ROLE OF CURLI FIMBRIAE IN MEDIATING THE CELLS OF ENTEROHEMORRHAGIC 

*Escherichia coli* TO ATTACH TO ABIOTIC AND BIOTIC SURFACES

by

DHARMENDRASINGH PAWAR

(Under the Direction of JINRU CHEN)

ABSTRACT

Cells of enterohemorrhagic *E. coli* (EHEC) express long, thin, wiry fimbriae know as curli. Three pairs of EHEC cultures, each with a curli-expressing and non curli-expressing strain were used in this study to examine the effect of incubation temperature and growth media on curli-expression, and the role of curli in attachment to abiotic and biotic surfaces. Growth of the cells on 3 growth media at 5 incubation temperatures revealed that curli is not expressed at 10°C, and curli phenotypic conversion took place under appropriate incubation conditions. Results of crystal violet binding assays indicate that cells of the curli-expressing strains attached better than did the cells of the non curli-expressing strains. The curli-expressing cells also attached more efficiently than the non curli-expressing cells on beef with the smallest differences of 0.12 and 0.22 Log_{10} CFU/cm² and the largest differences of 0.86 and 1.17 Log_{10} CFU/cm² on raw beef and beef salami, respectively.

INDEX WORDS: Curli, attachment, enterohaemorrhagic *Escherichia coli*, polystyrene, glass, stainless steel, rubber, beef, ready-to-eat
ROLE OF CURLI FIMBRIAE IN MEDIATING THE CELLS OF ENTEROHEMORRHAGIC

*ESCHERICHIA COLI* TO ATTACH TO ABIOTIC AND BIOTIC SURFACES

by

DHARMENDRASINGH PAWAR

B. Tech., Dairy Technology,
Gujarat Agricultural University, India, 2004

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial
Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2004
ROLE OF CURLI Fimbriae in Mediating the Cells of Enterohemorrhagic Escherichia Coli to Attach to Abiotic and Biotic Surfaces

by

Dharmendrasingh Pawar

Major Professor: Dr. Jinru Chen
Committee: Dr. Mark Harrison
Dr. Yao-wen Huang

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2004
DEDICATION

To my Mom, Dad, and Grandfather
ACKNOWLEDGEMENTS

My Dad always says, “If you want to reach the top, go step by step”. In my quest to reach the top, there are people who helped me, and now I would like to thank all of them, knowing very well that, mere words are not enough for whatever they did for me.

Dr. Jinru Chen, for accepting me as her graduate student and providing me invaluable suggestions in conducting the research. I appreciate her understanding, patience, and support.

Dr. Mark Harrison and Dr. Yao-wen Huang for serving on my advisory committee and providing valuable inputs.

Joy Adams for her friendship and technical assistance. Shiao Mei Lee, Julie Yeh, Denton Giles, Noah Giles, Sudeep Jain, and Suvang Trivedi for being helpful and encouraging lab mates.

Jerry Davis for teaching me statistical analysis and Dr. John Shields for help with scanning electron microscopy.

The faculty and staff of Department of Food Science and Technology in Athens and Griffin campuses.

Abhay Shah, Dr. Sivakumar Pattathil, Supriyo Ghosh, and Rashmi Deshpande for their social activities to turn my graduate studies into one wonderful adventure.

My parents, grandparents, Tinu, and Minu for their unconditional love, support, and perennial encouragement.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS.............................................................................................................v  
LIST OF TABLES......................................................................................................................viii  
LIST OF FIGURES. ..................................................................................................................ix  

CHAPTER  
1 INTRODUCTION..................................................................................................................1  
2 LITERATURE REVIEW..........................................................................................................3  
   Enterohemorrhagic Escherichia coli (EHEC).........................................................................3  
   Factors affecting bacterial attachment..............................................................................8  
   Mechanism of attachment on food contact surfaces.........................................................17  
   Properties of surface-associated cells.............................................................................19  
   Objectives of the study.......................................................................................................19  
   References..........................................................................................................................21  

3. PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF CURLI-EXPRESSING  
   ENTEROHEMORRHAGIC Escherichia coli.........................................................................39  
   Abstract..............................................................................................................................40  
   Introduction.......................................................................................................................41  
   Materials and methods....................................................................................................42  
   Results..............................................................................................................................46  
   Discussion.........................................................................................................................48  
   Acknowledgements...........................................................................................................50
LIST OF TABLES

Table 3.1: Quantification of curli expressed by EHEC cultures........................................53

Table 3.2: The effect of 3 growth media and 5 incubation temperatures on rate of curli
expression of EHEC cultures..................................................................................54

Table 4.1: Correlation between the populations of EHEC cells and the optical densities of the
crystal violet extracted from the EHEC cells that attached to the polystyrene
surfaces..................................................................................................................75

Table 4.2: The $P$ values that reflect the differences in EHEC attachment on abiotic surfaces in
the long- and short-term attachment studies.......................................................76

Table 4.3: The effect of cell-surface contact time on the efficiency of EHEC attachment to
abiotic surfaces in the long-term attachment study.............................................77

Table 4.4: The effect of cell-surface contact time on the efficiency of EHEC attachment to
abiotic surfaces the short-term attachment study..............................................78

Table 5.1: Attachment of curli-expressing and non curli-expressing EHEC cells at different
attachment times to raw beef..............................................................................95

Table 5.2: Attachment of curli-expressing and non curli-expressing EHEC cells at different
attachment times to beef salami........................................................................96
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Quantification of curli expressed by EHEC cultures</td>
<td>55</td>
</tr>
<tr>
<td>3.2</td>
<td>Scanning electron micrographs of curli-expressing cells 5-11C⁺ (A) and 7-52C⁺ (C) and as well as non curli-expressing cells 5-11C⁻ (B) under the magnification of 10,000 times</td>
<td>56</td>
</tr>
<tr>
<td>3.3</td>
<td>Growth of curli-expressing and non curli-expressing EHEC cells on MGA and casamino acid agar</td>
<td>57</td>
</tr>
<tr>
<td>3.4</td>
<td>PCR amplification of a 417-bp csgA product from curli-expressing and non curli-expressing EHEC cells</td>
<td>58</td>
</tr>
<tr>
<td>4.1</td>
<td>Scanning electron micrographs of curli-expressing (A) and non curli-expressing cells (B) of E. coli O157:H7 5-11</td>
<td>79</td>
</tr>
<tr>
<td>4.2</td>
<td>Long-term attachment study</td>
<td>80</td>
</tr>
<tr>
<td>4.3</td>
<td>Short-term attachment study</td>
<td>81</td>
</tr>
<tr>
<td>5.1</td>
<td>Beef and salami slices used in the attachment studies</td>
<td>97</td>
</tr>
<tr>
<td>5.2</td>
<td>Light micrographs of curli-expressing (A), and non curli-expressing (B) EHEC cells used in beef attachment studies</td>
<td>98</td>
</tr>
<tr>
<td>5.3</td>
<td>Removal of EHEC cells that attached on the surfaces of agar plates</td>
<td>99</td>
</tr>
<tr>
<td>5.4</td>
<td>Total plate counts of EHEC cells that attached on the surface of TYE agar plates</td>
<td>100</td>
</tr>
<tr>
<td>5.5</td>
<td>Attachment of E. coli 7-57C⁺ and 5-9C⁻ (A), 7-52C⁺ and 7-52C⁻ (B), as well as 5-11C⁺ and 5-11C⁻ on raw beef</td>
<td>101</td>
</tr>
</tbody>
</table>
Figure 5.6: Attachment of *E. coli* 7-57C$^+$ and 5-9C$^-$ (A), 7-52C$^+$ and 7-52C$^-$ (B), as well as 5-11C$^+$ and 5-11C$^-$ beef salami………………………………………………………102

Figure 5.7: Scanning electronic micrographs of *E. coli* cells attached to the surface of raw beef…………………………………………………………………………………103
CHAPTER 1
INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) have emerged as foodborne pathogens of global importance (Bell, 2002). More than 160 serotypes of EHEC have been identified (Tkalcic, 2001). The illnesses caused by EHEC include hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura (Fey *et al*., 2000; Karmali, 1989; Sussman, 1997). Raw and ready-to-eat beef products have been implicated in the outbreaks of EHEC infections (Blanco *et al*., 1996; CDC, 1995; Duffy *et al*., 2000). A survey conducted in the Northwestern regions of the U.S. revealed that the prevalence of EHEC O157:H7 in pre-eviscerated, post-eviscerated, and post-processed beef carcasses was 43% (148 of 341), 18% (59 of 332), and 2% (6 of 330), respectively (Elder *et al*., 2000). *E. coli* O157:H7 cells attached to the processing surfaces are possible source of beef contamination (Farrell *et al*., 1998; Flores and Tamplin, 2002).

EHEC attachment to meat as well as to other surfaces is a complex process that can be influenced by the properties of both EHEC and their contact surfaces. Certain EHEC strains express a thin, wiry, aggregative surface fiber, known as curli (Olsen *et al*., 1989; Olsen *et al*., 1993; Uhlich *et al*., 2001). Expression of curli is observed under conditions like low osmotic pressure, nutritional starvation, and lower than optimal growth temperatures (Olsen *et al*., 1989; Olsen *et al*., 1993a). Previous studies have demonstrated that the curli-expressing *E. coli* O157:H7 cells are more virulent and pathogenic than non-curli expressing cells (Uhlich *et al*., 2001, 2002). However, the role of curli in attachment to abiotic and biotic surfaces has not been determined. In this study, we evaluated the effect of growth media and incubation temperature
on curli expression, and the efficiency of attachment of the three pairs of EHEC cultures, each with a curli-expressing and a non curli-expressing strain (O157:H7 7-57C\(^+\) and 5-9C\(^-\), O111:H-5-11C\(^+\) and 5-11C\(^-\), as well as O103:H2 7-52C\(^+\) and 7-52C\(^-\)) to polystyrene, glass, stainless steel, rubber surfaces as well as to raw beef and beef salami.
CHAPTER 2
LITERATURE REVIEW

I. ENTEROHEMORRHAGIC *ESCHERICHIA COLI* (EHEC)

A. General microbiological characteristics

*Escherichia coli* are gram-negative, asporogenous, and facultative anaerobic rods. Most *E. coli* strains are not pathogenic; however, some have been known to cause mild to severe diseases. Diarrheagenic *E. coli* are comprised of seven different groups which include enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), diarrhea-associated hemolytic *E. coli* (DHEC), and cytolethal distending toxin (CDT)-producing *E. coli* (Clarke, 2001). This method of classification is sometimes misleading because some *E. coli* strains fall under more than one group (Salyers and Whitt, 1994). A more realistic method of classification is by the identification of *E. coli* surface antigens such as somatic (O), flagellar (H), and capsular (K) antigens (Jay, 2000). Thus far as many as 167 O antigens, 53 H antigens, and 72 K antigens have been identified in *E. coli* (Lior, 1994).

*E. coli* O157:H7 is a member of EHEC which produces Shiga toxins similar to the toxin produced by *Shigella dysenteriae* type 1 (Calderwood et al., 1996). The pathogen is similar to other serotypes of *E. coli* with the exception of its inability to ferment sorbitol within 24 h (Ratnam et al., 1988; Wells et al., 1983), the lack of β-glucuronidase (Doyle and Schoeni, 1984; Ratnam et al., 1988), and the production of enterohemolysin (Beutin et al., 1989).
B. Association with illness

Two outbreaks of severe bloody diarrheal syndrome occurred in both Oregon and Michigan in 1982. The outbreaks were linked to the consumption of hamburgers from a fast food restaurant in these states (Riley et al., 1983). *E. coli* O157:H7 was later isolated from the beef patties involved in the outbreaks. In addition to *E. coli* O157:H7, *E. coli* O103:H2, O111:H-, and several other serotypes have also been found to cause hemorrhagic colitis (HC). These serotypes are placed into the group of EHEC along with *E. coli* O157:H7.

Human infection of *E. coli* O157:H7 can be asymptomatic or lead to the development of a variety of symptoms including watery diarrhea, bloody diarrhea, HC, hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP) (Griffin et al., 1988). The incubation period of *E. coli* O157:H7 infection ranges from 3 to 4 days with approximately 25-75% of infected patients developing watery diarrhea. The disease may resolve without further progression but in some individuals it may progress to bloody diarrhea with small or large amounts of blood in the stool. HUS may develop in 4 to 10% of the patients with bloody diarrhea.

C. Pathogenesis

The virulence factors of EHEC include Shiga toxins, hemolysin, intimin, serine protease, type III secretion proteins, and O157 lipopolysaccharide O-side chain. EHEC produces more than one type of Shiga toxins (Nataro and Kaper, 1998). The genes for the toxins are located on losogenic λ-phage (O'Brien et al., 1984). Shiga toxins are classified into two major groups, Stx1 and Stx2 (Scotland et al., 1985). Shiga toxins contain two subunits, A and B (Calderwood et al., 1987). Subunit A is an N-glycosidase and specifically removes one adenine base from the 60S ribosomal subunit, inhibiting protein synthesis (Tarr, 1995). The B subunit is a pentamer that
binds to the host cell receptors for Shiga toxins, glycolipid globotriaosylceramide (Gb3). The receptors for Shiga toxin are abundant in the cortex of the human kidney (Sandvig and van Deurs, 1996; Schmidt et al., 1996) and the toxins, hence, cause severe kidney damage and even kidney failure.

*E. coli* O157:H7 strains carry a 60-kb plasmid which encodes putative virulence factors, EHEC-hemolysins, and serine protease (Toth et al., 1990). EHEC hemolysins belong to the family of pore-forming proteins. The toxic effects of the hemolysins include changing the permeability of cytoplasmic membranes of the mammalian cells. Serine protease is encoded by *espP*, which cleaves pepsin A and human coagulation factor V. Patients suffering from HC often experience prolonged blood clotting (Elliott et al., 1998).

EPEC strains were the initial group that was recognized to produce A/E lesions. Certain strains of EHEC were later found to be able to cause A/E lesions as well (Francis et al., 1986; Sherman et al., 1988). The genetic elements that are responsible for the formation of the A/E lesion are located on the locus of enterocyte effacement (LEE) pathogenicity island (Donnenberg et al., 1997). The LEE pathogenicity island encodes intimin, translocated intimin receptor (Tir), and Type III secretion system. Intimin is the adherence factor for *E. coli* O157:H7 while the Tir protein triggers host-signaling events and involves in the development of A/E lesions. The type III secretion proteins and lipopolysaccharide are involved in the adhering of EHEC to the host epithelial cells and exports virulence factors directly to eukaryotic cells (Bilge et al., 1996; Cohen and Giannella, 1992; Jarvis and Kaper, 1996).

