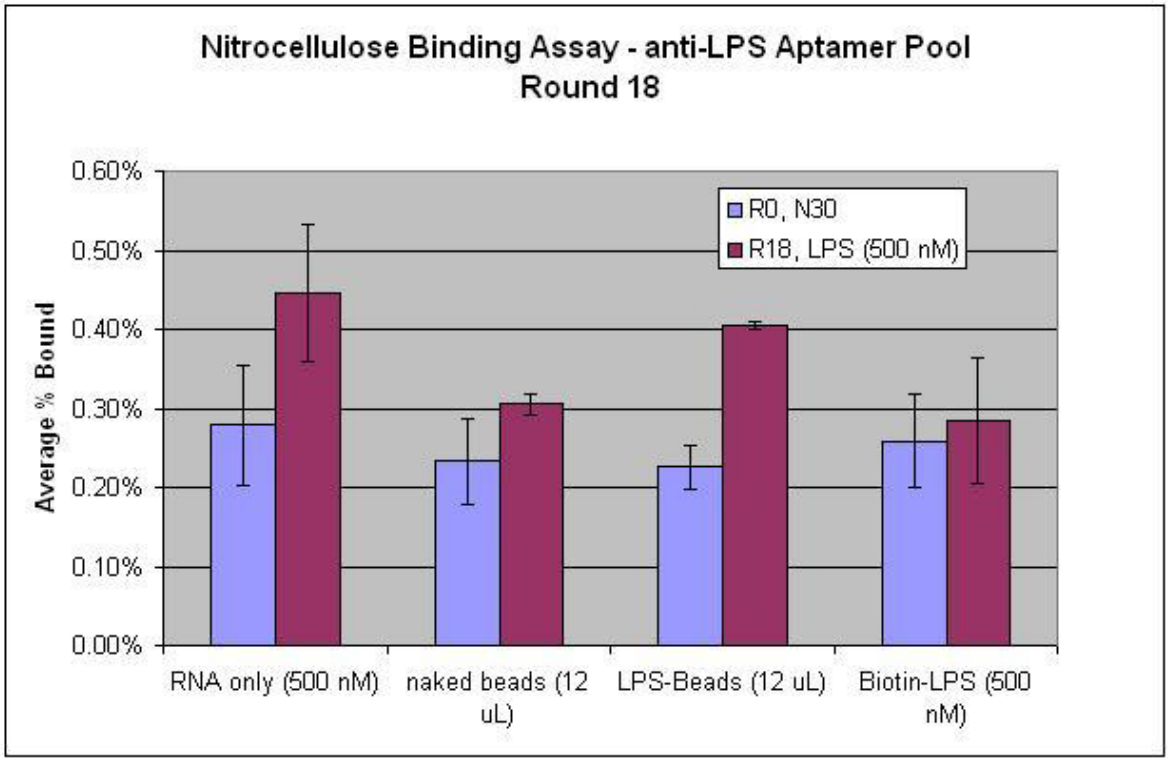


Figure 4. Binding assay performed at round 18 of automated RNA SELEX.

Percent bound indicates the percent of a radiolabeled starting pool (round 0 or round 18 pool) bound target in a nitrocellulose membrane presentation assay.

Figure 5. DNA SELEX with nitrocellulose-immobilized LPS.

