CHARACTERIZATION OF CELLULOSE, AN EXTRACELLULAR POLYSACCHARIDE PRODUCED BY SHIGA TOXIN PRODUCING *ESCHERICHIA COLI*

by

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(Under the direction of JINRU CHEN)

ABSTRACT

Shiga toxin producing *Escherichia coli* (STEC) are known to have several defense mechanisms, one of which is the production of protective, extracellular substances such as the production of cellulose, a long-chain polysaccharide of glucose with β-1, 4 glycosidic bonds. This study was undertaken to determine the physiological conditions favoring the production of cellulose and to prepare pairs of STEC strains, comprising of a cellulose producing parent and a spontaneous cellulose deficient mutant, useful for research studies designed to address the roles of cellulose in protecting the cell of STEC under stress. It was found that the cultural conditions that favored the production of cellulose by STEC included a 28^{o}C incubation temperature, aerobic atmosphere and presence of 2% of ethanol on Luria Bertani no salt agar with a pH value of 6.0 and a water activity of 0.99. Two pairs of STEC strains were prepared and characterized. The two members within each pair shared the same serotypes and similar PFGE profiles. Profound morphological differences were however, found between the two types of cells. The two members of each STEC pair shared similar growth characteristics except under extreme stress in the phenotypic microarray study. All these STEC strains were subjected to oxidative (10, 20 and 30 mM H_2O_2), osmotic (1, 2 and 3 M NaCl) and acidic (pH 3.0, 3.5 and 4.5) stress as well as

chlorine treatment (25, 50 and 100 µg/ml NaOCl). Cells of cellulose producing strains were found to be relatively more resistant than those of the cellulose deficient strains.

INDEX WORDS: Shiga toxin producing *E. coli*, Bacterial cellulose, Scanning electron microscopy, Transmission electron microscopy, Phenotype microarray, Colanic acid, Hydrogen peroxide, Sodium chloride, Acetic acid, Sodium hypochlorite

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

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2009

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May 2009

ACKNOWLEDGEMENTS

I would like to acknowledge my major professor, Dr. Jinru Chen for her invaluable guidance, constant encouragement, and financial support throughout my Ph.D. program. I am very grateful to Dr. Joseph F. Frank, Dr. Robert D. Phillips, Dr. Mark A. Harrison, and Dr. Yao-Wen Huang for serving on my advisory committee. Appreciation is extended to many friends in the Department of Food Science and Technology for all their friendship and invaluable help. Finally, I want to thank my family including my wife and my precious daughter, for all their love and support.

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CHAPTER 1

INTRODUCTION

Shiga toxin producing *Escherichia coli* (STEC) is one of the most important groups of bacterial pathogens in the United States as well as many other countries in the world. Although the occurrence of STEC related foodborne illness has been slightly decreased in the recent past, the impact of the illness on public health cannot be overlooked. STEC causes severe gastroenteritis that may progress to hemorrhagic colitis and hemolytic-uremic syndrome in individuals with compromised immune systems (Nataro and Kaper 1998).

Gram negative bacterial cells are known to produce several extracellular polysaccharides (EPS), one of which is cellulose. Cellulose is a β-1,4 long chain polysaccharide of glucose, enabling bacterial cells to interact with one another (Romling et al. 2000) and to form biofilms (Danese et al. 2000). Cellulose was previously known to be synthesized by plant pathogenic bacteria such as *Gluconoacetobacter, Rhizobium* and *Agrobacterium* (Ross et al. 1991; Masaoka et al. 1993). Recent studies have shown that some members of the *Enterobacteriaceae* such as *Enterobacter sakazakii* (Grimm et al. 2008), *Salmonella* Typhimurium (Zogaj et al. 2001) and *E. coli* (Da Re and Ghigo 2006) are also capable of producing cellulose. The amounts of cellulose produced by these organisms are however, relatively low. Knowledge of environmental conditions and media compositions that can enhance the production could significantly benefit cellulose and biofilm research.

In addition to cellulose, some strains of STEC are known to produce copious amounts of another EPS that is comprised of colanic acid (CA) (EPS-CA). Although the EPS-CA has been shown to protect the cells of STEC against oxidative stress (Chen et al. 2004), osmotic shock (Hiramatsu et al. 2005) and treatments with organic acids (Mao et al. 2006), the role of cellulose and cellulose in conjunction with the EPS-CA in protecting the cells of STEC has not been appropriately defined. In order to address the protective role of cellulose and cellulose in conjunction with the EPS-CA, the use of only wild type STEC strains may not be adequate.

The objectives of the present study include:

1. To determine the influence of cultural conditions and media composition on the production of cellulose by STEC strains, and to formulate a new microbiological medium that can significantly enhance the production of cellulose by STEC.
2. To select, and characterize the morphological, serological, physiological and genetic differences between, wild type strains and their cellulose deficient mutants.
3. To investigate the role of cellulose and cellulose in conjunction with the EPS-CA in protecting the cells of STEC against adverse environmental conditions such as osmotic, oxidative and acid stress as well as chlorine treatment.

CHAPTER 2

LITERATURE REVIEW

1. Shiga toxin producing *Escherichia coli* (STEC)

Shiga toxin producing *Escherichia coli* (STEC) is one group of *E. coli* strains that produce Shiga toxins, which are similar to the cytotoxins produced by Type I *Shigella dysenteriae* (O'Brien et al. 1982). The cytotoxins were originally named as verotoxins because their toxicities to vero cells or African green monkey kidney cells (Karmali et al. 1983). Enterohemorrahgic *E. coli* (EHEC) is a subgroup of STEC, and it shares epidemiological, clinical and pathogenic similarities with STEC (Beutin 2006). By definition, the group of EHEC includes the STEC strains that cause diarrhea in humans, carry *eae* gene for bacterial adherence and a 60 MDa plasimd encoded EHEC-*hly*A gene for disruption of cell membrane permeability (Nataro and Kaper 1998). EHEC serotype O157:H7 was first identified as a cause of foodborne outbreaks associated with undercooked ground beef in 1982 (Riley et al. 1983). The infectious dose of *E. coli* O157:H7 is reportedly to be under 100 cells (Tilden et al. 1996). STEC causes severe gastroenteritis in humans, and the illness may progress to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). The latter can result in acute renal failure in children and sometimes in adults (Karch et al. 2005; Razzaq 2006). STEC with O157 serotype is not able to utilize sorbitol, and this characteristic has been used as means of isolating the organism (March and Ratnam 1986). In addition, there have been a meaningful number of reported outbreaks due to non-O157 STEC such as those with serotype O26, O111 and O128 in recent years (Bettelheim

2007), and these outbreaks were responsible for 20-50% of STEC related infections annually in the United States (Mead et al. 1999).

Ruminants are a natural reservoir of STEC, and food or water contaminated with cattle manure have been traced as a source of human STEC infections (Gyles 2007). Foods are the most prevalent vehicle of STEC outbreaks, and more than a half of the infections occurred between 1982 and 2002 in the United States related to the consumption of contaminated food (Rangel et al. 2005). The food vehicles involved in STEC related outbreaks of infections range from meats including undercooked beef and ready-to-eat sausages, dairy products such as raw milk and cheese, to fresh produce including lettuce, spinach, cantaloupes, alfalfa sprouts and radish sprouts. Acidic foods such as unpasteurized apple cider and juice have also been involved in *E. coli* O157:H7 associated outbreaks (Gyles 2007), which demonstrates the ability of the pathogen to survive in acidic environment (Conner and Kotrola 1995; Erickson and Doyle 2007). Once STEC is consumed by susceptible humans, it survives the acidic stomach, passes through the small intestine, colonizes in the large intestine where adherence and effacement occur, damages blood vessels, and affects kidneys and central nervous system through the circulation of Shiga toxins (Gyles 2007).

Shiga toxins are the major virulence factor of STEC. There are two types of Shiga toxins, Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). The toxins are 70 kDa proteins with an A1B5 structure in which a monomeric A subunit is noncovalently associated with a pentamer of B subunits (Strockbine et al. 1986; O'Brien and Holmes 1987). Most STEC strains produce Stx2, but some strains also produce Stx1. Between the A subunits of Stx1 and Stx2, there is 56% homology in their amino acid sequences (Paton and Paton 1998). The cluster of B subunits binds to glycolipid receptors, globotriaosylceramide on the surface of host cells, enabling the

toxin molecule to internalize (Gyles 2007). After binding to its receptor, Shiga toxins are transported through clathrin-coated pits inside the membrane by receptor-mediated endocytosis (Lauvrak et al. 2004). In most cases, the toxin molecules are destroyed by lysosomal vesicles in the cytosol, however in susceptible cells, the toxins are transported to the Golgi apparatus and the endoplasmic reticulum (Gyles 2007). The A subunit is responsible for the enzymatic activity to cleave a specific adenine base from the 28 S rRNA, thereby inhibit protein synthesis, leading to apoptosis in intestinal epithelial cells (Smith et al. 2003). In addition to toxin damages, colonization of STEC cells in the intestine is involved with the disease development prior to toxin activities. The colonization of STEC cells begins with adherence to intestinal epithelial cells. The attaching and effacing gene (*eae*) has a critical role in this process, enabling STEC to form a characteristic attaching and effacing lesion on intestinal epithelial cells (Kaper et al. 2004). The locus of enterocyte effacement (LEE), a pathogenicity island possessed by the *eae*-positive STEC, encodes genes for the formation of attaching and effacement lesion (Spears et al. 2006). The pathogenicity island is a distinct cluster of genes that do not have identical percentages of guanine (G) and cytosine (C) (%GC) content as the remainder of the *E. coli* genome (Hacker and Kaper 2000). The pathogenicity island is incorporated into the genome of pathogenic microorganisms by horizontal gene transfer, and is usually absent from non-pathogenic strains of the same or closely related species. LEE consists of 5 major operons, including a type III secretion system, a protein translocation system, an adherence system including an outer membrane protein, intimin and its receptor, translocated intimin receptor and effector proteins that are also translocated by the secretion system. Type III secretion system is a molecular syringe structure that injects secreted proteins from inside the bacterial cytoplasm directly into the cytoplasm of the host cell through the apparatus (Barba et al. 2005; Naylor et al.