**D. Modes of transmission**

*E. coli* O157:H7 can be transmitted through the consumption of contaminated food or water, person-to-person transmission, and contact with farm animals. Epidemiological studies
have shown that undercooked ground beef and ready-to-eat beef products such as salami have been involved in outbreaks of *E. coli* O157:H7 infections (Blanco et al., 1996; CDC, 1995; Duffy et al., 2000). Dairy and beef cattle are reservoirs of *E. coli* O157:H7 and cattle fecal shedding is a source of direct and indirect environmental contamination (Rahn et al., 1997). Contamination of meat and meat products can be caused by the exposure of beef carcasses to dry and wet fecal materials in a slaughter line (Elder et al., 2000). Furthermore, contaminated grinding equipment in a processing plant itself is a point of concern for contamination. Routine cleaning and sanitation methods sometimes fail to completely remove *E. coli* O157:H7. Meat grinders are a possible source of contamination of large quantities of ground meat (Farrell et al., 1998).

Roast beef (Rodrigue et al., 1995), deer jerky (Keene et al., 1997b), pre-cooked ground beef (Belongia et al., 1991), raw milk (Keene et al., 1997a; CDC, 1996), raw goat milk (Bielaszewska et al., 1997), and goat cheese (Belongia et al., 1991) have all served as a vehicle for *E. coli* O157:H7 infections. Cross contamination of properly processed meat, pasteurized milk, and yogurt have also been linked to the illness associated with *E. coli* O157:H7 (CDC, 1995; Morgan et al., 1993; Upton and Coia, 1994). Outbreaks of *E. coli* O157:H7 infection are associated with consumption of vegetables such as leaf lettuce (Ackers et al., 1998) and mesclun lettuce (Hilborn et al., 1999), fruit juices such as apple cider (Besser et al., 1993), as well as cantaloupe. *E. coli* O157: H7 has been shown to be able to survive in drinking water for several weeks even at low temperatures (Artz and Killham, 2002). It is therefore, not surprising that outbreaks of *E. coli* O157:H7 infection have been linked to tap water (Swerdlow et al., 1992), ice (CDC, 2000), well water (CDC, 1999) and swimming pool water (Keene et al., 1994).
HUS has occurred in daycare facilities (Belongia et al., 1993; Reida et al., 1994) and institutions for mentally retarded persons (Pavia et al., 1990). The fecal oral route may be the cause of the spread in such places due to poor hygiene. There have been cases of *E. coli* O157:H7 infection among school children that had visited farms and had close contact with farm animals (Crump et al., 2002; Renwick et al., 1993).

**E. Survival in food**

*E. coli* O157:H7 was found to be able to survive in beef patties for at least 9 months at –20°C (Doyle and Schoeni, 1984). The pathogen could not grow at 2-5°C but formed long filaments. The *E. coli* O157:H7 strain used in the study was not resistant to heat and had a D value of <1 min at 63°C in ground beef with a fat content up to 20%. The D values of the *E. coli* O157:H7 strain at 57.2, 60, 62.8, or 64.3°C are 270, 45, 24, and 9.6 min, respectively (Doyle and Schoeni, 1984). The effectiveness of heat inactivation was influenced by product pH, water activity, humectants, fat content, and prior history of stress. Heating of food to an internal temperature of at least 68.3°C for several seconds ensured complete inactivation of *E. coli* O157:H7 (Meng et al., 2001).

The minimum pH for *E. coli* O157:H7 growth is 4.0 to 4.5. The survival of the pathogen is however, influenced by the type and concentration of acid present in a particular food product. *E. coli* O157:H7 survived for 10-13 days at 8°C and 2-3 days at 25°C (Zhao et al., 1993). Hot sprays (55°C) of acid did not have adverse effect on *E. coli* O157:H7. On the contrary, the presence of acetic (pH 5.2), citric (pH 4.0), lactic (pH 4.7), malic (pH 4.0), mandelic (pH 5.0), and tartaric (pH 4.1) acid enhanced the survival of *E. coli* O157:H7 at 4°C compared to the unacidified control (Conner and Kotrola, 1995). Penetration of *E. coli* O157:H7 to deeper layers
of muscle tissues and attachment of *E. coli* O157:H7 to collagen fibers may be responsible for the reduced effectiveness of the acid treatment (Fratamico *et al*., 1996).

**II. FACTORS AFFECTING BACTERIAL ATTACHMENT**

**A. Cell Surface Structures**

1. **Curli**

   Olsen *et al*., (1989) found that approximately half of the bovine mastitis and fecal isolates of *E. coli* tested in their study were able to bind $^{125}\text{I}$-labeled fibronectin. This binding was observed when cells were grown on colonization factor antigen (CFA) agar at temperatures between 26° and 32°C. The electron microscopy of these *E. coli* cells revealed thin, wiry, coiled surface fimbriae of various lengths, known as curli (Olsen *et al*., 1989). Growth *in vitro* at 37°C inhibits the expression of curli in most *E. coli* strains (Olsen *et al*., 1993a). The diameter of curli under higher magnification of transmission electronic microscope is ~2 to ~12 nm (Chapman *et al*., 2002; Olsen *et al*., 1989). Curli has the property to laterally aggregate and form thick bundles (Olsen *et al*., 1989). The cross-sectional diameter of laterally aggregated curli is 60 nm under a scanning electron microscope (Prigent-Combaret *et al*., 2000). Curli binds Congo red dye and curlinated cells of *E. coli* form dark red colored colonies on CFA agar supplemented with the dye (Hammar *et al*., 1995). Curli are highly stable and treatment with 90% formic acid is required to depolymerize curli into soluble subunits (Hammar *et al*., 1996).

   **a. Structure of curli**

   The genes for curli synthesis are encoded by two divergently transcribed operons, *csgBA* and *csgDEFG* (Hammar *et al*., 1995). The *csgBA* encodes the major subunit protein CsgA (curlin) and CsgB (Olsen *et al*., 1993a). The amino acid sequences of CsgA and CsgB reflect a tertiary structure, and are made up of basic unit of β-strand. Overall, CsgB shares 49% of
similarity and 30% identity with CsgA and hence is speculated to be a minor component of curli (Arnqvist et al., 1994).

The csgDEFG operon is upstream of csgBA and is in opposite transcriptional direction of csgB and csgA. The csgD, csgE, csgF, and csgG encode polypeptides of 216, 129, 138, and 277 amino acids, respectively (Hammar et al., 1995) and are involved in curli formation since polar mutations in any one of the genes abolish the expression of curli (Hammar et al., 1995). The CsgD is a transcriptional regulator (Uhlich et al., 2001), and CsgG is an outer membrane lipoprotein, which is required for maintaining the stability of CsgA and CsgB complex by preventing them from premature proteolysis. In this sense, the CsgG functions as a chaperone protein that encapsulates and escorts the CsgA and CsgB complex (Loferer et al., 1997). The CsgE is also as a chaperone-like protein whereas, the CsgF is a nucleator protein and is required for proper nucleation of CsgA on CsgB (Chapman et al., 2002).

b. Biosynthesis of curli fimbriae

The CsgA and CsgB proteins secreted in a soluble form are required for the formation of curli. The secreted CsgA precipitates on CsgB anchored on cell membrane (Bian and Normark, 1997). The CsgB is believed to impose a conformational alteration on CsgA by protein interaction during CsgA polymerization and curli assembly. Neither CsgA⁻CsgB⁺ nor CsgA⁺CsgB⁻ mutant cells express curli. However, when both are grown together, curli is formed on CsgA⁻CsgB⁺ but not on CsgA⁺CsgB⁻ cells (Hammar et al., 1996). This extracellular complementation strongly suggests that the assembly of curli takes place extracellularly.

c. Regulation of curli expression

Curli expression is regulated by temperature, osmotic pressure, and other growth conditions (Olsen et al., 1989). Curli biosynthesis takes place at temperatures below 37°C on
low osmolarity growth medium (Olsen et al., 1989). The csgA transcription occurs after 48 h of growth and is repressed by the addition of 0.35 M NaCl to growth medium (Olsen et al., 1993b). Curli is not expressed in exponentially growing bacteria (Olsen et al., 1993a). The expression of curli in the stationary-growth phase is induced by the stationary-phase-specific sigma factor, RpoS. It is speculated that the transcriptional activation of csgA by RpoS in the late stationary phase is to increase the cell’s ability to survive in the nutritional limiting situation (Olsen et al., 1993a). Transcription of csgA becomes independent of sigma factor in the absence of the histone-like protein H-NS, a DNA-binding protein that can affect either positively or negatively the transcription of a number of E. coli genes (Hammar et al., 1996). Interestingly, RpoS and HN-S double mutants retain growth phase control of csgA transcription, indicating the existence of elements other than sigma factor in the regulatory response (Olsen et al., 1993b). Increased osmolarity has been reported to inhibit the transcription of curli genes (Olsen et al., 1993b).

The two component system OmpR/EnvZ is implicated in the regulation of curli biogenesis (Dorel et al., 1999). OmpR is a cytoplasmic protein, known to positively regulate curli expression by binding to the csgDEFG operon. The EnvZ is a sensor protein and senses external osmolarity and mediates phosphorylation of OmpR, which may change its binding affinity for different sites in the target promoters. A low intracellular concentration of phosphorylated OmpR corresponds to the low-osmolarity state. This in turn activates the transcription of both csgBA and csgDEFG, thus curli biosynthesis takes place (Pratt and Silhavy, 1994; Russo and Silhavy, 1991; Vidal et al., 1998).

d. Role of curli fimbriae

Curli binds to several matrix and plasma proteins such as fibronectin, laminin, plasminogen, tissue plasminogen activator (Olsen et al., 1989), major histocompatibility
complex (MHC) class I molecules, and H-kininogen (Ben Nasr et al., 1996), and collagens (Olsen et al., 1993a). Curli has two binding sites, which include the 24 amino acid residues on the N-terminal, and the 26 amino acid residues on the C-terminal end of the curli protein (Olsen et al., 2002). The binding of curli to human contact phase proteins leads to the release of inflammatory molecules, causing the onset of pain, fever, oedema, and hypotension in patients infected with the pathogen (Ben Nasr et al., 1996).

Curli has two binding sites, which include the 24 amino acid residues on the N-terminal, and the 26 amino acid residues on the C-terminal end of the curli protein (Olsen et al., 2002). The binding of curli to human contact phase proteins leads to the release of inflammatory molecules, causing the onset of pain, fever, oedema, and hypotension in patients infected with the pathogen (Ben Nasr et al., 1996).

Curli has two binding sites, which include the 24 amino acid residues on the N-terminal, and the 26 amino acid residues on the C-terminal end of the curli protein (Olsen et al., 2002). The binding of curli to human contact phase proteins leads to the release of inflammatory molecules, causing the onset of pain, fever, oedema, and hypotension in patients infected with the pathogen (Ben Nasr et al., 1996).

2. Other cell surface structures

Fimbriae or pili are thread-like projections attached to the outer membrane of bacterial cells. They are 0.5 to 10 μm long, 7-11 nm thick, and may be rigid or flexible (Ofek and Doyle, 1994). Fimbriae are made up of repeating subunits of proteins (Finlay and Falkow, 1989; Sharon and Lis, 1989). The amino acids of the subunits have non-polar side chain, which imparts hydrophobicity to the cells (Corpe, 1980). Fimbriae expression in Salmonella enterica serotype Enteritidis is temperature-dependent, maximum levels of SEF 14 and SEF 17 fimbriae are expressed at 37° and 25°C, respectively (Woodward et al., 2000).
express SEF14 and SEF17 fimbriae adhered poorly than fimbriae expressing cells to polystyrene microtiter plates (Woodward et al., 2000).

Flagella are surface structures on the bacteria that aid cells to move around in an aqueous environment to overcome nutritional limitation. In addition, they serve as adhesive structures to assist cells in attaching to abiotic surfaces (Moens and Vanderleyden, 1996). In a study involving flagellated and non-flagellated *Pseudomonas aeruginosa*, the adherence of the bacterial cells to the surface of stainless steel decreased by 90% when flagella was not present (Stanley, 1983). In another study, motile *E. coli* had five-time greater attachment rate than did the non-motile *E. coli* (McClaine and Ford, 2002). The placement of flagella on the cell and swimming speed of the cell plays a role in the attachment of *Vibrio alginolyticus* to glass for example polar flagellated cells attached faster attachment than did lateral flagellated cells (Kogure et al., 1998).

Piette and Idziak, (1991) observed that flagellated *Pseudomonas flurorescens* attached in larger numbers than did deflagellated cells to tendon slice. This was due to the ability of the flagellated cells to reach tendon slice (Piette and Idziak, 1989). In another study, there was 90% reduction in the attachment of *Salmonella choleraesuis* subsp. *choleraesuis* after mechanical removal of the flagella (Bouttier et al., 1997). Treatment of the tissue with a concentrated suspension of flagella or treatment of the bacteria with antisomatic serum did not reduce the attachment of *S. choleraesuis* to tissues, indicating the absence of specific attachment sites for flagella or antigen O on the beef tissue surface (Bouttier et al., 1997). Lillard (1986) showed that *Salmonella enterica* serovar Typhimurium cells lacking fimbriae and flagella attached to poultry skin in equal number to those of *S. Typhimurium* possessing these surface appendages.
However, Farber and Idziak (1984) observed that the motile bacteria attached to meat tissue in high numbers but at lesser strength than did the non-motile bacteria.

B. Bacterial surface charge and hydrophobicity

Bacterial surface hydrophobicity varies between species and is influenced by growth medium, cell age, and cell surface structures such as fimbriae (Gibbons et al., 1983; Hejazi and Falkiner, 1997), polypeptides (Jenkinson, 1986), and proteins (Dankert et al., 1986). The surface hydrophobicity of bacteria is an important factor, which determines the types of surface to which bacterial cells may attach. Generally, bacteria with hydrophobic characteristics prefer to attach to hydrophobic surfaces while those with hydrophilic characteristics prefer to attach to hydrophilic surfaces (Hogt et al., 1983; Vacheethasanee et al., 1998). Hydrophobic bacteria usually attach more efficiently than do hydrophilic bacteria (van Loosdrecht et al., 1987a, b). The uropathogenic *E. coli* cells that attached to surface of tubular medical devices were more hydrophobic than were the *E. coli* that did not attached to the same surface (Harkes et al., 1992b). Cells of *Staphylococcus epidermidis* used in the study of Hogt et al. (1983) were more hydrophobic and attached better to surfaces of polytetrafluorethylene-co-hexafluorprpylene (FEP)-flurocarbon and cellulose acetate in comparison to the cells of *Staphylococcus saprophyticus*.