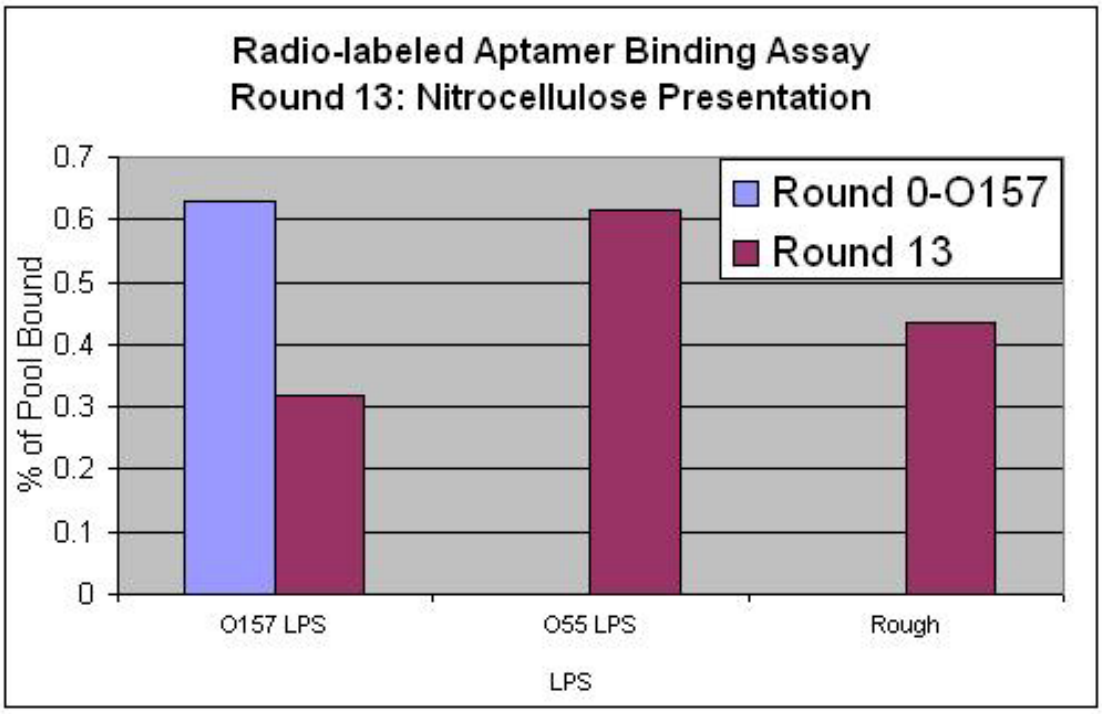


Figure 5. Binding assay performed at round 13 for nitrocellulose-presented LPS. Percent bound indicates the percent of a radiolabeled starting pool (round 0 or enriched pool) bound target in a nitrocellulose membrane presentation assay.

Figure 6. DNA SELEX with whole bacterial cells, round 13.