2005). The attaching and effacement lesion helps STEC form a pedestal structure and mediate intimate adherence to the epithelial cells (Gyles 2007).

2. Biofilms and their importance to the food safety

Bacterial cells such as those of STEC can either live as individual free floating cells (planktonic) in suspensions or attach to solid surfaces (sessile) in biofilms (Chmielewski and Frank 2003). The biofilms are a community of bacterial cells immersed in an organic matrix that are attached to solid surfaces (Branda et al. 2005). Environmental conditions leading to the transition from planktonic to sessile growth vary depending on bacterial strains (Dewanti and Wong 1995; Mittelman 1998; O'Toole and Kolter 1998; Pratt and Kolter 1998). Biofilm formation has discrete steps including initial attachment to surfaces, microcolony formation and maturation into biofilm (Davey and O'Toole 2000). The initial attachment is influenced by the physicochemical properties of microbial cellular surfaces, and the process proceeds in two steps, reversible attachment followed by irreversible attachment (van Loosdrecht et al. 1990; Kumar and Anand 1998). The reversible interaction between bacteria and a substratum is weak, and involves the van der Waals attraction forces, electrostatic forces and hydrophobic interactions (Kumar and Anand 1998; Chmielewski and Frank 2003). The attached cells at this stage can be easily removed by rinsing (Marshall et al. 1971). Transition from reversible to irreversible attachment needs the participation of extracellular polymers produced by bacterial cells (Stoodley et al. 2002).

Biofilms formed by bacterial cells on food and food contact surfaces may serve as a potential source of cross-contamination for food products. The contaminated foods subsequently provide health risks to consumers and serve as vehicles for foodborne diseases (Carpentier and

Cerf 1993; Kumar and Anand 1998). Bacterial cells embedded in biofilms are more resistant to antibacterial agents because the cells are protected by the biofilm surface structure limiting the penetration of antibacterial agents (Evans and Holmes 1987; Kumar and Anand 1998; Frank 2001).

3. Extracellular polysaccharides (EPS) and their relationship to biofilm formation

Biofilm development into structured forms by most bacterial pathogens depends on the EPS produced by microbial cells (Branda et al. 2005). It is believed that most bacteria can express EPS under appropriate environmental conditions, but the production is usually lost on microbiological media in a laboratory setting (Junkins and Doyle 1992). The composition of bacterial EPS is highly diverse in terms of chemical and physical properties, ranging from neutral to highly charged polyanionic macromolecules comprised of sugars such as pentoses, hexoses, amino sugars and uronic acids or non-sugar components such as acetic acid, succinic acid, pyruvic acid, phosphoric acid and sulfuric acid (Kenne and Lindberg 1983; Sutherland 2001). Most biofilm-associated cells appear to grow relatively slowly to enhance EPS production (Evans et al. 1990). The EPS with β-1, 3- or -1, 4-linked backbone structures provide biofilms with the mechanical stability against shear forces (Mayer et al. 1999). The production of the EPS comprised of colanic acid (EPS-CA) enables the cells of *E. coli* to build mature biofilms with three-dimensional architecture (Danese et al. 2000). The EPS-CA is however, not involved in the initial attachment process. Some plant pathogenic bacteria such as *Gluconoacetobacter xylinus* have been previously known to produce extracellular cellulose (Ross et al. 1991). Most recently *S.* Typhimurium and *E. coli* were found to produce cellulose as a major component of their EPS (Austin et al. 1998; Danese et al. 2000; Zogaj et al. 2001;

Solano et al. 2002). The EPS mediates cell-cell interactions which affect the maturation of single species or mixed species biofilms (Whittaker et al. 1996; Davey and O'Toole 2000). Quorum sensing molecules such as acyl homoserine lactones in *E. coli* and *Pseudomonas* are involved in the regulation of complex mushroom structures of biofilms formed by *P. aeruginosa* and in the positive regulation of EPS production in *P. syringae* (Quinones et al. 2005). Biofilm structure development has been shown to be related to the EPS production in *P. aeruginosa* (Davies et al. 1998; De Kievit et al. 2001; Hall-Stoodley and Stoodley 2002; Shin and Huang 2002).

3.1. Cellulose

Cellulose is a long-chain polysaccharide of glucose with β-1, 4 glycosidic bonds. It is the primary structural component of plant cell walls which accounts for more than half of the organic material on the earth. Bacterial cellulose has greater mechanical strength than plant cellulose since it exhibits higher degree of polymerization and crystallinity. The fibrils of bacterial cellulose are embedded with lignin, hemicellulose and waxy substances (Bertocchi et al. 1997).

3.2. Fundamental chemistry of cellulose

Cellulose molecules contain 300 to over 15,000 glucose residues (Horton et al. 2002). Cellulose has many hydroxyl groups, and hydrogen bonding has a critical influence on its structure. The polymers of β-D-glucose have a series of rigid pyranose rings in a chair conformation, and are joined by an oxygen atom connecting two adjacent carbon atoms, which allow free rotation. The β-glycosidic bonds in β-D-glucose polymers cause a stiff elongated

conformation, and each glucose residue is rotated 180^{o} relative to its neighbor, providing a straight, extended chain, which is less flexible than molecules comprised of α-linked glucopyranose such as amylose. The β linkage's straight chain enables cellulose molecules to exist side by side via hydrogen bonds yielding a fiber of high tensile strength (Stryer 1988; Lehninger et al. 1993).

The molecular weights of cellulose including that of algal, bacterial and higher plants range from 300,000 to over 1,000,000, and its specific gravity ranges from 1.27 to 1.61. Cellulose decomposes at 260-270°C, and is generally insoluble in water, but dissolved in concentrated (>64% (w/w)) zinc chloride solution and in caustic alkalis containing carbon disulphide (Kawagoe and Delmer 1997; Kroon-Batenburg and Kroon 1997; Davidson et al. 2004b). Cellulose forms a partial crystalline structure, known as cellulose microfibril. For any given cellulose material, the portion of it that does not dissolve in a 17.5% solution of sodium hydroxide at 20°C is α-cellulose, the portion that dissolves and then precipitates upon acidification is β-cellulose, and the proportion that dissolves but does not precipitate is γ-cellulose (Zugenmaier and Sarko 1976; Matthysse et al. 1981; MacCormick et al. 1993). Natural cellulose has cellulose I structure with all β–glucose chains unidirectionally aligned in parallel arrangement, and after the mercerization (swelling) process in alkali conditions, it may be converted to the more stable cellulose II structure bearing antiparallel polyglucan chains. The crystalline structure of cellulose I consists of two allomorphs, cellulose I_{α} (triclinic crystal) derived mainly from bacteria and algae, and I_{β} (monoclinic crystal) derived from plants. These may be distinguished by the technique of solid state nuclear magnetic resonance (NMR) (Gardner and Blackwell 1974; Zugenmaier and Sarko 1976).

3.3. Microbial cellulose

A variety of bacterial cells are capable of producing cellulose as an extracellular matrix (de Rezende et al. 2005; Seto et al. 2006; Da Re et al. 2007; Grimm et al. 2008). Among cellulose producing bacteria, *Acetobacter, Agrobacterium, Rhizobia* and *Sarcina* are the best known species (Delmer 1999). *Acetobacter xylinus* produces a limited amount of cellulose as an extracellular pellicle (Glaser 1958). For the members of *Enterobacteriaceae*, cellulose biosynthesis is sometimes accompanied by the expression of curli, a proteinaceous projection on the surface of the bacteria cells (Bokranz et al. 2005; Anriany et al. 2006). Cellulose non-covalently binds with curli, forming a highly inert, hydrophobic extracellular matrix, which provide structural stability to bacterial cells (Zogaj et al. 2001). The expression of cellulose is complicated and seems to be influenced by temperature, pH, atmospheric conditions and nutrient composition in media (Zogaj et al. 2003). Cellulose produced by bacterial cells is usually quantified using a colorimetric assay (Updegraff 1969). After non-cellulosic materials are extracted with the acetic acid/nitric acid reagent, cellulose is quantified with anthrone in a sulfuric acid solution at elevated temperature. The heated strong acid hydrolyzes the glycosidic bonds of cellulose to yield glucose units which bind to anthrone molecules.

The macroscopic morphology of bacterial cellulose has been determined by ^{13}C NMR (Kono et al. 2002; Nakai et al. 2002), X-ray diffraction (Watanabe et al. 1998; Bootten et al. 2008), electron microscopy (Watanabe et al. 1998; Nakai et al. 2002) and Fourier transform infrared spectroscopy (FT-IR). Carbon-13 NMR measures the carbon atoms in the cellulose structure. X-ray diffraction has been used to confirm the mass fractions of cellulose I_α and I_β and crystallinity, because X-ray crystallography gives the most descriptive results in spite of its complicated data interpretation (Kroon-Batenburg and Kroon 1997; Davidson et al. 2004a).

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) techniques have frequently been used to study the surface structure of cellulose (Fromm et al. 2003; Zhou et al. 2005). The FT-IR is the technology that can be used to estimate the fraction of I_α and I_β in a cellulose molecule (Akerholm et al. 2004; Oh et al. 2005; Verhoef et al. 2005).

The genes for cellulose biosynthesis (*bcs*) were first found in *Acetobacter xylinus* (Aloni et al. 1982; Ross 1987; Ross et al. 1991). The *bcs* operon encodes 4 primary proteins: the catalytic subunit (BcsA, cellulose synthase), the regulatory subunit (BcsB, cyclic-di-GMP binding protein), the cellulase encoding subunit (BcsZ, endoglucanase) and the oxidoreductase subunit (BcsC) (Ross et al. 1991; Solano et al. 2002). The *csgD* (curli subunit gene D) in *E. coli* or *agfD* (thin aggregative fimbriae gene D) in *Salmonella* positively regulates the expression of cellulose. The expression of CsgD initiates the biosynthesis of cellulose in the stationary phase of growth (Romling et al. 2000; Bokranz et al. 2005). Upon transcriptional activation by CsgD, a GGDEF domain protein, AdrA stimulates the production of cyclic-di-GMP (cGMP) (Mayer et al. 1991; Simm et al. 2004). The complex of cGMP and its binding protein, BcsB regulates the activity of cellulose synthase, BcsA to polymerize cellulose using UDP-glucose as precursors (Zogaj et al. 2001).