Cell surface charge affects bacterial adhesion especially in the initial step of colonization (Jucker et al., 1996). Bacterial particles obtain electrical charges through ionization of their surface chemical groups (Mozes and Rouxhet, 1990) and these ionized groups attract oppositely charged groups in the surroundings and form electric double layers. The surface charges on bacterial cells can be characterized by isoelectric points (Harden & Harris, 1953) and the electrokinetic potentials. The latter is also known as zeta electric potentials (Gilbert et al., 1991;
van Loosdrecht et al., 1987b). Bacteria are usually negatively charged in aqueous suspension (Hogt et al., 1985), but the strengths of the surface charges vary between species and strains and are influenced by environmental conditions (Dankert et al., 1986). The zeta potentials of enteropathogenic E. coli were in the range of –26 to –30 mV (Mangia et al., 1995). The higher the surface charges the more hydrophilic the bacterial surfaces, but in some instances, a hydrophobic bacterium may have high surface charge (Dankert et al., 1986).

Electrostatic and hydrophobic interactions may play a role in attachment of bacteria to meat. Dickson and Crouse (1989) observed an increase in attachment of Salmonella to meat when they applied electric currents to meats, indicating influence of electric charge on cell attachment to meat. In contrary, Bouttier et al., (1997) observed that electrostatic interactions were not responsible for attachment of S. choleraesuis to beef surface.

C. Material characteristics

The material properties such as surface chemical composition, surface roughness, surface morphology or configuration, and surface hydrophobicity or wettability all play a role in adherence of bacteria to surface (An et al., 1995; Hogt et al., 1985; Pringle and Fletcher, 1986; Reynolds and Wong, 1983). The material surface is often altered under practical conditions because of the deposition of food and formation of biofilms. Food deposits provide source of nutrition and act as the attachment sites for the bacteria (Frank, 2001).

Bacterial adhesion to a surface is affected by the chemical composition of surface materials (An et al., 1993). Kielemoes and Verstraete (2001) studied the influence of copper-alloying in the stainless steel on microbial colonization. It was found that the presence of copper in the steel matrix impeded the adhesion of microorganisms during an initial attachment period (48 h), but the impedance of copper disappeared after longer attachment periods (120 h). In an
another study, Arnold and Bailey (2000) observed that attachment of microorganisms to rubber was significantly less than stainless steel and other surfaces used in the poultry industry.

Surface finish affects bacterial attachment. Increased bacterial colonization was observed on glass or polystyrene with increase in surface roughness (Baker and Greenham, 1988). Lesser bacteria adhere to electrochemically treated stainless steel than on sand blasted or sanded type of surface finish (Arnold and Bailey, 2000; Arnold and Silvers, 2000). Rough surface provides greater surface area and the crevices in the roughened surface provides a favorable niche for colonization.

Surface morphology or configuration is described in terms of patterns of material surface, such as non-filament surface, braided surface, porous surface, or grid-like surface (An and Friedman, 2000). Ultra smooth surfaces do not allow bacterial adhesion and biofilm deposition (McAllister et al., 1993). It has been observed that bacteria applied to wooden cutting boards are not recovered in same levels as that applied to plastic cutting boards because the bacteria gets entrapped into the pores of wood (Ak et al., 1994). Boucher et al., (1998) studied the survival of Campylobacter jejuni in association with wood, polyurethane foams, and sintered glass discs. It was found that C. jejuni was able to survive in wooden cutting boards but not in other tested surfaces because of protection provided by the pores in the wood (Boucher et al., 1998).

Metal and polymers have different surface properties. Metal surface have high surface energy, are negatively charged, and hydrophilic, while polymers have low surface energy, are less electrostatically charged, and hydrophobic (An and Friedman, 2000). Bacteria attach with different efficiencies depending upon the material hydrophobicities (Fletcher and Loeb, 1979; Hogt et al., 1983; Satou et al., 1988). Pseudomonas bacteria attached more to hydrophobic plastics with little or no surface charge (Teflon, polyethylene, polystyrene, and polyethylene
terephthalate); moderately to hydrophilic metals with positive or neutral surface charge, and very little to hydrophilic, negatively charged substrata (glass, mica, oxidized plastics) (Fletcher and Loeb, 1979; Hogt et al., 1983; Satou et al., 1988).

The connective and adipose tissues are found between the skin and the skeletal tissues of animals. The connective tissues are composed of collagen I, laminin, fibronectin, and the glycosaminoglycans (hyaluronic acid, chondroitin sulfate, heparan sulfate) (Medina, 2001). Elastin constitutes <5% of the total connective tissues, while collagen is the major protein component comprising 10-30 mg/g of wet muscle weight and is 40% of the dry weight of the extracellular matrix (Bailey and Light, 1989). Collagen also accounts for 70-80% of animal skin (Kadler, 1994). *E. coli* O157:H7 strains have been reported to attach to collagen and fibronectin on the meat tissues (Fratamico et al., 1996). On using the surface plasmon resonance biosensor for studying interactions of immobilized *E. coli* O157:H7 with collagen, laminin and fibronectin, it was found that collagen had greater binding capacity with immobilized *E. coli* O157:H7 than did laminin and fibronectin.

**D. Environmental conditions**

Bacterial adherence depends on the surface properties of three phases involved, i.e., the surface tensions of the adhering particles, substrate, and suspending medium (Absolom et al., 1983b). Properties of the suspending medium such as: type of medium, shear stress of the flowing medium (Duddriege et al., 1982), temperature, time of contact, concentration of bacteria in the suspending liquid, presence of antimicrobial elements and surface tension of the medium will influence bacterial adhesion (Absolom et al., 1983).

In a study conducted to study the effect of temperature on attachment, it was observed that *E. coli* attached more efficiently at 22°C than at 37°C and 42°C on Penrose rubber drains.
(Guo et al., 1993). Biofilm formation on stainless steel by \( \text{S. Typhimurium, } Listeria \)
\text{monocytogenes, } \text{E. coli O157:H7, Pseudomonas fragi, and Pseudomonas fluorescens} \) strains as
affected by various growth medium was studied (Hood and Zottola, 1997). The medium that had
the highest observed level of adherent cells was different for each microorganism. The increase
in the number of adherent cells over time was seen with \( \text{S. Typhimurium in diluted meat juice} \)
(DMJ), \( \text{E. coli O157:H7 in tryptic soy broth (TSB), diluted TSB (dTSB), and DJM, P. fragi in} \)
reconstituted skim milk (RSM), and \( \text{P. fluorescens in RSM} \) (Hood and Zottola, 1997).

Ionic strength of the surrounding medium contributes to the electrostatic interaction
between the bacteria and the surface. An increase in adhesion of \( \text{P. fluorescens} \) was observed to
tendon slice with increase in ionic strength ranging from 5 to 100 mM, but at ionic strength
outside this range the attachment decreased (Piette and Idziak, 1992). The increase in adhesion
is due to the decrease of the electrically diffuse double layer on the surface of the bacteria, which
is created by ions in the suspending solution. The negatively charged cells can approach the
negatively charged surface by overcoming the electrostatic repulsive force, either by attractive
van der Walls forces or by surface appendages (Yada and Skura, 1982).

III. MECHANISM OF ATTACHMENT TO FOOD CONTACT SURFACES

The attachment of bacteria can occur to almost any surface in any environment in which
living microorganisms are present. The surfaces may be animate or inanimate, living or dead
and/or organic or inorganic (Kumar and Anand, 1998). In food processing environments,
organic molecules like proteins from milk and meat are deposited on the surface during the
transport of fluid or while conveying the food product (Kumar and Anand, 1998). The deposited
layer thus formed is called conditioning film (Frank, 2001). The extent of deposition depends
upon the rate of transport and the extent of adsorption (Characklis, 1983). The conditioning film
has higher levels of nutrients than the liquid phase and provides a rich source of nutrient favoring bacterial growth. The conditioning film alters the physico-chemical properties of materials viz., hydrophobicity, electrostatic charges, and surface free energy, which subsequently affect the microbial adhesion (Dickson and Koohmaraie, 1989). The adsorbed proteins to the surface can either promote or inhibit bacterial adhesion. Whey proteins promote attachment of several milk-associated microorganisms to stainless steel, rubber and glass surfaces (Speers and Gilmour, 1985). Fletcher (1976) observed that albumin, gelatin, fibrinogen, and pepsin inhibited the attachment of pseudomonads to polystyrene. Milk components such as casein and lactoglobulin have been found to inhibit the attachment of *L. monocytogenes* and *S. Typhimurium* to stainless steel and buna-N rubber (Helke et al., 1993).

The adhesion of bacteria to solid surfaces can be described in a two-phase process including an initial, instantaneous, reversible phase and a time dependent irreversible phase (Vidal et al., 1998). The bacteria attach with the surface in the reversible phase by long range van der Walls forces and electric double layer forces. These forces can be easily destroyed by fluid shear force such as rinsing (Marshall et al., 1971). The attachment of microorganisms in this phase is affected by hydrophobicity, electronic charge, and motility of the cells as discussed earlier (van Loosdrecht et al., 1990). The reversible phase converts to irreversible phase when the attached bacteria produce exopolysaccharide (EPS). The forces involved in this phase include, dipole-dipole interactions, hydrogen, ionic and covalent bonding and hydrophobic interaction (Kumar and Anand, 1998). The EPS forms a bridge between the bacterial cell and the attached surface (Frank, 2001). In this phase, stronger forces such as scrubbing or scrapping are required to remove the bacterial cells (Marshall et al., 1971). Boyd and Chakrabarty, (1995) showed that exopolysaccharide biosynthesis genes are induced upon attachment of bacteria to the
substratum. The irreversibly attached bacteria procure nutrients from the conditioning film and the surrounding fluid environment. The bacteria grow under this condition and form microcolonies (Kumar and Anand, 1998). The attached bacteria produce additional EPS, which helps the microcolony to survive the change in the environment (Kumar and Anand, 1998).

IV. PROPERTIES OF SURFACE-ASSOCIATED CELLS

Surface associated bacterial cells may have higher tolerance to sanitizers and heat, and may be difficult to remove due to exopolymer production (Frank, 2001). Lee and Frank (1991) studied the effect of hypochloride on *L. monocytogenes* cells adhered to stainless steel and found that four-hour attachment time was long enough to confer the cells resistance against chlorine. Attached *L. monocytogenes* cells were also resistant to quaternary ammonium, acidic, and anionic sanitizers (Frank and Koffi, 1990). Dhir and Dodd (1995) reported the increased resistance of *S. Enteritidis* to sanitizers upon attachment to glass or stainless steel surface. According to the same authors surface-associated bacterial cells are more thermotolerant than were the free living cells. This thermotolerance was due to enhanced synthesis of disaccharide trehalose, which preserves the membrane function and integrity (Hengge-Aronis *et al*., 1991). The attached cells were metabolically more active than were the detached cells. Exopolymers are known to provide a strong bond between bacteria and the contact surfaces (Frank, 2001). The production of exopolymer is activated as a response to attachment (Roberson and Firestone, 1992).

V. OBJECTIVES OF THE STUDY

The objectives of this study are to:

1. Determine the genotypic and phenotypic characteristics of curli-expressing and non curli-expressing cells of EHEC.
2. Evaluate the role of curli in assisting the cells of enterohemorrhagic *Escherichia coli* (EHEC) in attaching to abiotic surfaces and to determine the influence of cell-surface contact time on the efficiency of the attachment.

3. Evaluate the role of curli in the attachment of EHEC on growth media as well as on raw and ready-to-eat beef products.
References:


Attachment of *Salmonella choleraesuis choleraesuis* to beef muscle and adipose tissues.
*J. Food Prot.* **60**:16-22.


Nucleotide sequence of the Shiga-like toxin genes of *Escherichia coli*. *Proc. Natl. Acad.

Calderwood, S.B., Asheson, D., Keusch, G.T., Barrett, T.J., Griffin, P.M., Strockbine, N.,
Swaminathan, B., Kaper, J.B., Levine, M., Kaplan, B., Karch, H., O'Brien, A.D., Obrig, T.,

CDC (1995) *Escherichia coli* O157:H7 outbreak linked to commercially distributed dry-cured


CDC (1999) Outbreak of *Escherichia coli* O157:H7 and *Campylobacter* among attendees of the


Chapman, M.R., Robinson, L.S., Pinkner, J.S., Roth, R., Heuser, J., Hammar, M., Normark, S.,
and Hultgren, S.J. (2002) Role of *Escherichia coli* curli operons in directing amyloid


*Escherichia coli* O157:H7 strain associated with an outbreak of hemorrhagic colitis. 

influencing attachment of *Escherichia coli* O157:H7 to beef tissues and removal using 

Gibbons, R.J., Etherden, I., and Skobe, Z. (1983) Association of fimbriae with the 
hydrophobicity of *Streptococcus sanguis* FC-1 and adherence to salivary pellicles. *Infect. 

and adhesion of *Escherichia coli* and *Staphylococcus epidermidis*. *J. Appl. Bacteriol.* 
71:72-77.

fibers mediate internalization of *Escherichia coli* by eukaryotic cells. *Infect. Immun.* 
69:2659-2665.

Griffin, P.M., Ostroff, S.M., Tauxe, R.V., Greene, K.D., Wells, J.G., Lewis, J.H., and Blake, 

of *Escherichia coli* to Penrose rubber drains--an *in vitro* study. *Zentralbl. Bakteriol.* 
278:73-82.