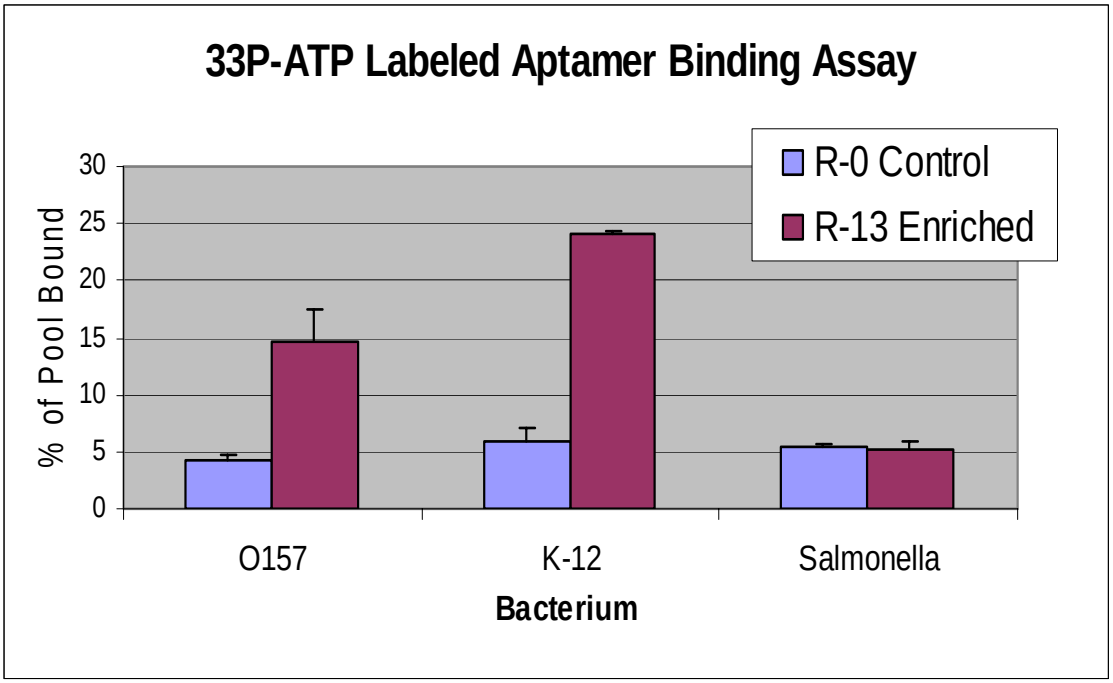


Figure 6. Binding assay for whole bacterial cell aptamer selections at round 13. Percent bound indicates the percent of the beginning radiolabeled round 0 or round 13 enriched pool that bound various targets in a centrifugation-based assay. Error bars represent one standard deviation from the mean.

Figure 7. Evolutionary relationships of enriched species, round 13.

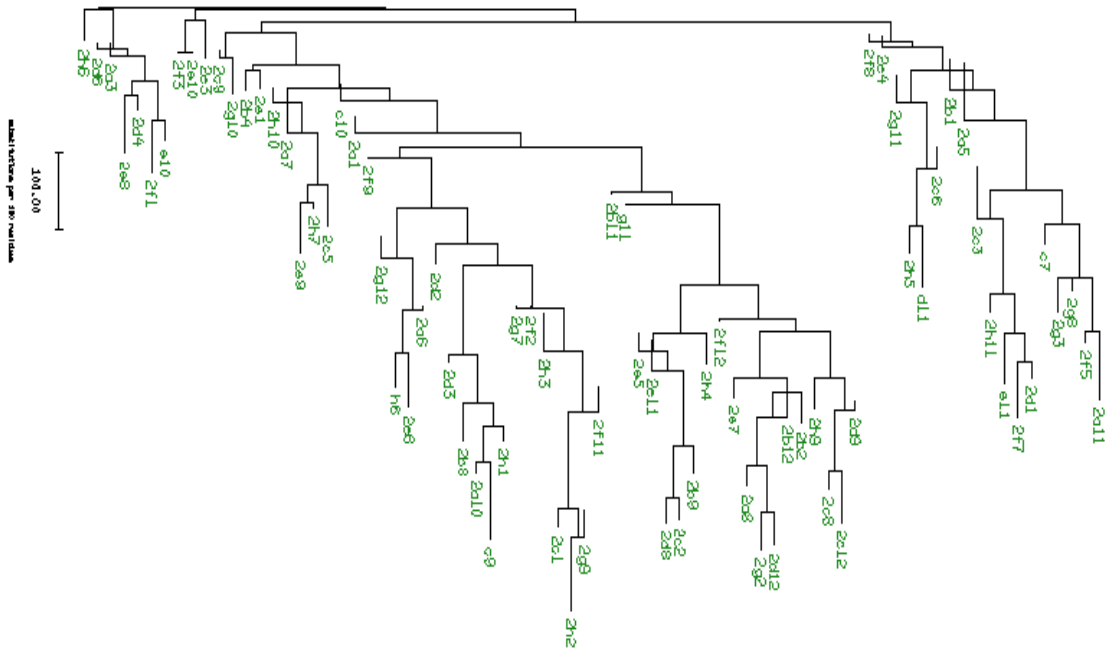


Figure 7. Phylodendritic tree computed by “GrowTree” (accelrys GCG). Six broad families of sequences were visually appreciated.

Figure 8. Evolutionary relationships of enriched species, round 19.