3.4. Significance of bacterial cellulose production

Cellulose produced by *S*. Enteritidis does not play a significant role in the virulence of the pathogen (Solono et al. 2002). The EPS however, protects the cells of *Streptococcus thermophilus* against mechanical and chemical damages (Broadbent et al. 2003). In *E. coli*, biofilm formation is related to the expression of cellulose (Brombacher et al. 2006; Da Re and Ghigo 2006; Uhlich et al. 2006; Wang et al. 2006; Weber et al. 2006). Cellulose synthesis in *G.*

xylinus is associated with specific adhesion in the periplasmic space between the inner and outer membranes (Ross et al. 1991). Plant pathogenic bacteria such as *A. tumefaciens* (Matthysse and McMahan 1998) and *R. leguminosarum* bv. *trifolii* (Ausmees et al. 1999) utilize cellulose to adhere to plant tissue. Cellulose enhances the attachment and colonization of *G. xylinus* (Williams and Cannon 1989) and *S. enterica* (Barak et al. 2007) to plants and of *S*. Typhimurium (Ledeboer and Jones 2005) to animal tissues. The EPS is believed to have a long term role in the adhesion of cells to the surface of leafy vegetables such as parsley, although cellulose does not confer any advantage during the initial step of the adhesion to produce surfaces (Lapidot et al. 2006).

3.5. Significance of the EPS-CA production and its impact on food safety

The EPS-CA is a polyanionic heteropolysaccharide containing a repeat unit with D-glucose, L-fucose, D-galactose and D-glucuronic acid that contains O-acetyl and pyruvate side chains (Grant et al. 1969). The genetic determinant for EPS-CA biosynthesis resides on the *wca* (*cps*) gene cluster which is responsible for the polymerization, modification and transport of the EPS-CA (Stevenson et al. 1996; Stout 1996). The gene cluster is tightly regulated by a complex signal transduction cascade governed by the *rcs* (regulator of capsule synthesis) phosphorelay system (Majdalani and Gottesman 2005). While normally not expressed in planktonic cultures, a number of culture conditions induce the production of the EPS-CA on solid surfaces, and these conditions include incubation temperatures below 25°C on minimal growth media (Hagiwara et al. 2003).

The EPS-CA is not directly involved in the pathogenesis of *E. coli* (Russo et al. 1995), but is related to survival strategy outside the host (Whitfield and Roberts 1999). Although not

required for the initial attachment of bacterial cells to surface, the EPS-CA plays an important role in the development of the three-dimensional structure of biofilms (Danese et al. 2000). The EPS-CA is widely found in *E. coli* as well as in other species of the family of *Enterobacteriaceae* (Roberts 1996). It provides protection against desiccation (Ophir and Gutnick 1994), acid (Lee and Chen 2004), high concentrations of detergents (Rajagopal et al. 2002), and β-lactams (Sailer et al. 2003) as well as osmotic and oxidative stress (Chen et al. 2004).

4. Techniques useful for characterization of cellulose and cellulose-producing bacteria

Transmission electron microscopy (TEM), a reliable tool for detailed morphology analysis of materials with a diameter less than μm (Tanem et al. 2005) and scanning electron microscopy (SEM), a valuable technology to investigate the surface morphology of biological materials (Mathew and Dufresne 2002) are used in this project to study the morphology of cellulose producing STEC. Phenotype microarray (PM) is used to study the growth characteristics of cellulose producing STEC and their cellulose deficient mutants.

4.1. Visualization of bacterial cellulose structure using electron microscopy

Electronic microscope has been used to study the structural attributes of cellulose produced by various bacterial species (Hirai et al. 2002; White et al. 2003; Romling and Lunsdorf 2004) including *Salmonella* (Jain and Chen 2006), *Acetobacter xylinum* (Hirai et al. 2002) and *S. enterica* serovar Typhimurium (Romling and Lunsdorf 2004). Bacterial cellulose is light-scattering and difficult to stain and identify by optical microscopes, although direct microscopic observation is a simpler approach to visualize the structures surrounding bacterial cells

(Beveridge et al. 2007). The basic steps of TEM include thin sectioning, chemical fixation using glutaraldehyde and osmium tetroxide, dehydration by acetone and staining using uranyl acetate or lead citrate. Although some of these steps could create potential artifacts, TEM remains to be a good tool to study prokaryotic structures in great details (Geesey et al. 1977; Costerton et al. 1978).

4.2. Phenotype Microarray (PM)

The PM technology is able to determine mutational effects on a genome-wide scale (Zhou et al. 2003), analyze the effects of loss of gene function (Bochner et al. 2001), correlate phenotypes with genotypes to determine genetic relationships among variants (Morales et al. 2005), and compare antibiotic resistance patterns within subpopulations (Guard-Bouldin et al. 2007). The technology has been used to find new functions of genes by comparing mutants with wild type cells (Tracy et al. 2002; Bochner 2003; Pruss et al. 2003; Clemmer et al. 2006), to screen superior chitinase producing *Trichoderma harzianum* for industrial scale enzyme production (Nagy et al. 2007), to characterize carbon/nitrogen balance regulatory elements in *P. aeruginosa* (Li and Lu 2007), to elucidate the physiological role of the nitrogen-metabolic phosphotransferase system in *E. coli* K-12 (Koo et al. 2004; Lee et al. 2005), to observe the physiological changes that lead to the development of *in vivo* antibiotic resistance in small colony variants of *Staphylococcus aureus* (von Eiff et al. 2006), to identify the genes responsible for the utilization of pyrimidine as nitrogen sources in *E. coli* K-12 (Loh et al. 2006), and to identify the carbon sources that trigger b-N-acetylglucosaminidase (NAGase) formation in *Hypocrea atroviridis* (Seidl et al. 2006).

PM tests are performed in 96-well microplates containing different nutritional substances, inhibitory agents or antibiotics. The PM microplates with inoculated bacterial cells are incubated over a period of time during which the rate of cell respiration is measured by a fluorescence indicator, tetrazolium. When the reduced nicotineamide adenine dinucleotide (NADH) generated in citric acid cycle is oxidized as an electron donor, the oxygen molecule, an electron acceptor is reduced to water. Meanwhile, the electron transport couples the biochemical reaction to proton gradients to generate ATP energy. The oxidized form of tetrazolium violet is reduced when NADH is oxidized, and its color changes to purple. This change of color is irreversible, and accumulates over a period of incubation time (Bochner et al. 2001).

5. Stress response of STEC

Food industries are in need of a variety of antimicrobial interventions to inactivate foodborne pathogens. These interventions impose biological, chemical or physical stresses to bacterial cells (Rowbury 1993). Antibiotics and competitive microflora are considered as biological stresses. Oxidizing agents, organic or inorganic acids and sanitizing agents are chemical stresses. Increased osmotic pressures and reduced or elevated temperatures provide physical stresses to bacterial cells (Rowbury 2001). Most *E. coli* including STEC have developed a number of mechanisms to combat these stresses (Law 2000).

5.1. Oxidative stress

Oxidative stress can be described as an excess amount of prooxidants in bacterial cells (Farr and Kogoma 1991). Oxygen toxicity occurs when the oxidative stress is beyond the capacity the cell defense system can withstand (Farr and Kogoma 1991). Molecular oxygen cannot easily

oxidize other molecules by accepting a pair of electrons since the pair of electrons in an orbital has antiparallel spins. Its reactivity is elevated upon the acceptance of one, two or three electrons to form a superoxide radical anion, hydrogen peroxide and hydroxyl radical, or when it experiences a spin flip to become singlet oxygen (Hill and Allen 1978). Active oxygen molecules are able to cause damage to DNA, RNA, protein and lipids. Hydroxy radicals attack the sugar moiety of DNA leading to strand breaks (Von Sontag 1979). Intermediate organic radicals from lipid peroxidation react with DNA leading to alkylation of bases and interstrand or intrastrand cross-links (Segerback 1983; Summerfield and Tappel 1983). Lipid peroxy radicals abstract a hydrogen atom from an unsaturated fatty acid constituting cellular membrane resulting in shorter fatty acid chains (McElhaney 1985). Altered fatty acid chains cause bacterial membranes to become more fluid and to lose a structural integrity. Loss of structural integrity will break proton gradients affecting intracellular pH followed by further production of hydroperoxy radical (Farr et al. 1988).

Bacterial defense against the damaging effects of active oxygen can be divided into two categories, prevention and repair. The prevention of oxidative damage is achieved by destroying the reactive oxygen molecules, and the repair of oxidative damage is attainable by restoring the molecules that are deteriorated by oxidative stress (Farr and Kogoma 1991).

Bacteria use both enzymatic and nonenzymatic reactions to respond to oxidative stress. The enzymatic components include superoxide dismutases, catalases, glutathione synthetase, glutathione reductase, glutathione peroxidase and NADH-dependent peroxidases while the nonenzymatic components include glutathione, thioredoxin, ubiquinone, and menaquinone (Lunn and Pigiet 1987). Superoxide dismutases (SODs), such as Mn-containing SOD and Fe-containing SOD, catalyze superoxide radicals into hydrogen peroxide and oxygen molecules

with the input of protons. Catalases decompose hydrogen peroxide into molecules of water and oxygen (Wang 1955). Peroxidases and glutathione system require a reducing power from NADH or NADPH as an electron source to function properly in destroying hydrogen peroxide and disulfide bridges; on the other hand, catalases can destroy hydrogen peroxide in an energy-starved cell (Heimberger and Eisenstark 1988). These defensive mechanisms have been found in several bacterial species. Superoxide dismutase and peroxidase conferred the cells of STEC resistance to hydrogen peroxide (Kim et al. 2006). Peroxidase was found to protect *Campylobacter jejuni* under highly aerobic (21% oxygen) and microaerophilic (10% oxygen) conditions (Atack et al. 2008). Catalases played a role in protecting the planktonic and biofilm cells of *P. aeruginosa* against 1 h treatment with 50 mM H_2O_2 (Elkins et al. 1999). The same enzyme from *Bacillus subtilis* was able to confer an 800-fold increase in the survival of *Lactococcus lactis* in the presence of 4 mM H_2O_2 during an hour period of time (Rochat et al. 2005).