CHAPTER 3

Phenotypic and genotypic characterization of curli-expressing enterohemorrhagic

*Escherichia coli*¹

Abstract

Certain enterohemorrhagic *Escherichia coli* (EHEC) express a thin, wiry, aggregative surface fiber, known as curli, to assist them in attaching to solid surfaces. This study was undertaken to characterize and compare the genotypic and phenotypic differences of curli-expressing and non curli-expressing EHEC cells. Wild type *E. coli* O111-H- 7-57 and O157-H7 5-9, with and without the ability to express curli, respectively, were included in the study. Also included were *E. coli* O103:H2 7-52 and O157:H7 5-11, capable of forming both curli-expressing (C⁺) and non curli-expressing (C⁻) colonies on tryptone yeast extract agar (TYE) supplemented with Congo red dye. Quantification of curli expressed by EHEC cells was accomplished with a Congo red binding assay. The growth requirements of the cells of both curli phenotypes were compared on casamino acid and minimal glucose (MGA) agar as well as on the GN2 microtiter plates supplied by Biolog. The rates of curli phenotypic conversion were determined by growing the EHEC cultures on 3 media at 5 incubation temperatures. Polymerase chain reaction (PCR) was performed to determine the prevalence of *csgA* in curli-expressing and non curli-expressing EHEC cells. The results of the Congo red binding assay indicated that the quantities of curli expressed by EHEC cells were inversely proportional to the amounts of free Congo red in the supernatants of the cell suspensions. Two non curli-expressing variants were more fastidious than were their curli-expressing counterparts and failed to grow on MGA although they were capable of growing on casamino acid agar. Both curli-expressing and non curli-expressing cells of EHEC carried *csgA*, the structural gene for a major subunit of curli protein. Curli phenotypic conversion took place spontaneously during growth and was influenced to a certain extent by growth media and incubation temperatures.

*Key words:* Curli, EHEC, *E. coli* O157:H7, *csgA*, phenotypic conversion
1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) has been one of the greatest microbial challenges of the past two decades. The illnesses caused by this group of pathogen range from asymptomatic infection, mild uncomplicated diarrhea, and bloody diarrhea to hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura (Karmali, 1989; Sussman, 1997; Fey et al., 2000). Human infections caused by EHEC may be transmitted through foods that are contaminated with the pathogen. EHEC cells adhered to the solid surfaces in a processing environment could be a possible source of food contaminations.

Cells of certain EHEC isolates O157:H7 express a thin, wiry, aggregative fiber, known as curli, on their surfaces. The surface fiber was found to play a significant role during primary adhesion of *E. coli* to inert surfaces (Vidal et al., 1998; Prigent-Combaret et al, 2000; 2001; Cookson et al., 2002). Recent research conducted in our laboratory revealed that curli was able to mediate the attachment of *E. coli* to the surfaces of polystyrene, glass, and stainless steel (Pawar and Chen, Unpublished). Curli-expressing cells of some EHEC strains attached to these surfaces much more efficiently than did their non curli-expressing counterparts.

Curli expression in *E. coli* is controlled by two divergently transcribed operons, *csgBA* and *csgDEFG*. The *csgA* encodes a major subunit of curli protein while the *csgB* encodes a cell surface protein, which acts as a nucleator for CsgA monomer and is responsible for polymerization of curli fimbriae (Hammar et al., 1995; Bian and Normark, 1997). The CsgD is a transcriptional activator necessary for curli expression, and CsgG is an outer membrane lipoprotein involved in extracellular stabilization of CsgA and CsgB (Loferer et al., 1997). The CsgE and CsgF is a chaperone-like and a nucleator protein, respectively, and both proteins are required for proper nucleation of CsgA on CsgB (Chapman et al., 2002).
While csg genes are ubiquitous among *E. coli*, only half of the avian isolates tested in a study expressed curli (Maurer et al., 1998). La Ragione et al. (1999) reported that the lack of ability to express curli in *E. coli* O78:K80 was due to an insertion in csgB or a defect on rpoS. The work of Uhlich et al. concluded that the inability to express curli by *E. coli* O157:H7 was not caused by a large DNA deletion or insertion but instead by point mutations in the csgD promoters (Uhlich et al., 2001).

The objective of this study was to determine the genotypic and phenotypic characteristics of curli-expressing and non curli-expressing cells of EHEC.

2. Materials and methods

2.1. Bacterial strains and growth conditions

*E. coli* O157:H7 5-9 and 5-11, O103:H2 7-52, and O111:H- 7-57, all from our laboratory collections were used in the study. The cultures were maintained at -20°C in 15% glycerol and were sub-cultured twice prior to use. Tryptone yeast extract agar (TYE; 0.1% tryptone, 0.05% yeast extract, and 1.5% agar) supplemented with 20 mg/l of Congo red (Sigma Aldrich Co, St. Louis, MO) and 40 mg/l of coomassie brilliant blue (Sigma) was used to grow the EHEC cultures. Cells of 5-11 and 7-52 formed both curli-expressing and non curli-expressing colonies on TYE indicator agar at 28°C. The curli-expressing and non curli-expressing variants of the 2 strains were purified and used as separate cultures in the study. The curli-expressing variant of 5-11 was designated 5-11C⁺ and the non curli-expressing variant as 5-11C⁻. Similarly, the curli-expressing and non curli-expressing 7-52 were named 7-52C⁺ and 7-52C⁻, respectively.

2.2. Quantification of curli

Curli expressed by the cells of EHEC was quantified using the procedure of Gophna et al. (2001) with some modifications. Three EHEC pairs, 7-57C⁺ and 5-9C⁻, 5-11C⁺ and 5-11C⁻, as
well as 7-52C$^+$ and 7-52C$^-$, were grown on TYE agar plates at 28°C for 24, 48, and 72 h, respectively. The cultures were collected with 0.85% NaCl, and the optical densities of the cell suspensions were adjusted to 1.00 ± 0.03 at wavelength 600 nm. One and a half ml of each suspension was centrifuged at 16,000x g for 10 min. The supernatant was discarded and the cell pellet was re-suspended in 1.5 ml 0.002% Congo red solution (Sigma). The cell suspension was re-centrifuged using the conditions described above and the quantities of free Congo red were determined by measuring the absorbance of the supernatant at wavelength 500 nm.

Concurrently, the EHEC cultures were serially diluted and appropriate dilutions were plated on TYE indicator agar using an Autoplate® 4000 (Spiral Biotech, Inc., Bethesda, MA). The plates were incubated at 37°C for 24 h. The colony counts were then determined using an automatic colony counter (Q Count®, Spiral Biotech, Norwood, MD).

2.3. Scanning electron microscopy

The cultures of 7-52C$^+$, 7-52C$^-$, 5-11C$^+$, and 5-11C$^-$ were grown on TYE indicator agar plates at 28°C for 72 h. The cells were collected and fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (SCB; pH 7.4) at room temperature for 90 min. The fixed cells were washed 3 times with SCB, each for 10 min. Lipids on EHEC cells were fixed with 1% osmium tetroxide at room temperature for 1 h. The samples were washed twice with SCB and then dehydrated serially with 50, 70, 80, 90, and 100% ethanol, each for 15 min. The samples were stored at 4°C in 100% ethanol and were dried at critical point temperature with liquid CO$_2$ using a critical point dryer (Samdri model 780-A, Tousimis, Rockville, MD). The cells were then coated with gold in a sputter coater (Structure Probe, Inc., West Chester, PA) and were visualized using a scanning electronic microscope (LEO Electron Microscopy, Inc, Thornwood, NY).
2.4. Growth requirement

Casamino acid, and minimal glucose (MGA) agar were used to examine the growth requirement of curli-expressing and non curli-expressing EHEC cells. The casamino acid agar was prepared by dissolving 4 g casamino acids, 6.8 g NaH$_2$PO$_4$, 3.0 g K$_2$HPO$_4$, 0.5 g NaCl, 1.0 g NH$_4$Cl, and 15.0 g agar in 1 liter deionized water. The medium was sterilized at 121$^\circ$C for 15 min. After the solutions cooled to 45$^\circ$ - 50$^\circ$C, 20 ml of filter-sterilized 20% glucose, and 2 ml of sterile 1.0 M MgSO$_4$ were added. The compositions of MGA and casamino acid agar are similar except that the MGA does not contain the casamino acids prepared by acid hydrolysis of casein.

The cultures of 7-57 C$^+$, 5-9 C$^-$, 7-52C$^+$, 7-52C$^-$, 5-11C$^+$, and 5-11C$^-$ grown on TYE agar at 28$^\circ$C for 48 h, were streaked heavily on MGA and casamino acid agar. The inoculated plates were incubated at 28$^\circ$C for 72 h. The plates with the EHEC cultures were photographed using Gel Doc System 2000 (Biorad, Hercules, CA).

2.5. Substrate utilization

The cultures of 7-57C$^+$, 5-9 C$^-$, 7-52C$^+$, 7-52C$^-$, 5-11C$^+$, and 5-11C$^-$ were inoculated on TYE agar and incubated for 18 h at 28$^\circ$ or 37$^\circ$C. A colony of each culture was picked and dispersed into an inoculating fluid (0.40% NaCl, 0.03% Pluronic F-68, 0.02% Gellan Gum; Biolog, Hayward, CA) to achieve a transmittance of 61% at a wavelength of 590 nm. A volume of 150 µl of the inoculating fluid was inoculated onto a GN2 microtiter plate (Biolog, Hayward, CA). The microplate was pre-coated with appropriate concentrations of 95 substrates, including carbohydrates, carboxylic acids, polymers, amines or amides, amino acids, and other miscellaneous substances. A tetrazolium salt at oxidized state was used as an indicator of a positive reaction. The tetrazolium salt functions as electron acceptors that are reduced in the
presence of cellular respiratory enzymes to form colored formizan compounds (Jambour, 1954). The inoculated microplates were incubated at 28°C for 72 h or 37°C for 24 h. The results were documented based on visual determination of the color change.

2.4. PCR amplification of csgA

EHEC cultures were grown in 1.5 ml TYE broth (0.1% tryptone, 0.05% yeast extract) in Eppendorf tubes at 28°C for 24 h. The cultures were centrifuged at 16,000x g for 2 min. The supernatants were discarded and the cell pellets were washed twice with sterilized deionized water. After the final wash, the cells were re-suspended in 100 µl of sterile deionized water and boiled for 10 min. Centrifugation was repeated under the conditions described above. The DNA in the supernatants was used as templates in PCR amplification. The reaction mix consisted of 5 µl of 10X PCR buffer, 0.2 µl of Taq DNA polymerase (1U/µl), 5 µl of template DNA, 5 µl of dNTP mix (10 mM), 2 µl of csgA primers (5’-GCA ATC GTA TTC TCC GGT AG and 5’ GAT GAG CGG TCG CGT TGT TA), and 32.6 µl of sterile deionized water. All reagents used in the PCR were purchased from Roche Molecular Biochemicals (Indianapolis, IN). The csgA primers were commercially synthesized by GIBCO-BRL. The reaction was carried out in a DNA thermal cycler (Model 480; Perkin Elmer, Norwalk CT) under the following conditions: 1 cycle of 94°C for 5 min, 30 cycles of 94°C for 1 min, 68°C for 1 min and 72°C for 1 min followed by 1 cycle of 72°C for 10 min. The csgA PCR products were then subjected to electrophoresis on 1% agarose. The agarose gel was stained with 1 µg/ml ethidium bromide and was photographed using Gel Doc system 2000 (Biorad, Hercules, CA).

2.5. Phenotypic conversion

A single colony of 5-11, 5-11C⁺, 5-11C⁻, 7-52, 7-52C⁺, or 7-52C⁻ on TYE indicator agar was transferred into TYE broth, beef extract broth (0.1% desiccated beef extract), and beef
extract peptone broth (0.1% desiccated beef extract and 0.05% peptone), respectively. The inoculated broth was incubated at 37°C for 6 h with shaking at 150 rpm in an incubation shaker (New Brunswick Scientific Co., Inc, Edison, NJ). The resulting cultures were serially diluted and 0.1 ml of appropriate dilutions were spread plated on TYE, beef extract, or beef extract peptone agar supplemented with 20 mg/l of Congo red (Sigma) and 40 mg/l of coomassie brilliant blue (Sigma). The plates were incubated at 10°, 15°, 22°, 28, or 37°C for 10, 5, 3, 2, and 1 d, respectively. The curli-expressing and non curli-expressing colonies were enumerated. The percentage ratios of curli-expressing to non curli-expressing colonies were calculated.

2.6. Statistical analysis

Two replicates of each experiment were performed and every test in each individual experiment was conducted in duplicate. The data collected in the study was analyzed using general linear model procedure and Statistical Analysis Software (SAS, 1999). Significant differences between mean values were determined based on a 95% confidence level.

3. Results

As with any other amyloid protein, curli has a high affinity to Congo red. A Congo red binding assay was, therefore, utilized to measure the quantities of curli expressed by the EHEC cells. The binding of curli fibers to Congo red leads to a decrease in the concentrations of the free-formed dye in the supernatants, which in turn results in a decrease in the optical densities at wavelength 500 nm. Based on the results summarized in Table 3.1, E. coli O111-H- 7-57C+ expressed the greatest amounts of curli compared to other strains tested. In addition, the differences in curli expression between the 2 members of all 3 EHEC pairs were statistically different, except for the cultures 7-52C+ and 7-52C- grown at 28°C for 48 h (Table 3.1).
It is evident that significantly greater amounts of curli were expressed by the cells of 7-52C−, 5-11C+, and 5-11C− grown for 72 h compared to the cultures grown for 24 and 48 h at 28°C (Fig. 3.2, Table 3.1). Prolonged incubations from 24 and 48 to 72 h significantly enhanced the expression of curli on EHEC. The difference in curli expression between the 24- and 72-h cultures of 7-52C− was statistically significant. However, the differences between the 24- and 48-h, as well as the 48- and 72-h cultures were statistically insignificant (Table 3.1). Furthermore, the lengths of incubation time have no statistical effects on the expression of curli by 7-57C+ and 5-9C− (Table 3.1).

Curli appeared as thin and coiled filaments around the EHEC cells under SEM with an average width of 29.98 ± 1.45 nm (Fig. 3.2A). No similar structure was detected in the non-curli expressing cells (Fig. 3.2B). Extended incubation on TYE indicator agar at 28°C induced the production of excessive amounts of curli, which formed interconnecting mesh between the EHEC cells (Fig. 3.2C).

The non curli-expressing EHEC 5-9C− and 7-52C− appeared to have higher growth requirements than did their curli-expressing counterparts. The 2 variants failed to grow on MGA although they were capable of growing on casamino acid agar (Fig. 3.3B and 3.3C). The non curli-expressing mutant of 5-11, however, is less fastidious and grew just as well on MGA as it did on casamino acid agar (Fig. 3.3A). L-alaninamide was found to be the unique substrate utilized by 7-52C+ and 5-11C+ at 28°C. In addition, the cells of curli-expressing cultures were able to metabolize Tween 40, Tween 80, glycogen, and bromosuccinic acid, in comparison to 7-52C− and 5-11C−, at 37°C.