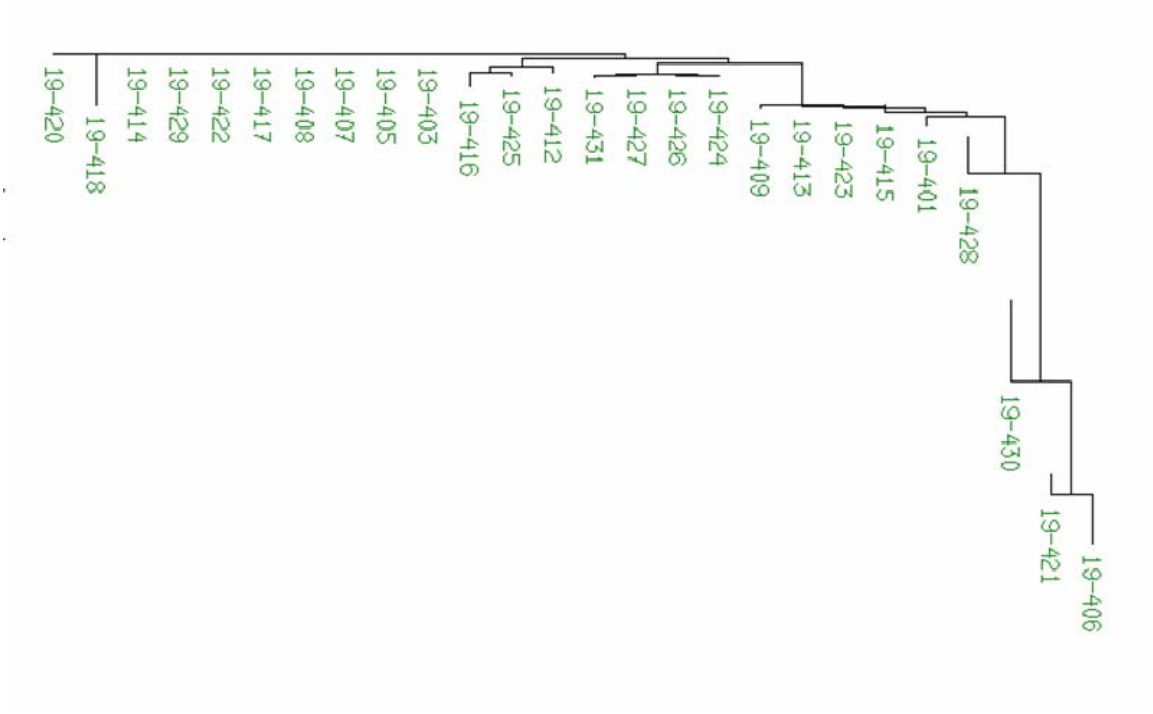


Figure 8. Phylodendritic tree computed by "GrowTree" (accelrys GCG). One dominant, highly conserved family and one smaller family of sequences was visually appreciated.

Figure 9. Sequence alignment of 26 sequences, round 19, whole cell selection.