In addition to the enzymatic or nonenzymatic responses to oxidative stresses, some other factors are known to protect bacterial cells against oxidative stress. EPS produced by *V. cholerae* O1 strain TSI-4 (Wai et al. 1998) and *E. coli* O157:H7 (Chen et al. 2004) protected the cells against oxidative stress. Global regulators such as SpoT in *Vibrio* spp. and ArcA in *S.* Enteritidis are also known to be involved in cells' response to oxidative stress (Lu et al. 2002; McDougald et al. 2002).

5.2. Osmotic stress

Increasing the osmotic pressure has often been used to limit microbial growth by lowering water activity (a_w), the amount of available water to a microorganism (Csonka 1989). The

minimal water activity required for microbial growth relies on the kinds of solutes present such as sugars, salts and glycerol (Sperber 1983). Many bacteria have their optimum growth rates at a_w 0.990 and 0.995 (Beales 2004). Exposure of cells to a_w values outside of this range may inhibit essential cellular functions such as active transport of carbohydrates (Roth et al. 1985) and DNA replication (Meury 1988).

Microbial cells preserve a higher internal osmotic pressure than that of surrounding environment at a normal range of water activities, which is known as turgor pressure (Gutierrez et al. 1995). When the turgor pressure is challenged by the increased osmotic pressure outside the cells, bacterial cells turn on the osmoregulation system to maintain the internal osmotic pressure by the accumulation of compatible solutes or changes in the membrane lipid composition (Gutierrez et al. 1995; Russell et al. 1995). Compatible solutes are highly water-soluble metabolites, which are accumulated by biosynthesis or by uptake from the environment, including sugars, amino acids and cations from the degradation of carbohydrates or proteins (Csonka and Hanson 1991). Lipid composition in the bacterial membrane is altered when external a_w is lowered by increase in the amount of solutes. The proportion of anionic phospholipids such as diphosphatidylglycerol and phosphatidylglycerol is increased compared to neutral, zwitterionic lipids such as phosphatidylethanolamine (McGarrity and Armstrong 1975). The phospholipid bilayer is able to keep the osmotic pressure from disrupting membrane function in the non-bilayer phase (Russell et al. 1995). Osmotic stress also affects genetic responses in Gram negative bacteria (Csonka 1989; Gutierrez et al. 1995). In *E. coli*, the accumulation of potassium and glutamate in the cell membrane is controlled to maintain turgor pressure. The decrease in turgor pressure activates the potassium transport system, thereby induces the expression of *kdp* gene encoding potassium uptake system Kdp (Booth et al. 1994).

Uptake of potassium glutamate is accompanied by the accumulation of trehalose, which prevents the accumulation of potassium glutamate, and inhibits enzyme function (Dinnbier et al. 1988). The glycine betaine transport is also known to be affected by changes in osmotic pressure in *E. coli* (Gutierrez et al. 1995). The capability of STEC, *Salmonella* spp. and *Shigella* spp. to survive in simulated dry foods had been described (Hiramatsu et al. 2005). Resistance to osmotic stress was found to be associated with EPS production in *E. coli* O157:H7 (Chen et al. 2004), *E. sakazakii* (Lehner et al. 2005), *V. cholerae* O1 (Wai et al. 1998), *Lactobacillus helveticus* ATCC 15807 (Torino et al. 2005) and *Azospirillum brasilense* (Fischer et al. 2003).

5.3. Acidic stress

Acidic stress is generally dependent on changes in the internal pH of the cytoplasm, rather than changes in the external pH. The mechanisms of bacterial inhibition by organic and inorganic acids are different. Organic weak acids can diffuse across cell membrane and dissociate in the cell to generate protons, lowering the intracellular pH (Bearson et al. 1997). Proton transport system is activated to pump protons out of the cell to maintain pH in the cell at the optimum range. This process is energy dependent, and repetitive usage of this pump depletes cellular energy, leading to cell death (Jay 2000). Inorganic strong acids denature enzymes on the cellular surface and lower the cytoplasmic pH by increasing proton permeability under extremely large pH gradients. As results, bacterial growth is retarded, and lag growth phase is extended because essential nutrients are not uptaken appropriately by reduced activity of transport system (Cheroutre-Vialette et al. 1998).

The adaptive responses of *E. coli* to low pH conditions have been investigated extensively. Gorden *et al*. (1993) found that nonpathogenic *E. coli* strains were less acid tolerant than

pathogenic strains. The mechanisms of acid tolerance varied depending on the growth phases and pH values. Most *E. coli* cells in the stationary phase were more resistant to acid stress than cells in the log phase (Buchanan and Doyle 1997). The acid tolerance in the stationary phase occurs regardless of the adaptation at milder pH prior to the exposure to extreme pH levels (Arnold and Kaspar 1995).

Three acid resistance systems have been found in the stationary phase of *E. coli* cells including an oxidative system, a glutamate decarboxylase system, and an arginine decarboxylase system (Lin et al. 1995; Chung et al. 2006). These systems depends on the alternative sigma factor σ^s which regulates the proteins associated with the glutamate decarboxylase acid resistance system, and assists the arginine decarboxylase acid resistance system to operate in maximum efficiency (Lin et al. 1996; Bhagwat 2003). The oxidative acid resistance system is induced in bacteria grown aerobically in rich media such as Luria Bertani broth (Lin et al. 1996), and it protects cells from acidic pH above 3.0 (Bhagwat et al. 2006). The other two other acid resistance systems are induced in minimal media supplemented with glutamate or arginine, and they confer protection to cells at pH below 3.0 (Lin et al. 1996; Bhagwat et al. 2006). These amino acid decarboxylation acid resistance systems maintain intracellular pH by consuming protons during decarboxylation and replacing decarboxylated end product for new substrates containing carboxyl end (Hall et al. 1995). The arginine decarboxylase and glutamate decarboxylase dependent acid resistance systems have been investigated in *E. coli* O157:H7 (Large et al. 2005) and the sigma factor and glutamate decarboxylase dependent acid resistance system in diarrheagenic *E. coli* (Bhagwat 2003).

Cellular response to one type of stress can provide enhanced resistance to other stresses (Volker et al. 1992; Gunasekera et al. 2008). As with osmotic stress, alteration in membrane

lipid composition may protect the bacterial cells against low pH. Exposure of cells to sublethal levels of acids can habituate the cells under lethal levels of acid (Goodson and Rowbury 1989).

5.4. Chlorine stress

Chlorine is a common and cost effective method for sanitizing food equipment and inactivating foodborne pathogenic microorganisms. When sodium hypochlorite is dissolved in water, an equilibrium reaction between hypochlorite ion and hypochlorous acid occurs. Hypochlorous acid is the form of free available chlorine that has bactericidal effect against microorganisms. The free available chlorine oxidizes the sulfhydryl groups of the enzymes in the cell membrane, followed by devastation of cellular activities (Bloomfield 1996). The effectiveness of chlorine treatment is determined by temperature, pH as well as the presence of organic materials and metals. Chlorine has the maximum solubility in water at near 4^{o}C. Organic materials competitively react with chlorine; which reduces concentration of available chlorine. Components detaching from tissues of cut meat or produce surfaces may neutralize some of active chlorines before they contact with microbial populations, and thus reduce the effectiveness (Adams et al. 1989; Kotula et al. 1997). The recommended pH range of a chlorine solution is from 6.5 to 7.5. Toxic chlorine gas would form at lower pH levels.

Chlorine-based sanitizers at 50 to 200 ppm are commonly used to rinse beef (Kenney et al. 1995), poultry carcasses (Sofos and Smith 1998), fresh fruit (Sanz et al. 2002) and fresh produce (Niemira 2008). Treatment with 50 ppm chlorine was found least effective in reducing aerobic plate counts, total coliform counts and *E. coli* counts on variety of beef products compared with treatment with 2% acetic, 2% lactic acid or 12% trisodium phosphate (Delmore et al. 2000). Some studies showed that treatments with 200 ppm and 500 ppm chlorinated water was

ineffective in significantly reducing bacterial populations on beef carcasses (Cutter and Siragusa 1995; Kenney et al. 1995). Marshall et al. (2005) however, found that treatment with 20 ppm chlorine at 20°C killed 1.48 log of *E. coli* O157:H7 on beef cattle. Bosilevac et al. (2005) found that a 200 ppm chlorine wash caused a 2.5 log reduction in coliform counts on cattle hides. In terms of produce, treatments with 20 ppm chlorine at 20 or 50°C were ineffective in reducing the populations of *E. coli* O157:H7 on lettuce (Li et al. 2001), and more than 50 ppm of chlorine did not cause significant reductions in total microbial counts in artichoke (Sanz et al. 2002). Internalization of *E. coli* O157:H7 into lettuce leaves diminished the efficacy of the treatment with 200 ppm (Nthenge et al. 2007) or 600 ppm chlorine (Niemira 2008).

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CHAPTER 3

INFLUENCE OF CULTURE CONDITIONS AND MEDIA COMPOSITION ON THE PRODUCTION OF CELLULOSE BY SHIGA TOXIN PRODUCING *ESCHERICHIA COLI* [1]

[1] B.Yoo and J. Chen. Submitted to *Applied and Environmental Microbiology*

ABSTRACT

Bacterial cellulose, a long-chain polysaccharide of glucose with β-1, 4 glycosidic bonds is known to play an important role during the formation of biofilm. In this study, over one hundred Shiga toxin producing *Escherichia coli* (STEC) strains in our culture collection were screened in order to select those that had different abilities to produce the polysaccharide. The influence of culture conditions and medium compositions on the production of cellulose by selected STEC strains was determined using a colorimetric assay. The experimental conditions evaluated in the study included incubation temperatures (15, 22, 28 and 37^{o}C), incubation atmospheres (aerobic, microaerophilic and anaerobic), medium compositions (ethanol and fructose), medium pH (5.0, 6.0 and 7.0) and medium water activities (0.96, 0.97, 0.98 and 0.99). Each tested sample had a duplicate, and each experiment conducted in the study was repeated at least three times. The results obtained were plotted using the Table Curve 3D software and analyzed using the Student *t* test of the Statistical Analysis Software at a 95% confidence interval. The tested conditions that favored the production of cellulose by the six STEC strains included a 28^{o}C incubation temperature, an aerobic atmosphere, and presence of 2% of ethanol in Luria Bertani no salt agar with pH 6.0 and a water activity of 0.99. The findings of the study will assist in formulating microbiological media useful for cellulose and biofilm research.