The 417-bp PCR product was amplified from all EHEC cultures tested in the study (Lane D to K, Fig. 3.4) and from the positive control cultures (Lane B, C, L, and M, Fig. 3.4). The
same product was, however, not detected in the negative control culture (Lane N, Fig. 3.4). These results suggest that both curli-expressing and non curli-expressing EHEC cells tested in the study carried \textit{csgA}, the structural gene for a major subunit of curli protein.

In the curli phenotypic conversion study, the wild type cells of 5-11 and 7-52 formed both curli-expressing and non curli-expressing colonies on TYE, BE, and BEP agar at all incubation temperatures except at \(10^\circ\text{C}\) (Table 3.2). The 5-11\textsuperscript{C\textsuperscript{-}} was stable and no curli phenotypic conversion was observed on all media and incubation temperatures (Table 3.2). The 7-52\textsuperscript{C\textsuperscript{-}} was stable on BEP at all incubation temperatures, on TYE at \(10^\circ\text{C}, 22^\circ\text{C}, 28^\circ\text{C}, \text{and } 37^\circ\text{C}\), as well as on BE at \(10^\circ\text{C} \text{ and } 37^\circ\text{C}\). However, 100\% of the colonies converted to curli-expressing on BE at \(22^\circ\text{C} \text{ and } 28^\circ\text{C}\) (Table 3.2). The 7-52\textsuperscript{C\textsuperscript{+}} was stable on BEP at \(15^\circ\text{C}, 22^\circ\text{C}, \text{and } 28^\circ\text{C}\), on TYE at \(22^\circ\text{C}, 28^\circ\text{C}, \text{and } 37^\circ\text{C}\), as well as on BE at \(37^\circ\text{C}\) (Table 3.2). The 5-11\textsuperscript{C\textsuperscript{+}} was stable on BEP at \(22^\circ\text{C}, 28^\circ\text{C}, \text{and } 37^\circ\text{C}\), and on BE at \(28^\circ\text{C} \text{ and } 37^\circ\text{C}\), but the two curli-expressing variants were not completely stable under other growth conditions (Table 3.2).

4. Discussion

Both wild type curli-expressing and non curli-expressing EHEC as well as the non curli-expressing spontaneous mutants and their curli-expressing EHEC parents were included in this study. Uhlich et al., (2001) researched the curli of \textit{E. coli} O157:H7 and concluded that the lack of curli expression on the surfaces of \textit{E. coli} O157:H7 was not caused by a large DNA deletion or insertion but by the mutations in the \textit{csgD} promoter. However, an interrupted \textit{csgB} and defective \textit{ropS} also altered the expression of curli in \textit{E. coli} O78:K80 (La Ragione et al., 1999).

The results of the Congo red binding assay presented in this study indicated that the curli-expressing cells of EHEC bound greater amounts of Congo red than did the non curli-expressing cells. Statistical differences were observed between the 2 members of all 3 EHEC pairs, except
for the 7-52C⁺ and 7-52C⁻ cultures grown at 28°C for 48 h. Gophna et al. (2001) reported that curli-expressing cells bound 10 times more Congo red than did the non curli-expressing cells. The samples with higher populations of bacterial cells was also noticed to have greater Congo red binding capacities (Gophna et al., 2001; Pawar and Chen, Unpublished).

Polymerized curli in *E. coli* MC4100 appeared as 4-12 nm wide fibers of varying lengths under transmission electron microscope (Olsen et al., 1989; Chapman et al., 2002) however, scanning electron microscope was used in this study. The diameter of the fimbriae was slightly larger with an average diameter of 29 nm.

Non curli-expressing *E. coli* O157:H7 5-9C⁻ and O103:H2 7-52 failed to grow on MGA although both were capable of growing on casamino acid agar at 28°C. The components that is lacking in the MGA compared to the casamino acid agar are the amino acids and minerals from acid hydrolysates of casein, suggesting that the growth of 5-9C⁻ and 7-52C⁻ is dependent upon the availability of these nutritional components in the environment. It was reported that an amino acid, glycine, makes up 11.5% of the amino acid residue in total *E. coli* proteins (Chirwa and Herrington, 2003). Curli protein, however, contains 1.7 times as much glycine than other *E. coli* proteins. Since *E. coli* expresses a great deal of curli, the cell’s ability to make or access to glycine, perhaps other amino acids, could have a significant impact on the expression of curli on EHEC cells. On testing 30 carbohydrates, 24 carboxylic acids, 5 polymers, 6 amines or amides, 20 amino acids, and 10 miscellaneous substances pre-coated on the Biolog GN2 microplates, Uhlich et al. (2001) concluded that curli-expressing variants used in their study were able to utilize arginine and/or pyruvate in addition to the substrate fermented by the non curli-expressing variant of *E. coli* O157:H7. In the present study, 7-52C⁺ and 5-11C⁺ were able to utilize L-alaninamide at 28°C, as well as Tween 40, Tween 80, glycogen, and bromosuccinic acid at 37°C
in comparison to their non curli-expressing counterparts. It is not clear, however, whether these substrate utilization profiles have any association with the expression curli on EHEC.

It was found in this study that EHEC cells of both curli phenotypes carry \( csgA \). Curli phenotypic conversion took place spontaneously and was influenced to a certain extent by the growth media and incubation temperatures. Incubation at 10\(^{\circ}\)C prohibited the cells of EHEC from expressing curli on their surfaces.

**Acknowledgements**

We thank Dr John Siels for his assistance with the SEM work. This study was made possible through the research funding provided by the National Cattlemen’s Beef Association.

**References**


Prigent-Combaret, C., Brombacher, E., Vidal, O., Ambert, A., Lejeune, P., Landini, P., Dorel, C.,
2001. Complex regulatory network controls initial adhesion and biofilm formation in

Developmental pathway for biofilm formation in curli-producing *Escherichia coli* strains:


Cambridge University Press.

Uhlich, G.A., Keen, J.E., Elder, R.O., 2001. Mutations in the *csgD* promoter associated with

Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert
180, 2442-2449.
Table 3.1
Quantification of curli expressed by EHEC cultures

<table>
<thead>
<tr>
<th>Culture age</th>
<th>7-52C⁺</th>
<th>7-52C⁻</th>
<th>5-11C⁺</th>
<th>5-11C⁻</th>
<th>7-57C⁺</th>
<th>5-9C⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>0.83 b</td>
<td>0.93 a</td>
<td>0.79 b</td>
<td>0.92 a</td>
<td>0.60 c</td>
<td>0.92 a</td>
</tr>
<tr>
<td>48 h</td>
<td>0.78 b</td>
<td>0.84 ab</td>
<td>0.76 b</td>
<td>0.92 a</td>
<td>0.45 c</td>
<td>0.91 a</td>
</tr>
<tr>
<td>72 h</td>
<td>0.72 b</td>
<td>0.71 a</td>
<td>0.68 b</td>
<td>0.89 a</td>
<td>0.42 c</td>
<td>0.87 a</td>
</tr>
</tbody>
</table>

*: Values in column not followed by the same upper case letter are significantly different with respect to incubation time; Values in rows not followed by the same lower case letter are significantly different with respect to cultures.
Table 3.2
The effect of 3 growth media and 5 incubation temperatures on rate of curli expression of EHEC cultures\(^a\)

<table>
<thead>
<tr>
<th>Incubation temperatures</th>
<th>Tryptone Yeast Extract Indicator Agar</th>
<th>7-52</th>
<th>5-11</th>
<th>7-52</th>
<th>5-11</th>
<th>7-52</th>
<th>5-11</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tryptone Yeast Extract Indicator Agar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Beef Extract Indicator Agar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Beef Extract Peptone Indicator Agar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10°C</td>
<td>WT(^b)</td>
<td>0:100</td>
<td>0:100</td>
<td>0:100</td>
<td>0:100</td>
<td>0:100</td>
<td>0:100</td>
</tr>
<tr>
<td></td>
<td>C(^+)</td>
<td>5:11</td>
<td>5:11</td>
<td>5:11</td>
<td>5:11</td>
<td>5:11</td>
<td>5:11</td>
</tr>
<tr>
<td>15°C</td>
<td>WT(^b)</td>
<td>-</td>
<td>-</td>
<td>79:21</td>
<td>98:2</td>
<td>0:100</td>
<td>0:100</td>
</tr>
<tr>
<td></td>
<td>C(^+)</td>
<td>-</td>
<td>-</td>
<td>91:9</td>
<td>98:2</td>
<td>0:100</td>
<td>0:100</td>
</tr>
<tr>
<td></td>
<td>C(^-)</td>
<td>52:48</td>
<td>100:0</td>
<td>0:100</td>
<td>0:100</td>
<td>75:25</td>
<td>96:4</td>
</tr>
<tr>
<td>22°C</td>
<td>WT(^b)</td>
<td>72:28</td>
<td>100:0</td>
<td>50:50</td>
<td>98:2</td>
<td>0:100</td>
<td>0:100</td>
</tr>
<tr>
<td></td>
<td>C(^+)</td>
<td>13:87</td>
<td>0:100</td>
<td>100:0</td>
<td>0:100</td>
<td>69:31</td>
<td>99:1</td>
</tr>
<tr>
<td></td>
<td>C(^-)</td>
<td>68:38</td>
<td>100:0</td>
<td>0:100</td>
<td>0:100</td>
<td>69:31</td>
<td>99:1</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>74:22</td>
<td>100:0</td>
<td>0:100</td>
<td>0:100</td>
<td>78:22</td>
<td>100:0</td>
</tr>
<tr>
<td>28°C</td>
<td>WT(^b)</td>
<td>73:27</td>
<td>100:0</td>
<td>47:53</td>
<td>100:0</td>
<td>0:100</td>
<td>0:100</td>
</tr>
<tr>
<td></td>
<td>C(^+)</td>
<td>30:70</td>
<td>0:100</td>
<td>100:0</td>
<td>0:100</td>
<td>65:35</td>
<td>100:0</td>
</tr>
<tr>
<td></td>
<td>C(^-)</td>
<td>74:22</td>
<td>100:0</td>
<td>0:100</td>
<td>0:100</td>
<td>78:22</td>
<td>100:0</td>
</tr>
<tr>
<td>37°C</td>
<td>WT(^b)</td>
<td>-</td>
<td>100:0</td>
<td>0:100</td>
<td>100:0</td>
<td>0:100</td>
<td>0:100</td>
</tr>
<tr>
<td></td>
<td>C(^+)</td>
<td>48:52</td>
<td>100:0</td>
<td>0:100</td>
<td>0:100</td>
<td>63:37</td>
<td>100:0</td>
</tr>
<tr>
<td></td>
<td>C(^-)</td>
<td>-</td>
<td>64:36</td>
<td>0:100</td>
<td>100:0</td>
<td>87:13</td>
<td>100:0</td>
</tr>
</tbody>
</table>

\(^a\): Expressed as the percentages of curli-expressing colonies to the percentages of non curli-expressing colonies.
\(^b\): Wild type culture.
\(^c\): The colors of the colonies were difficult to determine.
Fig. 3.1. Quantification of curli expressed by EHEC cultures. EHEC cultures were grown on TYE agar plates for 24, 48, or 72 h at 28°C. The cells were collected in 0.85% NaCl. The cell suspensions were centrifuged at 16,000x g for 10 min. The supernatants were discarded and the pellets were re-suspended in 1.5 ml 0.002% Congo red solution. The cell suspensions were re-centrifuged and the optical densities of the supernatants at 500 nm were measured.
Fig. 3.2. Scanning electron micrographs of curli-expressing cells 5-11C⁺ (A) and 7-52-C⁺ (C) and as well as non curli-expressing cells 5-11C⁻ (B) under the magnification of 10,000 times.
Fig. 3.3. Growth of curli-expressing and non curli-expressing EHEC cells on MGA and casamino acid agar. The growth of 5-11 (A), 7-57 & 5-9 (B), and 7-52 (C). Left: growth on MGA; Right: growth on casamino acid agar. On each plate on the left is non curli-expressing culture and right is curli expressing culture.
Fig. 3.4. PCR amplification of a 417-bp \textit{csgA} product from curli-expressing and non curli-expressing EHEC cells. Lane A: 100 bp DNA ladder; Lane B, C, L, and M are the PCR products amplified from 4 positive control cultures. Lane N is a negative control. PCR products amplified from 7-57, 5-9, 5-11, 5-11C\textsuperscript{+}, 5-11C\textsuperscript{-}, 7-52, 7-52C\textsuperscript{+}, and 7-52C\textsuperscript{-} are shown in Lane D, E, F, G, H, I, J, and K, respectively.
CHAPTER 4

Role of Curli Fimbriae in Mediating the Cells of Enterohemorrhagic *Escherichia coli* to Attach to Abiotic Surfaces\(^1\)

\(^1\)D.M. Pawar and J. Chen. To be submitted to Applied and Environmental Microbiology.
ABSTRACT

Bacterial association with solid surfaces is mediated by their surface appendages. One such appendage, curli, is suggested to play a role during bacterial attachment to surface and cell interaction during biofilm formation. The objectives of this study were to evaluate the role of curli in assisting the cells of enterohemorrhagic *Escherichia coli* (EHEC) in attaching to abiotic surfaces and to determine the influence of cell-surface contact time on the efficiency of the attachment. Three pairs of EHEC cultures, each with a curli-expressing and a non curli-expressing strain (O111:H- 7-57C⁺ and O157:H7 5-9C⁻, O157:H7 5-11C⁺ and 5-11C⁻, as well as O103:H2 7-52C⁺ and 7-52C⁻), were allowed to interact with polystyrene, glass, stainless steel, and rubber surfaces at 28°C for 24 h (short-term attachment) or 7 d (long-term attachment). The quantities of cells that attached to the surfaces were measured daily in the long-term study, and in 4 h intervals in the short-term attachment study. Quantification of the cells that attached to the surfaces was accomplished with a crystal violet binding assay. The results of the long-term attachment study indicated that 7-57C⁺ attached to the polystyrene and glass surfaces more efficiently (*P* < 0.05) than did 5-9C⁻. The curli-expressing variant of 5-11 possessed a better ability to adhere to the polystyrene and glass surfaces than did its non curli-expressing counterpart (*P*<0.05). The differences in attachment between 7-52C⁺ and 7-52C⁻ on polystyrene and stainless steel surfaces were statistically significant (*P*<0.05). The attachment of the pair on the glass surfaces, however, was statistically insignificant (*P* >0.05). In addition, the 2 members of all 3 EHEC pairs attached equally well to rubber surfaces (*P* >0.05). In the short-term attachment study, only the pair of 7-52 attached differently on glass and stainless steel surfaces (*P*<0.05). These results suggest that curli is an important cell surface component that mediates the initial interaction and subsequent adherence of some EHEC cells with/to certain abiotic
surfaces. Cell-surface contact time could have a significant influence on EHEC attachment to abiotic surfaces.