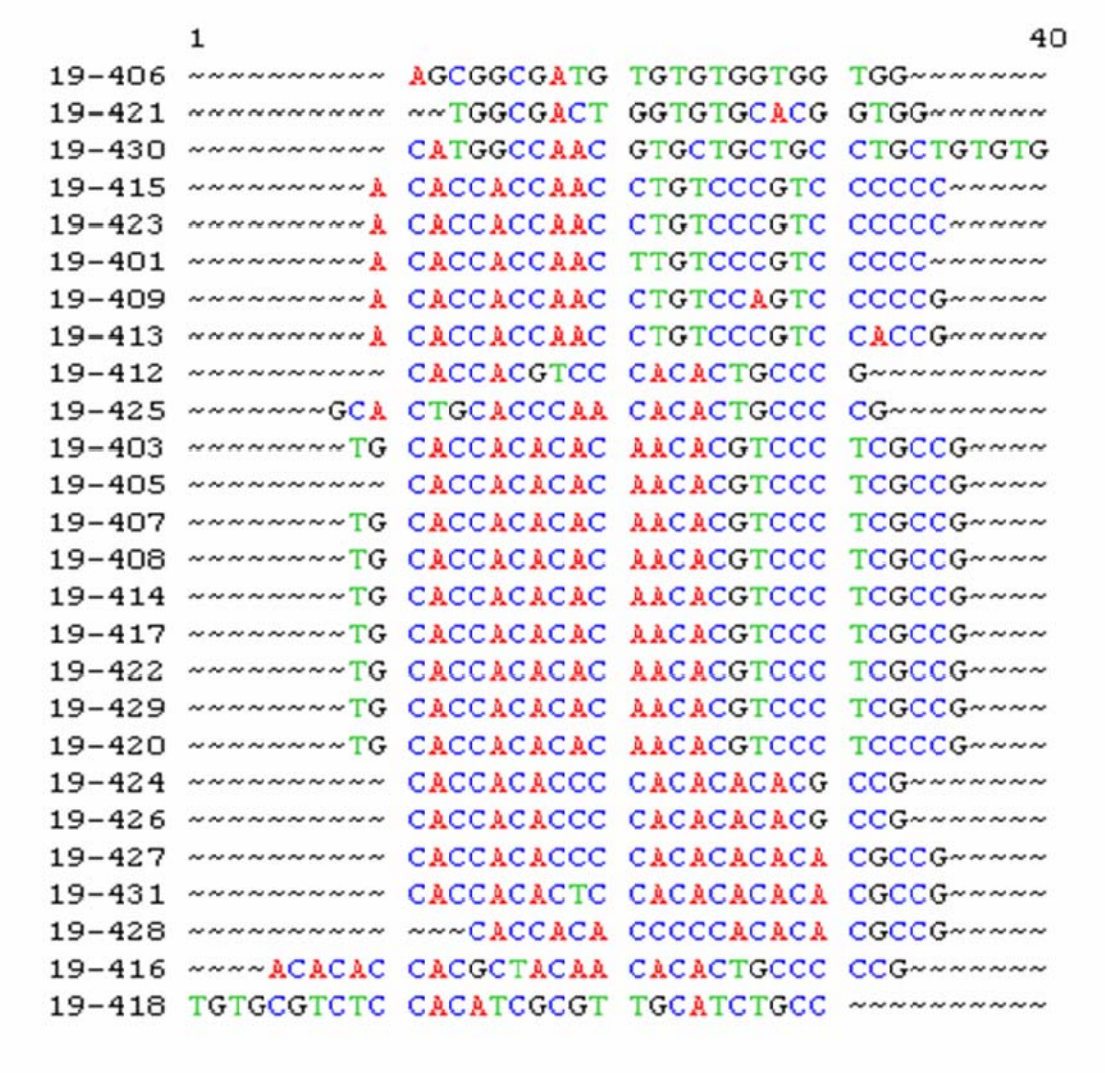


Figure 9. Sequence alignment computed by "Pretty" (accelrys GCG). Seven of these were identical 28-mers, and two additional sequences had only one mismatch or 5' deletion. The seven identical 28-mers were identical to the sequence repeated twice, 2A5, in the round 13 sequence analysis.

Figure 10. DNA SELEX with whole bacterial cells, round 19.