Shiga toxin producing *Escherichia coli* (STEC) is one of the most important groups of bacterial pathogens in the United States as well as many other countries in the world. Although the occurrence of STEC related illness has been slightly decreased in the recent past (6), the impact of the pathogen on public health cannot be overlooked. STEC causes severe gastroenteritis that may progress to hemorrhagic colitis and hemolytic-uremic syndrome in individuals with compromised immune systems (30). Outbreaks of STEC infections are mostly associated with serotype O157 (18). However, a considerable number of outbreaks (20-50%) reported in the United States were caused by non-O157 STEC, such as O26, O111 or O128 (4).

As many other bacteria, STEC can live as individual, free floating cells in suspensions (planktonic) or attach to solid surfaces forming biofilms (sessile) (9). The biofilms are a great concern of the food industry because they offer physical, mechanical and biological protection to bacterial cells. By definition, a biofilm is a community of bacterial cells immersed in an organic matrix that attaches to solid surfaces (5). Extracellular polysaccharides (EPS) produced by bacterial cells play a structural role in the formation of biofilms (5). Bacterial EPS are highly diverse in terms of chemical and physical properties, ranging from neutral to highly charged polyanionic macromolecules comprised of sugars, such as pentoses, hexoses, amino sugars and uronic acids or non-sugar components, such as acetic acid, succinic acid, pyruvic acid, phosphoric acid and sulfuric acid (23, 39). Some strains of *Salmonella* Typhimurium and *E. coli* are known to have the ability to produce cellulose as a major component of their EPS (43). Recent studies have shown that a variety of other bacterial cells including those of *Enterobacter sakazakii* (16) and *Lactobacillus mali* (34) are also capable of producing cellulose (12). In addition to contributing to biofillm formation, cellulose produced by STEC has been linked to

cells' tolerant to chlorine, indicating a possible role that cellulose plays during cell survival under stress (37).

Bacteria previously known to produce capacious amount of cellulose include *Gluconoacetobacter*, *Rhizobium* and *Agrobacterium* (33). In comparison, the amounts of cellulose produced by STEC cells are significantly lower. In order to better study the role and function of STEC cellulose, the environmental and nutritional conditions that influence the production of cellulose by STEC have to be determined. The goal of this research project was to determine the impact of selected culture conditions and medium compositions on the production of cellulose by the cells of STEC strains.

MATERIALS AND METHODS

STEC strains and their cellulose production. Approximately 115 strains of STEC from our culture collection were screened for their abilities to produce cellulose. Cultures of the STEC strains were grown on Luria-Bertani no salt (LBNS) agar supplemented with calcofluor white stain (200 mg/l) (Sigma-Aldrich Inc., St. Louis, MO) and grown at 28^{o}C for 48 h. Following the incubation, fluorescent colonies were selected under a long wave UV light in a multi-purpose imaging system (Kodak Digital Science Image Station 440 CF, Eastman Kodak Company, Rochester, NY). The calcofluor white stain has an excitation wavelength of 365-395 nm and an emission wavelength of 420 nm.

Quantitative evaluation of cellulose produced by STEC. Cellulose produced by STEC cells was quantified using a colorimetric assay developed by Updegraff with some modifications. STEC cultures were grown on LBNS agar plates at 28°C for 96 h. The cultures were harvested with 10 ml of buffered phosphate saline (PBS, pH 7.4) by gentle agitation at 20 × g for 25 min

with an Orbit shaker (Lab-line Instruments Inc., Melrose Park, IL) followed by gently scraping the cultures off the agar plates. The obtained cell suspensions (10 ml) were centrifuged (Beckman GS 6-R centrifuge, Beckman Instruments Inc., Palo Alta, CA) at 2,500 × g for 25 min. The cell pellet of each culture was collected after the supernatant fluid was discarded. Three ml of acetic-nitric reagent (150 ml of 80% acetic acid and 15 ml of concentrated nitric acid) was added to the cell pellet of each culture in a glass centrifuge tube (17 × 118 mm; Fisher Scientific, Fair Lawn, NJ) and mixed for 5 s vortexing. The centrifuge tubes were covered with aluminum foil and heated in a boiling water bath for 30 min. The contents in the tubes were re-centrifuged under the conditions described above. The cell pellet of each culture was collected after the supernatant fluid was discarded. The pellet was washed with 10 ml of distilled water after the centrifugation at 2,500 × g for 25 min. One ml of 67% sulfuric acid was then added and mixed with the cell pellet. The mixtures were allowed to stand for 1 h at room temperature. After dilution with 4 ml of distilled water, the mixtures in the centrifuge tubes were placed in an ice bath. Ten ml of anthrone (Sigma-Aldrich Inc., St. Louis, MO) reagent (0.2 g of anthrone in 100 ml of concentrated sulfuric acid) was added to each tube with intermittent mixing. The tubes were inverted gently and then heated in a boiling water bath for 16 min. The absorbance of each sample at 620 nm was recorded using the Novaspec II spectrophotometer (Pharmacia Biotech, Cambridge, UK). A standard curve of absorbance as a function of cellulose (Sigma-Aldrich Inc., St. Louis, MO) concentration was prepared. Cellulose produced by the cells of each STEC culture was expressed as μg per 10^{10} colony forming units (CFU) of STEC. The detection limit of the cellulose quantification assay was 0.003 μg/10^{10} CFU.

Influence of a single incubation condition or medium composition on cellulose production by STEC. Six strains of STEC having different ability to produce cellulose were

selected for the experiments. The STEC cultures were grown on LBNS agar at 28°C with different pH levels (5.0, 6.0 or 7.0), water activities (0.96, 0.97, 0.98 or 0.99), as well as fructose (0, 0.4, 1 or 2%, w/v) and ethanol concentrations (0, 1 or 2%, v/v), respectively. The cultures were incubated for 96 h under aerobic condition. Alternatively, the STEC strains were grown on LBNS agar with pH 7.0 and a water activity of 0.99 at 15, 22, 28 or 37°C. The cultures were incubated under aerobic, microaerophilic or anaerobic condition for 96 h. The anaerobic and microaerophilic atmospheres were obtained by using the BBL™ GasPak (Becton Dickinson and Co., Sparks, MD) and BBL™ CampyPak (Becton Dickinson and Co.), respectively. The resulting cultures were harvested with PBS buffer, and the amounts of cellulose produced by 10^{10} CFU of STEC were quantified as described above.

Interactive influence of incubation conditions and medium compositions on cellulose production by STEC. The response surface methodology was used to for the experiments. Bacterial strains selected for the experiments included STEC 46 and 49. The interactions used in the study were between incubation temperatures and atmospheres, medium pH levels and water activities, as well as fructose and ethanol concentrations. In the first set of interactions, STEC cultures were grown on LBNS agar with pH 7.0 and a water activity of 0.99. The STEC cultures were incubated for 96 h at 15, 22, 28 or 37°C under aerobic, microaerophilic or anaerobic condition. In the second set of interactions, the pH of LBNS agar was adjusted to 5.0, 6.0 and 7.0, respectively using 1 M HCl, and the medium water activities to 0.96, 0.97, 0.98 and 0.99, respectively, according to the method by Lebert (27). The STEC cultures were grown on each medium at 28°C under aerobic condition for 96 h. In the third set of interactions, LBNS agar with pH 7.0 and a water activity of 0.99 was used as an agar base into which different amounts of ethanol (0, 1 and 2%, v/v) or fructose (0, 0.4, 1 and 2%, w/v) were added. As in the second

set of interactions, the STEC cultures were grown on each medium at 28°C for 96 h under aerobic condition. Each of the resulting cultures was harvested with PBS and the amounts of cellulose produced by 10^{10} CFU of STEC were quantified as described above.

Statistical analyses. All tested samples in the experiments were duplicated and repeated for at least three times. Data obtained were plotted using the Table Curve 3D software (SYSTAT Software Inc., 2002), and analyzed using the Student *t* test of the Statistical Analysis Software (SAS Institute Inc., 2003) at a 95% confidence interval. Significant differences in the amounts of cellulose produced by the STEC cells were calculated by comparing the average absorbance of the anthrone solutions at 620 nm.

RESULTS

Influence of incubation temperatures. The average amount of cellulose produced by the six STEC strains at 28°C was significantly higher than the average amounts of cellulose produced at the other three incubation temperatures evaluated in the study ($P < 0.05$; Table 3.1). Under the other three incubation temperatures, the differences in the amounts of cellulose produced by the six STEC strains were statistically insignificant ($P > 0.05$).

STEC strain 46 produced 7.85 μg of cellulose/10^{10} CFU at 28°C, and this value was significantly higher ($P < 0.05$) than the amounts of cellulose produced by strain 17, 19, 50 and 117, respectively, and was not significantly different ($P > 0.05$) from the amount of cellulose produced by strain 49 at the same temperature (Table 3.1). Strain 46 also produced the highest amount of cellulose at 37°C. Strain 19 produced the highest amount of cellulose at 15°C, and sStrain 49 produced the highest amount of cellulose at 22°C (Table 3.1).

Influence of incubation atmospheres. Greater ($P < 0.05$) cellulose production was observed under aerobic, followed by microaerophilic condition (Table 3.1). Cells of the six STEC strains produced an average of 0.62 μg of cellulose per 10^{10} CFU of STEC under anaerobic incubation condition, which was significantly lower ($P < 0.05$) than the average amount of cellulose produced by the six STEC under the aerobic atmosphere.

Strain 46 and 49 produced 5.32 and 5.19 μg of cellulose/10^{10} CFU, respectively, significantly higher ($P < 0.05$) than the amounts of cellulose produced by strain 17, 19, 50 and 117 under the aerobic atmosphere. The amounts of cellulose produced by the latter four strains were not statistically different ($P > 0.05$). Strain 46 was also the greatest cellulose producer under the microaerophilic atmosphere. Its production was significantly higher than the amounts of cellulose produced by the other 5 strains tested in the study ($P < 0.05$). The average amount of cellulose produced by strain 49, under the anaerobic atmosphere, was significantly higher than the average amount of cellulose produced by strain 17 ($P < 0.05$), and was not significantly different ($P > 0.05$) from the average amounts of cellulose produced by the other strains tested in the study.