Key words: Curli, EHEC, attachment, abiotic surfaces.
INTRODUCTION

Cells of *Escherichia coli* may need to live for a considerable length of time during their life cycle outside animal hosts where the conditions could be less than optimal. Association with solid surfaces in an aqueous environment is a strategy that not only *E. coli* but other bacteria as well use to survive under sub-optimal conditions. *E. coli* cells associated with a surface generally clump together to form microcolonies or biofilm in order to maximize metabolic breakdown and stress management.

Vidal et al. (1998) believe that bacterial interaction with a surface is a two-stage process. The first stage is reversible and bacterial cells can easily be removed from the surface (22). Bacterial association with the surface becomes irreversible in the second stage. It is during this stage that cells multiply and produce extracellular polysaccharide that contributes to the formation of biofilm (15). In addition to polysaccharide, cells of *E. coli* produce a long, thin, and wiry surface fiber known as curli (14, 20, 21). Previous studies have revealed that curli is only expressed under low osmotic pressure, at ambient temperature, and during the stationary growth phase (13, 14).

Curli has a high affinity to Congo red dye (12). Differentiation of curli-expressing from non curli-expressing cells can, therefore, be accomplished by growth on microbiological media with no salt and containing the indicator dye (10). The curli-expressing cells form red, while non curli-expressing cells form colorless colonies on the indicator agar at 28°C.

Curli plays an important role in the adhesion of *E. coli* to its contact hosts (9). It interacts specifically with host matrix proteins such as fibronectin, laminin, and plasminogen to initiate adherence and colonization in the host (6, 13, 18). The curli-expressing variant of *E. coli* O157:H7 was more virulent than the non curli-expressing variant in a mouse model (21). Mice
challenged with curli-expressing cells had shorter survival times than those challenged with non
curli-expressing variant of the same strains. Recent research suggests that curli may also play a
role when cells attach to inert surfaces (15, 22). The aims of this study were to evaluate the role
of curli in assisting the cells of EHEC to attach to the surface of polystyrene, glass, stainless
steel, and rubber as well as to determine the influence of cell-surface contact time on the
efficiency of the attachment.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** EHEC strains 5-9 (O157:H7), 5-11
(O157:H7), 7-52 (O103:H2), and 7-57 (O111:H-), all from our laboratory collection were used in
this study. The cultures were retrieved from frozen storage prior to the experiment and were
cultured twice on tryptone yeast extract (TYE) indicator agar. The medium contained 0.1%
Bacto tryptone, 0.05% Bacto yeast extract, 1.5% Bacto agar, 40 mg/l of Congo red, and 20 mg/l
of coomassie brilliant blue (Sigma Aldrich Co, St. Louis, MO). Cells of *E. coli* O157:H7 5-11
and *E. coli* O103:H2 7-52 formed both curli-expressing and non curli-expressing colonies on
TYE indicator agar. The two variants were purified and used as separate cultures in the
attachment studies. The curli-expressing variant of 5-11 was designated as 5-11C⁺ and the non-
curli producing variant as 5-11C⁻. Similarly, the curli-expressing and non curli-expressing
variant of 7-52 was named as 7-52C⁺ and 7-52C⁻, respectively.

**Preparation of EHEC cultures for attachment studies.** One colony of each EHEC
culture on TYE indicator agar was transferred into 9 ml of TYE broth (0.1% Bacto tryptone and
0.05% Bacto yeast extract). The inoculated cultures were incubated at 28°C for 18 h. The
cultures were diluted (1:40) in fresh TYE broth and the resulting cultures were used in a long-
term attachment study (7 d at 28°C). The same diluted EHEC cultures were incubated at 28°C
for 3 d before being used in a short-term attachment study (24 h at 28°C). Uninoculated TYE broth was used as control throughout the study.

The EHEC cultures used in the attachment studies were serially diluted and appropriate dilutions were plated in duplicate on tryptic soy agar (TSA; Difco Laboratories, Sparks, MD) using an Autoplate 4000 (Spiral Biotech, Norwood, MS). Populations of cells in each culture were calculated so that approximately equal populations of cells of each EHEC culture would be used in the attachment studies.

**Abiotic surfaces.** Abiotic surfaces made of polystyrene, glass, stainless steel, or rubber were used in this study. Twenty-four well sterile tissue culture plates (Falcon 3847, Becton Dickinson Labware, Franklin Lakes, NJ) and sterilized SepCap vials (4.5 X 1.4 cm) (03-375-2A, Fisher Scientific Co. Fair Lawn, NJ) were used as polystyrene and glass surface respectively. Stainless steel (14-gauge and 2B finish; Impulse Manufacturing, Inc; Dawsonville, GA) and buna-n-rubber (1/8” and 70-Duro; Dixie Packing and Gasket Co., Lithia Springs; GA) coupons (2.0 x 5.0 cm²) were also included in the study. The coupons were washed with an alkaline detergent, TergA enzyme (Alconox, Inc., White Plains, NY), and rinsed thoroughly with deionized water before being autoclaved at 121°C for 30 min.

**Long-term attachment study involving polystyrene and glass surfaces.** The diluted EHEC cultures described above (2 ml) were placed into the glass vials and the wells of the tissue culture plates. The EHEC cells in the broth cultures were allowed to attach to the polystyrene and glass surfaces at 28°C for 7 d. The tissue culture plates and vials were covered with parafilm during the attachment to prevent the cultures from evaporating. The broth cultures were withdrawn after the attachment. The glass vials and the wells of the tissue culture plates were washed twice with 2 ml of 0.1% peptone water to remove loosely attached cells. The plates and
vials were then air-dried at 60°C for 2 h. The quantities of EHEC cells attached to both surfaces were determined daily using a crystal violet binding assay.

**Short-term attachment study involving polystyrene and glass surfaces.** Two ml of each culture were added to the glass vials and the wells of the tissue culture plates. The cells were allowed to attach for 24 h at 28°C. The tissue culture plates and glass vials were handled in the same manner as described above. The quantities of attached bacteria were measured in 4-h intervals using the crystal violet binding assay.

**Attachment studies involving stainless steel and rubber coupons.** Stainless steel or rubber coupons were placed horizontally in Petri plates (08-757-13, Fisher Scientific). Thirty ml of each EHEC culture was added into each Petri plate. The remainder of the long- and short-term attachment study was conducted in the same manners as described in the attachment studies involving polystyrene and glass surfaces.

**Crystal violet binding assay.** The EHEC cells that were attached to the polystyrene and glass surfaces were fixed by passing the plates and vials over the flame of a burner (Fisher Scientific) several times. The fixed cells on the surfaces were stained with 2 ml 1% crystal violet (C581-25, Fisher Scientific) at room temperature for 15 min. The wells of the tissue culture plates and glass vials were washed with deionized water until the wash water contained no visible stains. The wells and vials were dried for 2 h at 60°C and cooled to room temperature. Two ml of ethanol:acetone (80:20) mixture was added to the glass vials and wells of tissue culture plates to extract the crystal violet from the stained EHEC cells. The concentrations of the dye extracted from the cells were determined by measuring the absorbance of the ethanol and acetone solutions at wavelength 550 nm.
In the attachment experiments involving stainless steel and rubber surfaces, the loosely attached cells were removed by gently rinsing the coupons in 200 ml deionized water. The coupons were then dried at 60°C for 2 h. The cells on the stainless steel and rubber surfaces were fixed by passing the coupons over the flame of a burner several times. Five ml 1% crystal violet was used to stain the coupons at room temperature for 15 min. The excess dye on the coupons was subsequently washed off using deionized water. The coupons were air-dried at 60°C for 2 h. The amount of dye bound to the cells was extracted with 6 or 6.4 ml of ethanol and acetone solution for stainless steel and rubber coupons, respectively (volumes calculated based on the surface areas of stainless steel and rubber coupons). The concentrations of the extracted dye are expressed as the absorbance at 550 nm.

**Correlation of optical densities at 550 nm to plate counts.** The EHEC cultures (2 ml) were grown in TYE broth at 28°C for 72 h. The resulting cultures were serially diluted and appropriate dilutions were plated in duplicate on TSA using an Autoplate®4000 (Spiral Biotech, Inc., Bethesda, MD). The plates were incubated for 24 h at 37°C and the number of colonies on each plate was determined using an automatic colony counter (Q Count®, Spiral Biotech, Norwood, MS). Concurrently, 2 ml of each serial dilution was dispensed into the wells of a tissue culture plate and was allowed to dry for 8 h at 65°C. Crystal violet binding assay was performed subsequently to determine the amounts of crystal violet extracted from the EHEC cells.

**Statistical analysis.** Two replicates of each attachment study were performed and each experiment was conducted in duplicate. The study was set up by following the randomized complete block design and the data collected was analyzed by general linear model using the
Statistical Analysis Software (SAS, 1999). Significant differences among mean values were determined based on a 95% confidence level.

RESULTS

In addition to the 2 wild type EHEC strains, 7-57 and 5-9, also included in the study were 2 curli-expressing EHEC, 5-11C⁺ and 7-52C⁺, as well as their non curli-expressing mutants, 5-11C⁻ and 7-52C⁻. Both the curli-expressing and non curli-expressing variants of 5-11 and 7-52 carried csgA, the structural gene for a subunit of a major curli protein (Pawar and Chen, Unpublished). Scanning electronic micrographs revealed that the cells of curli-expressing variants of 5-11 and 7-52 had coiled fimbriae on their surfaces (Fig. 4.1).

EHEC cultures used in the long- and short-term attachment studies had similar cell populations. The average cell counts used in the long-term attachment study were 8.63 ± 0.08, 8.55 ± 0.15, 8.52 ± 0.15, 8.52 ± 0.15, 8.48 ± 0.13, 8.30 ± 0.22, and 8.37 ± 0.15 log_{10} CFU/ml in day 1 to 7, respectively. The average populations of EHEC cells in the short-term attachment study were 8.45 ± 0.10 log_{10} CFU/ml at 0 h and 8.36 ± 0.08 at 24 h.

The population of cells in the EHEC cultures correlated to the quantities of the crystal violet extracted from the cells that attached to the polystyrene surfaces (Table 4.1). The optical densities of the crystal violet solutions at 550 nm elevated as the population of cells in the EHEC cultures increased (Table 4.1). This correlation is critically important as it provides the scientific basis for using the optimal densities of crystal violet to reflect the efficiency of EHEC attachment on abiotic surfaces.

The results of the long-term attachment study revealed that 7-57C⁺ attached to the polystyrene and glass surfaces more efficiently than did 5-9C⁻ (P<0.05). The curli-expressing variant of 5-11 had a better ability to adhere to the polystyrene and glass surfaces than did its non
curli-expressing counterpart ($P<0.05$) (Fig. 4.2A and 4.2B; Table 4.2). The differences in attachment between the 2 members of another EHEC pair, 7-52C$^+$ and 7-52C$^-$, on polystyrene and stainless steel surfaces were statistically significant ($P<0.05$) (Fig. 4.2C; Table 4.2). The attachment of the pair on glass surfaces, however, was statistically insignificant ($P>0.05$) (Fig. 4.2B, Table 4.2). In addition, the 2 members of all 3 EHEC pairs attached equally well on rubber surfaces (Fig. 4.2D, Table 4.2).

Greater amounts of crystal violet were extracted from rubber, polystyrene, and stainless steel than from glass surfaces in the long-term attachment study (Figure 4.2). The highest optical densities of the crystal violet extracted from polystyrene, rubber, and stainless steel surfaces were 2.60 (Fig. 4.2A), 2.56 (Fig. 4.2D), and 2.51 (Fig. 4.2C) units, respectively. The same reading from the glass surface, however, was only 1.63 units (Fig. 4.2B).

In the short-term attachment study, the attachment of 2 EHEC pairs, 7-57C$^+$ and 5-9C$^-$ as well as 5-11C$^+$ and 5-11C$^-$, on polystyrene, glass, stainless steel, and rubber surfaces were statistically insignificant (Table 4.2). The additional pair, 7-52, attached equally well to polystyrene and rubber surfaces ($P>0.05$). The non curli-expressing variant, however, had greater attachment on glass and stainless steel surfaces ($P<0.05$) (Table 4.2), suggesting that cell surface appendages other than curli may also play a role in the attachment of EHEC on abiotic surfaces.

Cell-surface contact time appeared to have a significant influence on EHEC attachment to abiotic surfaces (Fig. 4.2 and 4.3; Table 4.3 and 4.4). The optical densities in the long-term attachment study (Table 4.3) were significantly higher than those obtained in the short-term attachment study (Table 4.4), except for the results obtained from the rubber surfaces.
DISCUSSION

Among the EHEC cultures used in the attachment studies, 7-57 and 5-9 were stable. Phenotypic conversion, changing from one curli phenotype to another, seldom occurred. In contrast, 5-11 and 7-52 were unstable and non curli-expressing mutants developed spontaneously during growth on TYE indicator agar at 28°C. Our preliminary research revealed that both the curli-expressing parents and their mutants carried the \( csg \) operon. This confirmed the results of Uhlich et al who found that the lack of curli expression in \textit{E. coli} O157:H7 was not due to large DNA deletion or insertion but a specific base pair change in one of the genes, \( csgD \), on curli operon (20). The non curli-expressing mutants used in our study developed spontaneously and the difference between the wild type parents and the mutants was limited to the expression of curli, it was, therefore, beneficial to include the cultures of both phenotypes in the attachment studies.

Biochemical, biophysical, and imaging analysis has concluded that curli produced by \textit{E. coli} were actually amyloid, extracellular deposits that are homogeneous and eosinophilic (7). Amyloid formation in eukaryotic cells is believed to be the result of a misguided protein-folding pathway. But the \( csg \) operon of \textit{E. coli} encodes a specific nucleation precipitation machinery to assemble curli. Like other amyloid fibers, curli induces a spectral change of a 10 \( \mu \)M Congo red solution with a maximum difference in absorbance between Congo red solution alone and Congo red bound to curli fiber at ~541 nm (7). The dyeing of amyloid is done by a process similar to the direct textile dyeing of cotton. The linearity of the dye configuration permits hydrogen bonding of the azo and amine groups of the dye to the carbohydrate hydroxyl groups of the amyloid substance (3).
Quantification of surface-associated cells in this study was accomplished by crystal violet staining, ethanol:acetone extraction, and optical density measurement. Crystal violet is a commonly used dye that stains the peptidoglycan of bacterial cell walls. Both gram-negative and gram-positive bacteria cell walls are composed of peptidoglycan and both take up the crystal violet. The gram negative bacteria, however, have a layer of lipopolysaccharide external to the cell wall, which is disrupted in the ethanol and acetone rinse, allowing the crystal violet to escape. The amounts of the leaked dye are theoretically proportional to the numbers of stained cells.