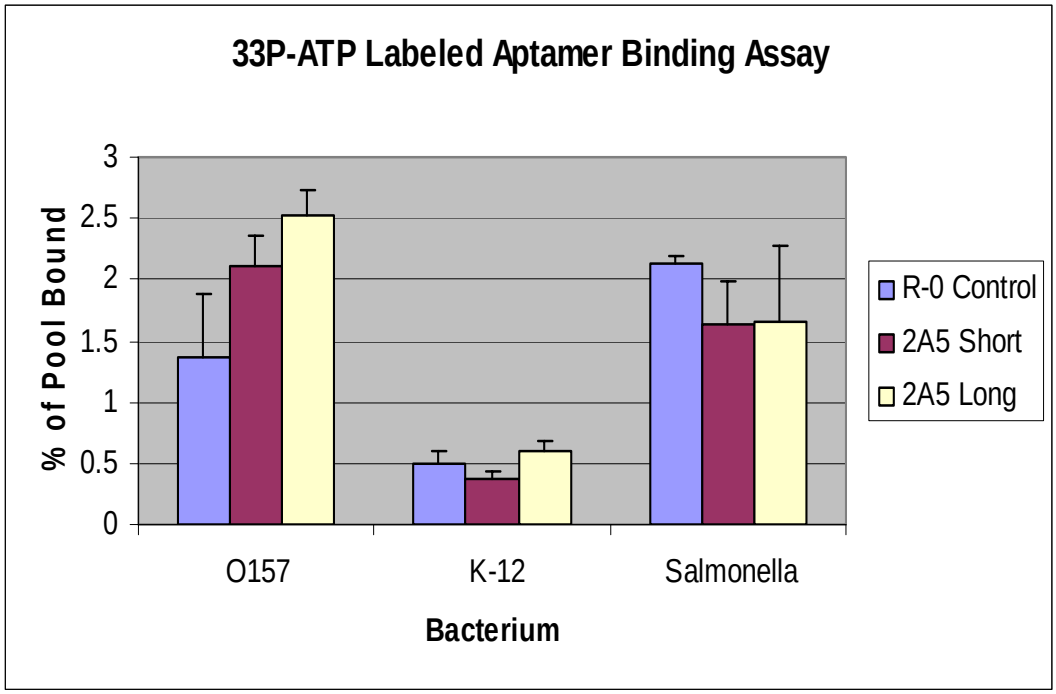


Figure 10. Binding assay for whole bacterial cell aptamer selections at round 19. Percent bound indicates the percent of the beginning radiolabeled round 0 or two lengths of 2A5, a round 19 enriched species, pool that bound various targets in a centrifugation-based assay. Error bars represent one standard deviation from the mean.

Round	LPS Target	Incubation	Other Comment
1	*E. coli* O157 43888	30 min	2X target available
2-6	*E. coli* O157 43888	30 min	Decreasing target conc.
7-9	*E. coli* O157 MA6	30-35 min	Increasing target conc
10-12	*E. coli* O157 43888	40-60 min	Positive selection
13-15A	*E. coli* O157 43888	4 - 45 min	To favor rapid binders
16A	*E. coli* O157 MA6	16 hrs	Prolonged incubation to
17A	*E. coli* O157 MA6	2 hrs	exclude species
18A-21A	*E. coli* O157 43888	4-10 min	PCR Cycles: 25,20,15,30
13B-14B	*E. coli* O157 43888	1 hr, 1wash	Began with archived R12
15B-17B	*E. coli* O157 MA6	45-60min	1 wash
18B-20B	*E. coli* O157 43888	1hr	2 washes
21B	*E. coli* O157 43888	1hr	3 washes

Table 1. Dynabead Presentation of LPS: Targets, incubations, and other parameters.

Round	LPS Target	Incubation	# of Washes
1-4	*E. coli* O157 43888	90-120 min	1
5-7	*E. coli* O157 43888	1 hr	2
8	*E. coli* O157 MA6	1 hr	1
9-11	*E. coli* O157 43888	1 hr	2
12	*E. coli* O157 MA6	1 hr	2
13	*E. coli* O157 43888	1 hr	3

Table 2. Nitrocellulose-presented LPS: Targets, incubation times, other parameters

Round	Target	Strategy/Comment	
1	*E. coli* K12 TOP10F'	Negative selection; 5 X 10^6 cells	
2-3	*E. coli* K12 TOP10F'	Negative selection; 1 X 10^5 cells	
4	*E. coli* O157 43888	4 X 10^4 cells; 1 wash	
5-7	*E. coli* O157 43888	4 X 10^4 cells; 2 wash	
8-10	*E. coli* O157 43888	4 X 10^4 cells; 3 wash	
11	*E. coli* O157 43888	3 X 10^4 cells; 3 wash	
12	*E. coli* O157 43888	2 X 10^4 cells; 3 wash	
13	*Y. pestis* KIM5 (avirulent)	1 X 10^6 cells; 3 wash	
14-19	Ec K-12 (1^{st}) & O157 (2^{nd})	Pool was incubated with K-12 first, then O157, followed by PCR.	K-12: 2 X 10^5 cells O157: 2 X 10^5 cells 3 washes

Table 3. Whole cell selections: Target presented and other strategies

VITA

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Doctor of Philosophy

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