Influence of medium water activities. The average amount of cellulose produced by STEC strains on LBNS agar with a water activity of 0.99 was 2.65 μg/10^{10} CFU, significantly higher ($P < 0.05$) than the average amounts of cellulose produced by the STEC strains on LBNS agar with a water activity of 0.96, 0.97 and 0.98, respectively (Table 3.1). The average amounts of cellulose produced by strain 46 and strain 49 were statistically higher ($P < 0.05$) than those produced by the other strains tested in the study (Table 3.1).

Influence of medium pH. The average amount of cellulose produced by the six STEC cultures was 4.29, 3.59 and 2.95 μg/10^{10} CFU on LBNS agar with pH 6.0, 7.0 and 5.0,

respectively (Table 3.1). The average amount of cellulose produced by the six STEC cultures on LBNS agar with pH 6.0 was significantly higher ($P < 0.05$) than the amount of cellulose produced on the same medium with pH 5.0, but was not significantly different ($P > 0.05$) from that produced at pH 7.0. The differences in the average amounts of cellulose produced by the STEC strains on LBNS agar with pH 5.0 *vs.* 7.0 were statistically insignificant ($P > 0.05$).

Strain 46 produced 9.98 μg of cellulose/10^{10} CFU on LBNS agar with pH 7.0, significantly higher than the amounts of cellulose produced by strain 49, 50, 19, 17 and 117 on LBNS agar with the same pH ($P < 0.05$) (Table 3.1). The differences in the amounts of cellulose produced by strain 17, 19, 50 and 117 at pH 7.0 were however, not statistically significant ($P > 0.05$). Strain 46 produced significantly higher ($P < 0.05$) amount of cellulose than strain 17, 19, 50 and 117 at pH 6.0, but the difference in the amounts of cellulose produced by strain 46 and 49 was not significant ($P < 0.05$). Strain 46 produced significantly higher ($P < 0.05$) amount of cellulose on LBNS agar with pH 7.0 than with pH 5.0, strain 19 produced the highest ($P < 0.05$) amount cellulose on LBNS agar with pH 6.0 among three pHs, and strain 17 produced the greatest ($P < 0.05$) amount of cellulose on LBNS agar with pH 5.0, followed by pH 6.0 and 7.0. On LBNS agar with pH 5.0, strain 49 produced an average of 7.00 μg of cellulose/10^{10} CFU, significantly higher ($P < 0.05$) than the average amount of cellulose produced by the other cultures except for strain 46.

Addition of fructose. The addition of fructose to LBNS agar did not seem to enhance the cellulose production by the tested STEC strains (Fig 3.1.A). The amounts of cellulose produced by the STEC strains decreased as the concentrations of fructose in LBNS agar increased. The average amount of cellulose produced by the tested STEC strains on LBNS agar supplemented with 0.4% fructose was not significantly different ($P > 0.05$) from the amount of

cellulose produced on the media without fructose addition, but was significantly higher ($P < 0.05$) than the amount of cellulose produced on the media supplemented with more than 0.4% fructose.

Strain 49 produced an average of 3.71 μg of cellulose/10^{10} CFU on LBNS agar supplemented with 0.4% fructose. This value was not significantly different ($P > 0.05$) from the average amount of cellulose produced by strain 46, but was significantly higher ($P < 0.05$) than the average amounts of cellulose produced by strain 50, 19, 117 and 17. On LBNS agar supplemented with 1% and 2% fructose, only strain 46 produced 0.01 μg of cellulose per 10^{10} CFU, and amount of cellulose produced by the other strains tested in the study was undetectable (< 0.003 μg of cellulose/10^{10} CFU).

Addition of ethanol. The addition of ethanol to LBNS agar significantly increased the production of cellulose by the six tested STEC strains ($P < 0.05$; Fig. 3.1.B). The average amounts of cellulose produced by the six STEC strains were 1.94, 2.83 or 4.03 μg/10^{10} cells on LBNS agar supplemented with 0, 1 and 2% ethanol, respectively. The average amount of cellulose produced by the tested STEC strains on LBNS agar with 2% ethanol was significantly higher than amounts produced on LBNS agar supplemented with 0 and 1% ethanol, respectively.

On LBNS agar with 1% ethanol, strain 49 and 46 produced 6.10 and 4.76 μg of cellulose/10^{10} CFU, respectively, which were significantly higher ($P < 0.05$) than the amounts of cellulose produced by the other strains tested in the study ($P < 0.05$). Similar phenomenon was observed on LBNS agar supplemented with 2% ethanol.

Interactive influences. The interactive influence of incubation temperature and atmosphere on the production of cellulose is shown in Fig. 3.2.A. The 28^{o}C incubation temperature and aerobic atmosphere were identified as a better incubation condition for the

production of cellulose by the STEC strains tested in the study. Fig. 3.2.B shows the influence of medium pH levels and water activities on the production of cellulose. The highest amount of cellulose was produced by the tested STEC strains grown on LBNS agar with pH 6.0 and a water activity of 0.99, while the lowest amount cellulose was produced on LBNS agar with pH 6.0 and a water activity of 0.96. The influence of ethanol or fructose addition to LBNS agar on the production of cellulose is shown in Fig 3.2.C. The results indicated that the tested STEC strains produced the highest amount of cellulose on LBNS agar supplemented with 2% ethanol and 0% fructose, and lowest amount of cellulose on LBNS agar supplemented with 0% ethanol and 2% fructose.

DISCUSSION

Previous studies (1, 8, 10) have mostly concentrated on cellulose produced by non-pathogenic bacteria such as *Gluconoacetobacter xylinus*. Recent studies revealed that cells of *Enterobacteriaceae* such as those of *S.* Typhimurium and *E. coli* were also capable of producing cellulose (43). Cells of *G. xylinus* were able to produce 4-5 g, and even up to ca. 10 g of cellulose per liter of cell culture (7). The average amount of cellulose produced by a strain of *S.* Typhimurium was ca. 8.57 μg per 10^9 CFU (19), which was much lower compared to the amounts of cellulose normally produced by *G. xylinus*. The small amounts of cellulose produced by *Salmonella* and *E. coli* are however, believed to have a significant role in the formation of biofilms and protection of cells against acid (28) and chlorine treatment (37) as well as desiccation (28, 39, 41).

The results of the present study showed that the addition of ethanol to LBNS agar significantly enhanced the production of cellulose by the tested STEC strains. This finding was

in agreement with what was reported in previous literatures (25, 38). In *Enterobacteriaceae*, ethanol is known to induce the expression of CsgD, which stimulates the transcription of AdrA, the *csgD* dependent regulator and activates the production of cellulose on the transcriptional level (15). Cellulose biosynthesis requires the participation of glucose, which is first phosphorylated *via* ATP consumption and then assembled into cellulose by four key enzymes including glucokinase, phosphoglucomutase, glucose-1-phosphate uridyltransferase and cellulose synthase (26, 33). Seto *et al.* (35) believed that ethanol serves as an energy source to generate ATP through oxidation to acetaldehyde or acetate by alcohol dehydrogenase or acetaldehyde dehydrogenase in the TCA cycle, thereby enhancing bacterial cellulose synthesis.

Addition of up to 2% fructose to LBNS agar in the present study had a negative effect on cellulose production by the tested STEC strains. This finding was contradictory to what was reported by Chao *et al*. (7) and Vandamme *et al*.(40). Fructose could be utilized as a carbon source for cellulose production. Successful conversion of fructose to cellulose in *G. xylinus* depends on the blockage of the metabolic flow toward pentose phosphate pathway by inhibiting glucose-6-phosphate dehydrogenase (3) or toward glycolysis by inhibiting phosphofructokinase (17). Both enzymes are allosterically inhibited by a high level of ATP (29, 42). This demand on cellular ATP to guide fructose to biosynthetic pathway to cellulose could create an additional burden to bacterial cells (33).

Römling *et al*. (32) found that the colonies of *S.* Typhimurium on LBNS agar display a temperature-dependent morphology change; and this colony morphology change was related to a higher level of cellulose production at 28^{o}C and lower level of cellulose production at 37^{o}C. Kader *et al*. found that *adrA* expression was activated at 28^{o}C in *S*. Typhimurium and reduced at

37°C (22). Incubation temperatures below 28°C were not favorable for cellulose production, and this might be due to limited cellular activities at these incubation temperatures (2).

It was found in the present study that water activities below 0.99 reduced the production of cellulose by the tested STEC strains. This phenomenon was probably caused by the elevated osmolarity in low water activity growth media. High osmolarity prevents the transcription of *csg*D (31), and the amount of cellulose produced by STEC cells would be therefore, reduced. Previous studies have shown that low osmolarity was required for cellulose production by *S.* Typhimurium and *E. coli* (32, 43). Gerstel and Römling (15) found that high salt conditions limited cellulose production in *S.* Typhimurium.

Oxygen tension is a significant factor for CsgD expression (14). In the present study, aerobic condition enhanced cellulose production compared to microaerophilic and anaerobic conditions. As stated previously, cellulose production requires the participation of ATP which is generated in the TCA cycle. This process may be deteriorated under low oxygen conditions, because oxygen is required as a final electron acceptor in the TCA cycle (33). Different observations have however, been reported in previous literatures. According to a study of Gerstel and Römling, the transcription of *csgD* promoter in *S*. Typhimurium increased under microaerophilic compared to aerobic or anaerobic atmosphere (15). This suggests that post transcriptional regulations may involve in cellulose biosynthesis (11, 36).

There was a significant difference in cellulose production on media with different pH levels. Responses of cellulose expression to changes in pH may be explained by changes in cell membrane fluidity at different pH levels. Lower membrane fluidity could interfere the secretion of cellulose through cell membranes (21). Studies on *G. xylinus* indicated that the optimal pH for cellulose production is strain-dependent and varied between 4.0 and 7.0 (13, 20). A pH range

from 5.0 to 8.0 was reported for a better cellulose production by *Gluconacetobacter* sp. RKY5 (24). Greater cellulose production by the tested STEC strain evaluated in the present study occurred at pH 6.0 which was in basic agreement with these previous observations.