The primary thermodynamic force to induce the attachment of bacterial cells to a solid surface from an aqueous environment is the hydrophobic effect (2). Metals and polymers reportedly have different surface properties (1). The metal surfaces are highly electronegative, hydrophilic and possess high surface energy. The polymers, however, are electrostatic, hydrophobic, and have low surface energy. Among the four types of surfaces tested in this study, rubber and polystyrene are hydrophobic with little or no surface charges. Stainless steel is, in contrast, hydrophilic with either positive or neutral surface charges. Glass surfaces are also hydrophilic but possess negative surface charges (8).

In the present study, the EHEC cultures attached more effectively on hydrophobic surfaces with a descending order of attachment on rubber, polystyrene, stainless steel, and glass. Similar results were observed in Pseudomonas spp. Greater adhesion was seen on hydrophobic plastic such as polyethylene, polystyrene, and polyethylene terephthalate, followed by hydrophilic metals and glass (8, 11, 17).

It was found in our study that cell-surface contact time had a significant influence on the efficiency of EHEC attachment to abiotic surfaces. Cells of EHEC attached better in the long-
term than in the short-term attachment study. This agrees with the results of Takeuchi and Frank who reported that the numbers of cells attached to the tested materials was influenced by the lengths of cell-surface contact time (Takeuchi and Frank, 2001).

ACKNOWLEDGEMENTS

This study was made possible through research funding provided by the National Beef Cattlemen’s Association. The authors wish to thank Jerry Davis for statistical support.
REFERENCES


TABLE 4.1. Correlation between the populations of EHEC cells and the optical densities of the crystal violet extracted from the EHEC cells that attached to the polystyrene surfaces

<table>
<thead>
<tr>
<th>EHEC population (Log$_{10}$ CFU/ml)</th>
<th>7-57C$^+$</th>
<th>7-57C$^-$</th>
<th>5-9C$^-$</th>
<th>5-11C$^+$</th>
<th>5-11C$^-$</th>
<th>7-52$^+$</th>
<th>7-52C$^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.0</td>
<td>9.12$^a$</td>
<td>2.557</td>
<td>2.587</td>
<td>2.599</td>
<td>2.623</td>
<td>2.544</td>
<td>2.627</td>
</tr>
<tr>
<td>8.0</td>
<td>8.12</td>
<td>0.479</td>
<td>0.534</td>
<td>0.690</td>
<td>0.556</td>
<td>0.486</td>
<td>0.486</td>
</tr>
<tr>
<td>7.0</td>
<td>7.12</td>
<td>0.192</td>
<td>0.199</td>
<td>0.251</td>
<td>0.233</td>
<td>0.188</td>
<td>0.188</td>
</tr>
<tr>
<td>6.0</td>
<td>6.12</td>
<td>0.096</td>
<td>0.068</td>
<td>0.091</td>
<td>0.086</td>
<td>0.126</td>
<td>0.126</td>
</tr>
<tr>
<td>5.0</td>
<td>5.12</td>
<td>0.045</td>
<td>0.048</td>
<td>0.049</td>
<td>0.045</td>
<td>0.053</td>
<td>0.053</td>
</tr>
</tbody>
</table>

$^a$ absorbance at 550 nm
<table>
<thead>
<tr>
<th>Surfaces</th>
<th>Long-term Attachment Studies</th>
<th>Short-term Attachment Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EHEC Cultures</td>
<td>EHEC Cultures</td>
</tr>
<tr>
<td></td>
<td>7-57C(^+) &amp; 5-9C(^-)</td>
<td>7-57C(^+) &amp; 5-9C(^-)</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>0.00</td>
<td>0.24</td>
</tr>
<tr>
<td>Glass</td>
<td>0.00</td>
<td>0.12</td>
</tr>
<tr>
<td>Stainless Steel</td>
<td>0.85</td>
<td>0.56</td>
</tr>
<tr>
<td>Rubber</td>
<td>0.08</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>7-52C(^+) &amp; 7-52C(^-)</td>
<td>7-52C(^+) &amp; 7-52C(^-)</td>
</tr>
<tr>
<td></td>
<td>0.00</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>0.26</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>0.65</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>5-11C(^+) &amp; 5-11C(^-)</td>
<td>5-11C(^+) &amp; 5-11C(^-)</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>0.55</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>0.62</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>0.31</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>0.62</td>
<td>0.73</td>
</tr>
</tbody>
</table>

TABLE 4.2. The \( P \) values that reflect the differences in EHEC attachment on abiotic surfaces in the long- and short-term attachment studies.
### TABLE 4.3. The effect of cell-surface contact time on the efficiency of EHEC attachment to abiotic surfaces in the long-term attachment study

<table>
<thead>
<tr>
<th>Surface</th>
<th>Attachment Time (Days)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Polystyrene</td>
<td>0.38&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.88&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.06&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.22&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Glass</td>
<td>0.30&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.54&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.73&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.77&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Stainless Steel</td>
<td>0.82&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.08&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.20&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Rubber</td>
<td>2.54&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.53&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean absorbance at 550 nm in the same row that are not followed by the same letter are significantly different (P ≤ 0.05)
TABLE 4.4. The effect of cell-surface contact time on the efficiency of EHEC attachment to abiotic surfaces in the short-term attachment study

<table>
<thead>
<tr>
<th>Surface</th>
<th>Attachment Time(^a) (h)</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>20</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polystyrene</td>
<td></td>
<td>0.06(^d)</td>
<td>0.09(^d)</td>
<td>0.10(^cd)</td>
<td>0.33(^bc)</td>
<td>0.56(^ab)</td>
<td>0.58(^a)</td>
<td>0.65(^ab)</td>
</tr>
<tr>
<td>Glass</td>
<td></td>
<td>0.12(^f)</td>
<td>0.17(^ef)</td>
<td>0.22(^de)</td>
<td>0.28(^cd)</td>
<td>0.33(^bc)</td>
<td>0.39(^b)</td>
<td>0.49(^a)</td>
</tr>
<tr>
<td>Stainless Steel</td>
<td></td>
<td>0.49(^d)</td>
<td>0.47(^d)</td>
<td>0.59(^cd)</td>
<td>0.64(^c)</td>
<td>0.80(^b)</td>
<td>0.93(^ab)</td>
<td>1.05(^a)</td>
</tr>
<tr>
<td>Rubber</td>
<td></td>
<td>1.98(^d)</td>
<td>2.16(^c)</td>
<td>2.36(^ab)</td>
<td>2.28(^bc)</td>
<td>2.25(^bc)</td>
<td>2.36(^ab)</td>
<td>2.50(^a)</td>
</tr>
</tbody>
</table>

\(^a\)Mean absorbacne at 550 nm in the same row that are not followed by the same letter are significantly different (P ≤ 0.05)
FIG. 4.1. Scanning electron micrographs of curli-expressing (A) and non curli-expressing cells (B) of *E. coli* O157:H7 5-11.
FIG. 4.2. Long-term attachment study. Three pairs of EHEC cultures, 7-57C$^+$ (●) and 5-9C$^-$ (○), 5-11C$^+$ (▲) and 5-11C$^-$ (△), as well as 7-52C$^+$ (◼) and 7-52C$^-$ (◻) and control (●), were allowed to attach to polystyrene (A), glass (B), stainless steel (C), and rubber (D) to surfaces at 28°C for 7 d. The quantities of EHEC cells that attached to the surfaces were determined daily by a crystal violet binding assay.
FIG. 4.3. Short-term attachment study. Three pairs of EHEC cultures, 7-57C+ (●) and 5-9C- (○), 5-11C+ (▲) and 5-11C- (△), as well as 7-52C+ (■) and 7-52C- (□) and control (●), were allowed to attach to polystyrene (A), glass (B), stainless steel (C), and rubber (D) surfaces at 28°C for 24 h. The quantities of EHEC cells that attached to the surfaces were determined in 4-h intervals by a crystal violet binding assay.
CHAPTER 5

Attachment of Enterohemorrhagic Escherichia coli on a Growth Medium as well as on Raw and Ready-to-Eat Beef Products

---

ABSTRACT

Both raw and ready-to-eat beef have been linked to the outbreaks of enterohemorrhagic *Escherichia coli* (EHEC) infections. The contamination of these products in the beef processing environment could occur at any stage from slaughtering to packaging. Beef-contact surfaces with adhered EHEC cells are a likely source of the contamination. Research has revealed that a thin, wiry, and aggregative cell surface fiber, known as curli, enhances the attachment of EHEC to the surface of polystyrene, glass, and stainless steel. We evaluated in this study the role of curli in the attachment of EHEC cells to raw and ready-to-eat beef products. Beef from an eye round roast and beef salami were sliced and cut into 3 mm thick, 4.6 cm diameter circles. The meats were exposed for different lengths of time to six EHEC cultures, the curli-expressing and non curli-expressing variants of *E. coli* O157:H7 and O103:H2 as well as a wild type curli-expressing strain of *E. coli* 111:H- and a non curli-expressing strain of *E. coli* O157:H7. The beef slices were rinsed following the exposure and EHEC cells that attached to the surfaces of beef were enumerated. Two replications were performed and the data generated was analyzed statistically based on a 95% confidence level. The results indicated that certain curli-expressing strain/variants of EHEC attached, under appropriate conditions, more efficiently than did the non curli-expressing strains/variants. The differences in attachment were, however, minor with the smallest differences of 0.12 and 0.22 Log\(_{10}\) CFU/cm\(^2\) and the largest differences of 0.86 and 1.17 Log\(_{10}\) CFU/cm\(^2\) on raw beef and beef salami, respectively.

In separate experiments, the EHEC cultures were grown on tryptone yeast extract (TYE) agar at 28°C for 72 h and the ease of removing the EHEC cells from the surface of the agar was evaluated. The results indicated that the curli-expressing cells had a firmer association with, and
was more difficult to remove from, the surface of agar in comparison to the non curli-expressing cells.

Keywords: Curli, attachment, EHEC, *E. coli* O157:H7, raw beef, beef salami.
INTRODUCTION

Enterohemorrhagic \textit{Escherichia coli} (EHEC) is a group of important foodborne pathogens which are associated with severe enteric and systematic diseases such as hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP) \cite{10, 14, 23}. Outbreaks of EHEC infection have been linked to the consumption of undercooked ground beef as well as ready-to-eat beef products, such as salami \cite{2, 3, 8}. Contamination of beef in the processing environment can occur at any stage from slaughtering to packaging. A survey conducted in the Northwestern regions of the U.S. revealed that the prevalence of EHEC O157:H7 in pre-eviscerated, post-eviscerated, and post-processed beef carcasses was 43\% (148 of 341), 18\% (59 of 332), and 2\% (6 of 330), respectively \cite{9}.

EHEC attachment to meat as well as to other surfaces is a complex process that can be influenced by the properties of both EHEC and their contact surfaces. Cells of EHEC express curli, a thin, wiry, and aggregative surface appendage \cite{5, 19, 20, 24}, which appears as a ~ 4-12 nm protein fiber under a transmission electron microscope \cite{4, 17} and 29 nm under scanning electron microscope \cite{18}. Over-expressed curli often forms an interconnecting mesh between EHEC cells. Curli expression in \textit{E. coli} can be induced by low osmotic condition, nutritional starvation, and lower than optimal growth temperatures \cite{16, 17}.

Curli enables \textit{E. coli} to adhere to host matrix proteins such as fibronectin, laminin, and plasminogen \cite{1, 13, 16, 21}. A curli-expressing variant of \textit{E. coli} O157:H7 was found to be more virulent to mice than the non curli-expressing variant of the same strain \cite{24}. Mice challenged with curliated cells of \textit{E. coli} O157:H7 had shorter survival times than those challenged with non-curliated cells. The goal of this study was to evaluate the role of curli in the attachment of EHEC on a growth medium as well as on raw and ready-to-eat beef.
MATERIALS AND METHODS

**EHEC strains and growth conditions.** EHEC strains 5-9 (O157:H7), 5-11 (O157:H7), 7-52 (O103:H2), and 7-57 (O111:H-), all from our laboratory collections were used in this study. The cultures were retrieved from frozen storage prior to the experiment and were cultured twice on tryptone yeast extract (TYE) indicator agar. The medium contained 0.1% tryptone, 0.05% yeast extract, 1.5% agar, 40 mg/l of Congo red (Sigma Aldrich Co, St. Louis, MO), and 20 mg/l of coomassie brilliant blue (Sigma). Cells of *E. coli* O157:H7 5-11 and *E. coli* O103:H2 7-52 formed both curli-expressing and non curli-expressing colonies on TYE indicator agar. The two variants were purified and used as separate cultures in the attachment studies. The curli-expressing variant of 5-11 was designated as 5-11C+ and the non-curli producing variant as 5-11C−. Similarly, the curli-expressing and non curli-expressing variants of 7-52 were named 7-52C+ and 7-52C−, respectively.

**Removal of EHEC cells attached to the surface of growth media.** The EHEC cultures described above were streaked heavily on TYE agar plates. The inoculated plates were incubated at 28°C for 72 h. The plates were removed from the incubator following incubation and placed on an orbital shaker (Lab-Line Instruments Inc, Melrose Park, IL) before 15 ml of saline (0.85% NaCl) was added. The EHEC cells were removed by shaking at 50 rpm for 20 min at room temperature. The cells that became suspended in the saline solutions were collected and discarded. The plates were then rinsed five times, each with 200 ml of sterile deionized water. The cells that firmly attached to the media were scraped from the agar surfaces and suspended in 25 ml of peptone water. Appropriate dilutions of the cell suspensions were made and spirally plated on tryptic soy agar (TSA, Difco Laboratories, Sparks, MD) using an Autoplate®4000 (Spiral Biotech, Inc., Bethesda, MD). The plates were incubated for 24 h at 37°C. The colonies
were then enumerated using an automatic colony counter (Q Count®, Spiral Biotech, Inc., Norwood, MA).