Among the culture conditions evaluated in the present study, those that favor the production of cellulose by the tested STEC strains included a 28^{o}C incubation temperature, an aerobic atmosphere, and presence of 2% of ethanol in LBNS agar with pH 6.0 and a water activity of 0.99. The findings of the study will assist in formulating a microbiological media useful for cellulose and biofilm research.

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Table 3.1. Average amounts (μg/10^{10} CFU) of cellulose produced by each of the six selected STEC strains under different conditions.

Strain	Temperature (°C)				Atmosphere			Water activity				pH		
	15	22	28	37	Aerobic	Mic	Ana	0.96	0.97	0.98	0.99	5.0	6.0	7.0
17	1.60a[b]B[c]	0.79bD	0.48bB	0.92bB	1.37aB	0.41bB	0.04bB	0.01bD	0.01bC	0.01bD	0.41aCD	1.62aC	1.09bC	0.48cC
19	3.10aA	1.39bcC	2.16bB	0.67cB	2.71aB	2.20aB	0.67bAB	0.05bC	0.03bC	0.07bC	1.47aCD	0.99bC	3.89aB	2.16bC
46	1.72bAB	2.92bB	7.85aA	3.13bA	5.32aA	4.85aA	0.77bAB	0.23bB	0.28bA	0.32bA	7.13aA	5.37bAB	8.62abA	9.98aA
49	0.95cB	4.37abA	6.15aA	1.54bcB	5.19aA	1.44bB	1.15bA	0.38bA	0.18bB	0.10bB	5.18aB	7.00aA	7.35aA	6.31aB
50	1.20bB	3.27aB	2.34abB	1.20bB	2.69aB	1.63aB	0.66aAB	0.05bC	0.04bC	0.07bC	1.61aC	2.35aBC	4.08aB	2.34aC
117	1.73aAB	0.89bD	0.27bB	0.61bB	1.19aB	0.37bB	0.46abAB	<[d]0.003aD	<0.003aC	<0.003aD	0.10aD	0.40aC	0.68aC	0.27aC
Mean[a]	1.62b	2.27b	3.89a	1.53b	3.08a	1.82b	0.62c	0.12b	0.10b	0.10b	2.65a	2.95b	4.29a	3.59ab

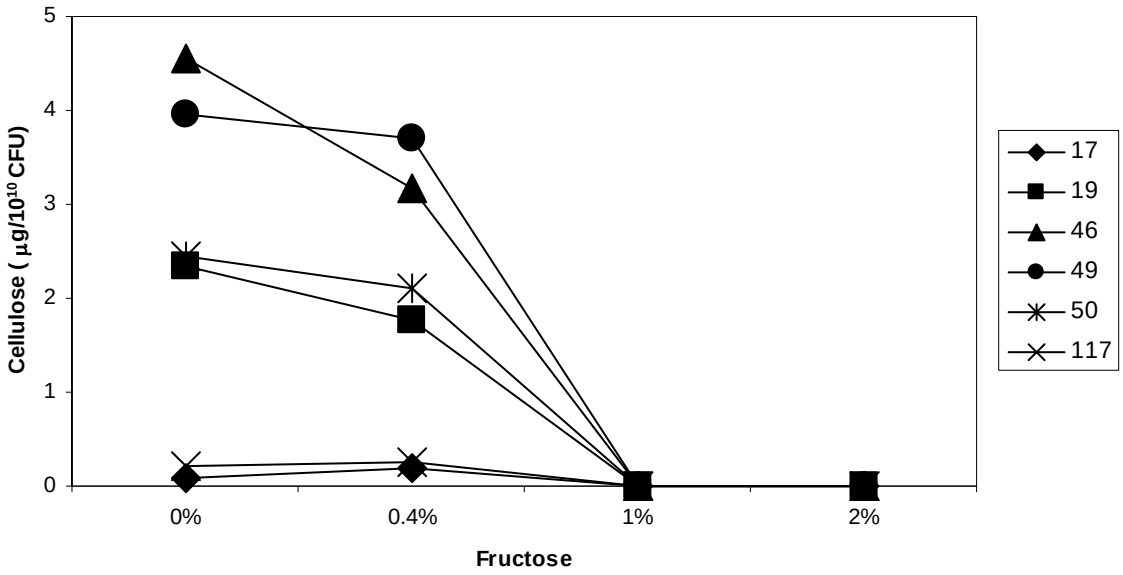
[a]Means are the average amounts of cellulose produced by all the strains used in the study under a specific condition.
[b]Values followed by the same lowercase letter in a same row are not significantly different with regard to conditions ($P > 0.05$).
[c]Values followed by the same uppercase letter in a same column are not significantly different with regard to cultures ($P > 0.05$).
[d]Detection limit is 0.003 μg/10^{10} CFU.

FIGURE LEGENDS

FIG. 3.1. Cellulose production by the six selected STEC strains on (A) LBNS agar supplemented with 0, 0.4, 1 or 2% (w/v) of fructose and (B) with 0, 1 or 2% of ethanol.

FIG. 3.2. Production of cellulose as influenced by (A) Incubation temperatures (15, 22, 28 and 37^{o}C) and atmospheres (aerobic, microaerophilic and anaerobic); (B) medium water activity (0.96, 0.97, 0.98 and 0.99) and pH 5.0, 6.0 and 7.0); (C) Addition of fructose (0, 0.4, 1 and 2%, w/v) and ethanol (0, 1 and 2%, v/v).

A.



B.