**Preparation of beef samples.** Beef from an eye round roast and beef salami slices were purchased from a supermarket in Griffin, GA one day before the experiment. The raw beef was stored at –20°C overnight. On the experiment day, the frozen beef was withdrawn from storage and cut into 3 mm thick slices using an electric slicer (Rival Manufacturing, ON). The beef salami (3 mm) was stored at 4°C prior to use. Both raw beef and salami slices were cut into 4.6 cm diameter circles using a cookie cutter (Fig. 1).

**Preparation of EHEC cultures for the attachment studies.** EHEC cultures were inoculated on TYE agar plates. The inoculated cultures were incubated at 28°C for 72 h. The resulting cultures were collected with 25 ml of saline. The cell suspensions were vortexed vigorously to break the clumped cells (Fig. 2). The optical densities of the cell suspensions were adjusted to 1.00 ± 0.03 at wavelength 600 nm. The suspensions were serially diluted and the appropriate dilutions were plated as described above. Population of cells in each culture was calculated so that approximately equal populations of cells of each EHEC culture would be used in the attachment studies.

Beef slices placed in a Petri plate were exposed to 25 ml of an EHEC culture (~10^9 CFU/ml) for 2, 15, 30, and 120 min, respectively, at room temperature. The slices were washed 5 times after the attachment, each for 1 min in 200 ml sterile water at 100 rpm. The washed beef and beef salami slices were mixed with 45 ml of 0.1% peptone water and stomached for 2 min at normal speed (Seward Limited, London). The homogenates were serially diluted and appropriate dilutions were plated as described above.
**Scanning electronic microscopy.** A piece of raw beef (3 mm thick, 15 mm in diameter) with attached EHEC cells was fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (SCB; pH 7.4) at room temperature for 90 min. The fixed cells were washed 3 times with SCB, each for 10 min. The lipids were fixed with 1% osmium tetroxide at room temperature for 1 h. The samples were washed twice with SCB and then dehydrated serially with 50, 70, 80, 90, and 100% ethanol, each for 15 min. The samples were then stored at 4°C in 100% ethanol and were dried at critical point temperature with liquid CO₂ using a Critical Point Dryer (Samdri model 780-A, Tousimis, Rockville, MD). The dehydrated samples were coated with gold in a sputter coater (Structure Probe, Inc., West Chester, PA). The coated samples were visualized using a scanning electron microscope (FE-SEM, LEO Electron Microscopy, Inc, Thornwood, NY).

**Statistical analysis.** Two replicates of each attachment study were performed and each experiment was conducted in duplicate. The study was set up by following the randomized complete block design and the data collected was analyzed by general linear model using the Statistical Analysis Software (22). Significant differences among mean values were determined based on a 95% confidence level.

**RESULTS AND DISCUSSION**

The adhesion of bacteria to animal tissues is a complicated phenomenon, which is far from being fully understood (6, 7, 11, 12). It is known however, that bacterial adherence to surfaces, including animal tissues, is a two-stage process (25). Although the association of bacterial cells with their contact surfaces can eventually become permanent, the initial contact is always reversible. Cells that loosely attached to the beef surfaces in the present study were removed by rinsing the beef with saline and sterile water. Cells that firmly associated with the surfaces of beef were enumerated subsequently.
It was noticed in our preliminary study that curliated cells of EHEC generally established a firm association with the surface of agar on which they were growing. In this study, the ease of removing the EHEC cells from their growth media was evaluated. After a TYE agar plate with a grown EHEC culture was rinsed with saline and sterile water, the non curli-expressing cells of 5-9 C−, 5-11, and 7-52 were effectively removed (Fig. 5.3 B and D), the curli-expressing cells; however, remained firmly attached to the surface of the agar plates (Fig. 5.3 A and C). Results of total plate counts revealed that approximately 3.67, 2.81, and 4.58 Log$_{10}$ CFU/cm$^2$ of 5-11C$^-$, 7-52C$^-$, and 5-9 C$^-$ were removed whereas only 1.01, 0.46, and 0.17 log$_{10}$ CFU/cm$^2$ of 5-11C$^+$, 7-52C$^+$, and 7-57C$^+$ were removed from the agar plates (Fig. 5.4). These results indicated that curli enhanced the attachment of EHEC to the surface of their growth medium.

Interestingly, the non curli-expressing cells that became suspended in the rinsing solutions often left a smear of cells on the surface of agar when the suspensions were removed. The efficiency of removal of the non curli-expressing cells might, therefore, have been underestimated. A better methodology is needed for more accurate and precise estimation of the efficiency of removal.

As stated previously, curli interacts specifically with host matrix proteins such as fibronectin, laminin, and collagen (1, 16). These proteins are the components of animal connective tissue, which can be found between muscle surface and the skin (15). The results of this study suggested that EHEC could interact with these host proteins and successfully attach on the surface of beef. The attachment of 5-11C$^+$ and 5-11C$^-$ on raw beef was statistically different when the cells were allowed to interact with the surface of beef for 2, 15, 30, and 120 min, respectively (Fig. 5.5 C; Table 5.1). The raw beef samples exposed to the cells of 7-57C$^+$ and 5-9C$^-$ for 120 min had significantly different populations of cells than those that were exposed for
2, 15, or 30 min (Fig. 5.5 A; Table 5.1). The cells of 7-52C⁺ and 7-52C⁻ only attached differently when they interacted with the surface of raw beef for 15 or 120 min (Fig. 5.5 B; Table 5.1). The attachment of 7-57C⁺ and 5-7C⁻ cells on beef salami was statistically different, regardless of the lengths of cell-surface contact time (Fig. 5.6 A; Table 5.2). The other 2 EHEC pairs, however, only attached differently when the cells interacted with the surface of salami for 2 or 120 min (Fig. 5.6 B and 5.6 C; Table 5.2). Compared to the agar, beef had a rough and uneven surface. The gaps between the muscle fibers were able to trap large numbers of EHEC cells (Fig. 5.7). The efficiency of attachment reflected by plate counts certainly had limitations. The method, however, is still the practical choice for study of bacterial attachment on biotic surfaces.

\textit{E. coli} O111-H- 7-57C⁺ expresses the greatest amounts of curli than any other strains tested in the study. Significant difference in attachment is often observed between the pair of 7-57C⁺ and 5-9C⁻. The 7-52 is an \textit{E. coli} O103:H2 isolate which behaved unpredictably in the attachment study involving glass and stainless steel surfaces (18). In this study, the 2 members of the pair attached differently on raw beef at 15 and 120 min of contact time (Fig 5.5 B; Table 5.2), on beef salami at 2 and 120 min of contact time (Fig. 5.6 B; Table 5.2).

This study demonstrated that curli, under appropriate conditions, enhanced the attachment of EHEC cells on the surface of raw and ready-to-eat beef products. It addressed the importance of control of curli expression on the surface of EHEC under food processing and storage conditions.

\textbf{ACKNOWLEDGEMENT}

This study was made possible through research funding provided by the National Beef Cattlemen’s Association. Statistical assistance was provided by Jerry Davis.
REFERENCES


Table 5.1. Attachment of curli-expressing and non curli-expressing EHEC cells at different attachment times to raw beef.*

<table>
<thead>
<tr>
<th>Attachment time</th>
<th>7-52C⁺</th>
<th>7-52C⁻</th>
<th>5-11C⁺</th>
<th>5-11C⁻</th>
<th>7-57C⁺</th>
<th>5-9C⁻</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 min</td>
<td>6.30 a B</td>
<td>6.03 ab B</td>
<td>6.26 a B</td>
<td>5.49 c B</td>
<td>6.28 a A</td>
<td>5.74 bc B</td>
<td>6.01 C</td>
</tr>
<tr>
<td>15 min</td>
<td>6.52 a B</td>
<td>6.05 a B</td>
<td>6.50 a AB</td>
<td>5.64 c B</td>
<td>6.45 a A</td>
<td>5.66 c B</td>
<td>6.13 B</td>
</tr>
<tr>
<td>30 min</td>
<td>6.50 ab B</td>
<td>6.04 cb B</td>
<td>6.58 a AB</td>
<td>5.87 c AB</td>
<td>6.49 ab A</td>
<td>5.70 c B</td>
<td>6.19 B</td>
</tr>
<tr>
<td>120 min</td>
<td>6.89 a A</td>
<td>6.77 a A</td>
<td>6.81 a A</td>
<td>6.55 b A</td>
<td>6.50 bc A</td>
<td>6.33 c A</td>
<td>6.63 A</td>
</tr>
<tr>
<td>Overall</td>
<td>6.55 a</td>
<td>6.22 b</td>
<td>6.54 a</td>
<td>5.89 b</td>
<td>6.43 a</td>
<td>5.86 c</td>
<td></td>
</tr>
</tbody>
</table>

*: Averages of total plate counts in column not followed by the same upper case letter are significantly different with respect to attachment time; Averages of total plate counts in rows not followed by the same lower case letter are significantly different with respect to cultures.
Table 5.2. Attachment of curli-expressing and non curli-expressing EHEC cells at different attachment time to beef salami*

<table>
<thead>
<tr>
<th>Attachment time</th>
<th>7-52C⁺</th>
<th>7-52C⁻</th>
<th>5-11C⁺</th>
<th>5-11C⁻</th>
<th>7-57C⁺</th>
<th>5-9C⁻</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 min</td>
<td>5.29 a C</td>
<td>4.98 b C</td>
<td>5.24 a C</td>
<td>4.86 bc B</td>
<td>5.30 a C</td>
<td>4.78 c AB</td>
<td>5.05 C</td>
</tr>
<tr>
<td>15 min</td>
<td>5.34 a C</td>
<td>5.05 a C</td>
<td>5.36 a C</td>
<td>4.89 a B</td>
<td>5.42 a BC</td>
<td>4.25 b B</td>
<td>5.07 C</td>
</tr>
<tr>
<td>30 min</td>
<td>5.69 a B</td>
<td>5.48 a B</td>
<td>5.80 a B</td>
<td>5.54 a AB</td>
<td>5.63 a BC</td>
<td>4.72 b AB</td>
<td>5.47 B</td>
</tr>
<tr>
<td>120 min</td>
<td>6.38 a A</td>
<td>6.15 b A</td>
<td>6.37 a A</td>
<td>5.99 c A</td>
<td>6.09 b A</td>
<td>5.41 d A</td>
<td>6.06 A</td>
</tr>
<tr>
<td>Overall</td>
<td>5.68 a</td>
<td>5.42 b</td>
<td>5.69 a</td>
<td>5.32 b</td>
<td>5.61 a</td>
<td>4.78 c</td>
<td></td>
</tr>
</tbody>
</table>

*: Averages of total plate counts in column not followed by the same upper case letter are significantly different with respect to attachment time; Averages of total plate counts in rows not followed by the same lower case letter are significantly different with respect to cultures.
FIGURE 5.1. Beef and salami slices used in the attachment studies.
FIGURE 5.2. Light micrographs of curli-expressing (A), and non curli-expressing (B) EHEC cells used in beef attachment studies.
FIGURE 5.3. Removal of EHEC cells that attached on the surfaces of agar plates. Residual cultures of *E. coli* 7-57 (A), 5-9 (B), 7-52C\(^+\) (C), and 7-52C\(^-\) (D) after the TYE indicator agar plates, on which the culture had been grown, were rinsed with 15 ml of 0.85% NaCl and rinsed 5 times, each with 200 ml of sterile deionized water.
Figure 5.4: Total plate counts of EHEC cells that attached on the surfaces of TYE agar plates. The cultures had been grown, were rinsed with 15 ml of 0.85% NaCl and rinsed 5 times, each with 200 ml of sterile deionized water and the total plate counts before and after washing the TYE agar plates were determined.
Figure 5.5: Attachment of *E. coli* 7-57C⁺ and 5-9 C⁻ (A), 7-52C⁺ and 7-52C⁻ (B,) as well as 5-11C⁺ and 5-11C⁻ (C) on raw beef.
Figure 5.6: Attachment of *E. coli* 7-57C⁺ and 5-9 C⁻ (A), 7-52C⁺ and 7-52C⁻ (B), as well as 5-11C⁺ and 5-11C⁻ (C) on beef salami.
FIGURE 5.7. Scanning electronic micrographs of *E. coli* cells attached to the surface of raw beef.
CHAPTER 6

CONCLUSIONS

The following conclusions were drawn from the studies described in Chapter 3, 4, and 5:

1. The quantities of curli expressed by EHEC cells were inversely proportional to the amounts of free Congo red in the supernatants of the cell suspensions. Two non curli-expressing variants tested in the studies were more fastidious than were their curli-expressing counterparts and both failed to grow on MGA although they were capable of growing on casamino acid agar. Both curli-expressing and non curli-expressing EHEC cells carried \textit{csgA}, the structural gene for curli protein. Curli phenotypic conversion took place spontaneously and was influenced to a certain extent by growth media and incubation temperatures.

2. The cells of 7-57C\textsuperscript{+} attached to the polystyrene and glass surfaces more efficiently ($P < 0.05$) than did the cells of 5-9C\textsuperscript{-}. The curli-expressing variant of 5-11 possessed a better ability to adhere to the polystyrene and glass surfaces than did its non curli-expressing counterpart ($P<0.05$). The differences in attachment between 7-52C\textsuperscript{+} and 7-52C\textsuperscript{-} on polystyrene and stainless steel surfaces were statistically significant ($P<0.05$). The attachment of the pair on the glass surfaces, however, was statistically insignificant ($P>0.05$). In addition, the 2 members of all 3 EHEC pairs attached equally well to rubber surfaces ($P>0.05$). In the short-term attachment study, only the pair of 7-52 attached differently on glass and stainless steel surfaces ($P<0.05$). These results suggest that curli is an important cell surface component that mediates the initial interaction and
subsequent adherence of some EHEC cells with/to certain abiotic surfaces. Cell-surface contact time could have a significant influence on EHEC attachment to abiotic surfaces.

3. Certain curli-expressing variants of EHEC attached, under appropriate conditions, more efficiently on the surfaces of raw and ready-to-eat beef products than did the non curli-expressing variants. However, the differences in attachment were minor, with the smallest differences of 0.12 and 0.22 $\log_{10}$ CFU/cm$^2$ and the largest differences of 0.86 and 1.17 $\log_{10}$ CFU/cm$^2$ on raw beef and beef salami, respectively. These results suggest that curli could play a role in mediating the initial interaction and subsequent adherence of some EHEC cells with/to beef surfaces. Cell-surface contact time could have a significant influence on EHEC attachment to these surfaces.