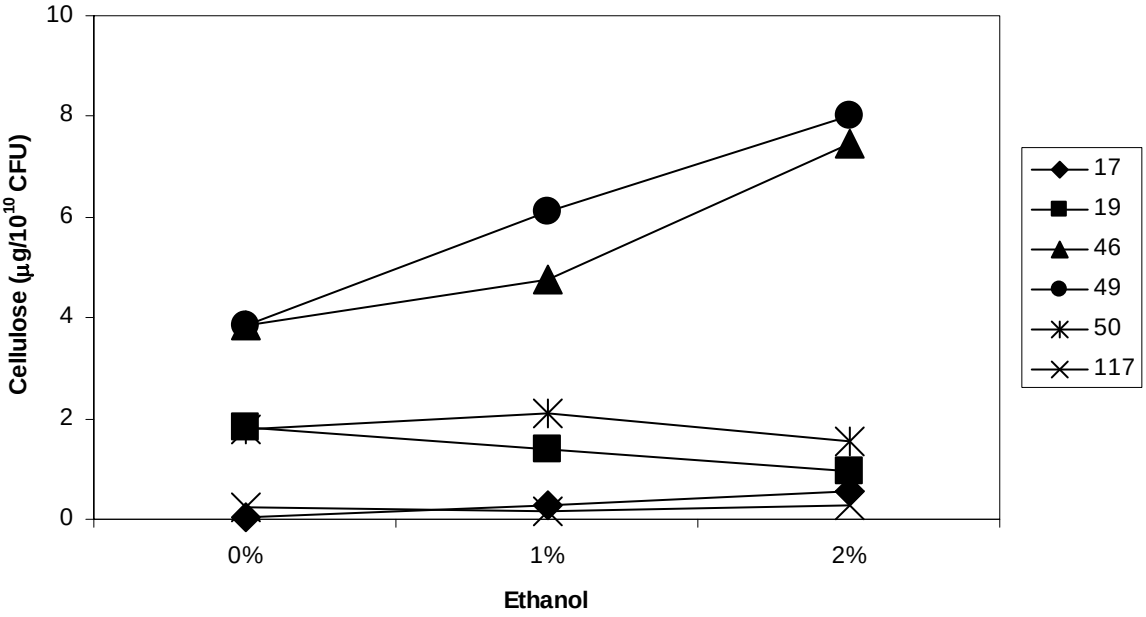


FIG. 3.1. Yoo and Chen

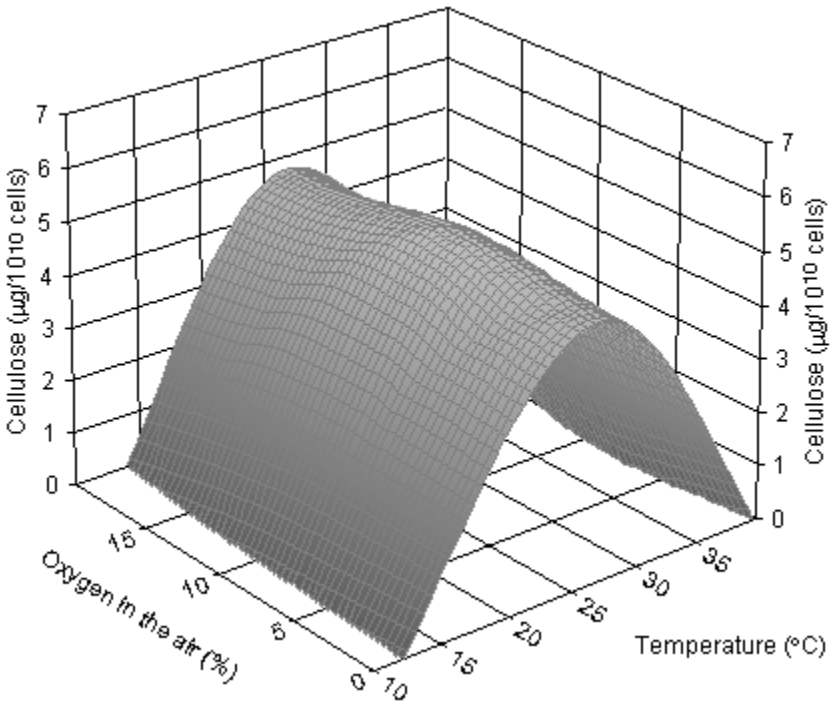


FIG. 3.2.A. Yoo and Chen

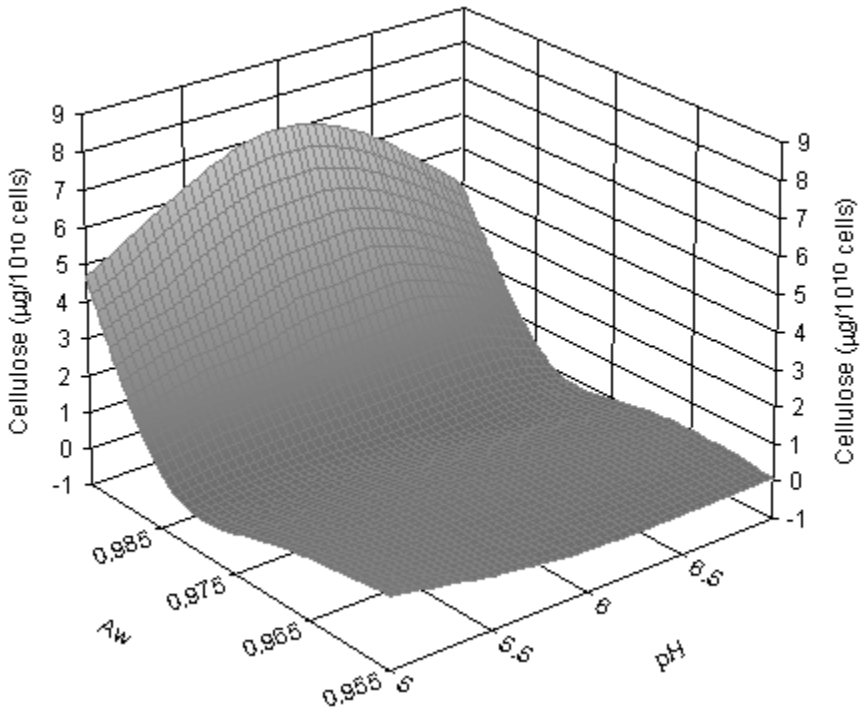


FIG. 3.2.B. Yoo and Chen

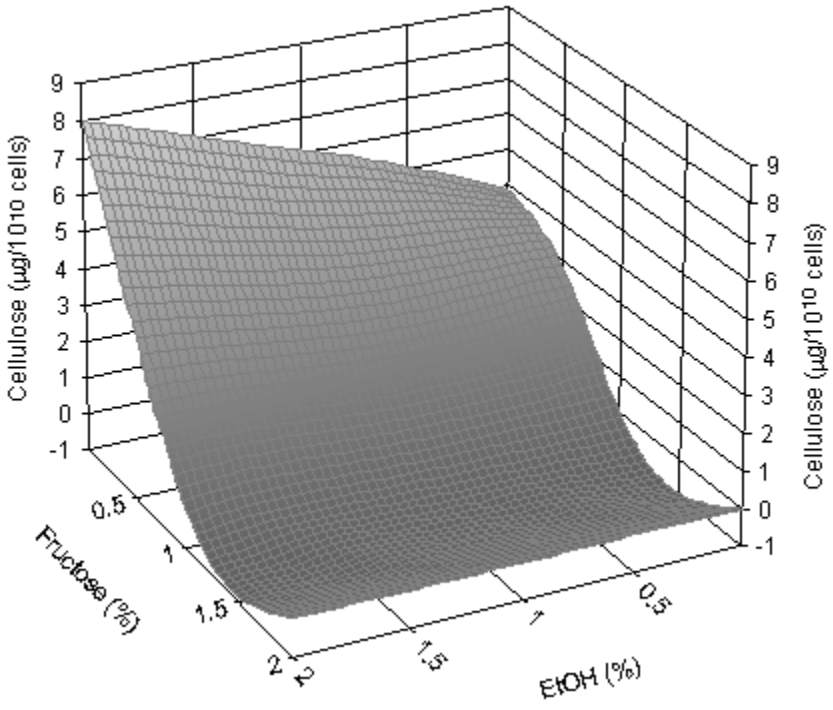


FIG. 3.2.C. Yoo and Chen

CHAPTER 4

SELECTION AND CHARACTERIZATION OF CELLULOSE DEFICIENT MUTANTS OF SHIGA TOXIN PRODUCING *ESCHERICHIA COLI* [1]

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ABSTRACT

Shiga toxin producing *Escherichia coli* (STEC) has been known to have several defense mechanisms, one of which is the production of protective extracellular substances including the production of cellulose. The goal of this study was to prepare pairs of STEC cultures useful for future studies designed to address the role of cellulose in protecting the cells of STEC against stress. Spontaneous cellulose deficient mutants, 19D and 49D were isolated, and the isolated mutants and their respective parents, 19B and 49B were characterized using serotyping, scanning and transmission electron microscopy as well as pulsed field gel electrophoresis. The growth characteristics of the STEC strains was studied using phenotypic microarray (PM). The cellulose deficient mutants and their respective parents shared the same serotypes and PFGE profiles. Profound morphological differences were however, found between the two types of cells. The overall results of PM indicated that 49B and 49D grew better ($P < 0.05$) than 19B and 19D in all three PM panels used in the study, probably due to the production of another polysaccharide, comprised of colanic acid, by the former two strains. The growth of 19B *vs.* 19D and 49B *vs.* 49D were only significantly ($P < 0.05$) different in the presence of two antibiotics, lincomycin and ceftriaxone, out of 24 antibiotics evaluated on the antibiotic panel. Among 90 tested substances on the osmolyte panel, strain 49D only had poorer ($P < 0.05$) growth than 49B in LBNS broth supplemented with 6% urea or 60, 80 and 100 mM sodium nitrite, respectively. In contrast to 49D, 19D had similar growth to 19B under these conditions. Among 96 tested acidulants on the pH panel, significant differences in the growth of 19B and 19D were only observed in LBNS broth with pH 4.5 and supplemented with L-histidine, hydroxyl-L-proline, L-ornithine or trimethylamine-N-oxide, and with pH 9.5 and supplemented with L-glutamine, L-methionine, hydroxyl-L-proline or anthranilic acid. Strain 49B only grew significantly ($P <$

0.05) better than 49D in LBNS broth with pH 9.5 and supplemented with L-phenylalanine, L-serine, L-threonine, hydroxy-L-proline or L-homoserine. Additionally, 19B and 49B grew significantly ($P < 0.05$) better than 19D and 49D, respectively in LBNS broth supplemented with X-caprylate. These results suggest that the two members of each STEC pair shared similar growth characteristics except under extreme stress. These strains could be useful in investigating the role of cellulose in protecting the cells of STEC against adverse environmental conditions.

Shiga toxin producing *Escherichia coli* (STEC) causes human diarrhea, which may proceed to the life-threatening hemolytic-uremic syndrome (9). STEC can form biofilms as one of cells' defense mechanisms against adverse environmental conditions. Extracellular polysaccharides (EPS) produced by the cells of STEC have been shown to play a structural role in the formation of biofilms (8). The composition of EPS is highly diverse, ranging from neutral to highly charged polyanionic macromolecules comprised of sugars such as pentoses, hexoses, amino sugars and uronic acids or non-sugar components such as acetic acid, succinic acid, pyruvic acid, phosphoric acid and sulfuric acid (24, 49). Certain members of the *Enterobacteriaceae* including *Salmonella* Typhimurium and *E. coli* have been known to have the ability to produce cellulose as a major component of their EPS (43, 61). Cellulose is a long-chain polysaccharide of glucose with β-1, 4 glycosidic bonds (4). Bactrial cellulose reportedly has greater mechanical strength than plant cellulose because it exhibits higher degree of polymerization and crystallinity (4).

Gluconoacetobacter is the best known group of microorganisms to produce cellulose, and have been extensively studied for cellulose production. There have been numerous reports on the development of spontaneous cellulose deficient mutants in *Gluconoacetobacter* strains. Cells of these strains reportedly had different morphological properties (40, 41, 47).

The ultimate goal of this study was to prepare pairs of STEC cultures useful for future studies designed to address the role of cellulose in protecting the cells of the pathogen under adverse environmental conditions. In such studies, the use of spontaneous cellulose deficient mutants seems necessary. Before the wild type and cellulose deficient cultures are used in these studies, their identities have to be confirmed and growth characteristics examined. The wild type STEC strains and their spontaneous mutants are supposed to have similar growth characteristics.

Otherwise, the differences in the growth patterns may introduce undesirable influence to the experiments.

The practical goals of this study were to isolate spontaneous cellulose deficient mutants, to confirm the identities of the selected mutants and their wild type parent strains using serotyping and pulsed field gel electrophoresis (PFGE), to observe the morphological differences between the mutant and parent cells using scanning and transmission electronic microscopy (SEM and TEM), and to determine the growth patterns of the STEC strains under optimal, suboptimal as well as stressful culture conditions using the Biolog® Phenotype Microarray™ (PM) technology.

MATERIALS AND METHODS

Selection of cellulose deficient mutants. Two wild type STEC strains, 19 and 49, were used in the study. Both of these strains were capable of producing cellulose. In addition to cellulose production, strain 49 also produced another extracellular polysaccharide comprised of colanic acid (EPS-CA). Spontaneous cellulose deficient mutants were selected by growing strain 19 and 49 on Luria Bertani no salt (LBNS) agar supplemented with 200 mg/ml of calcofluor white stain (Sigma-Aldrich Inc., St. Louis, MO). The inoculated plates were incubated at 28^{o}C for 96 h. Following the incubation, relatively brighter fluorescent colonies and dimmer to dark colonies on the agar plates were selected using a long wave UV lamp (UVGL-58, UVP, Upland, CA), and cultured separately. This process was repeated for at least four times in order to obtain pairs of STEC cultures producing distinct amounts of cellulose under the same incubation conditions. The brighter fluorescent colonies were cellulose producing, and were designated as 49B and 19B, respectively. The dimmer to dark colonies were cellulose deficient, and were designated as 49D and 19D, respectively. The amounts of cellulose and the EPS-CA produced

by the STEC cultures were determined using the procedures described below. Both types of cultures were stored at -30°C in LBNS broth supplemented with 15% glycerol (Fisher Scientific, Fair Lawn, NJ). All the cultures were purified before being used in the experiments.

Quantification of cellulose produced by STEC. Cellulose produced by STEC cells was quantified using a colorimetric assay developed by Updegraff with some modifications. STEC cultures were grown on LBNS agar (pH 6.0) plates supplemented with 2% EtOH at 28°C for 96 h. The cultures were harvested with 10 ml of buffered phosphate saline (PBS, pH 7.4) by gentle agitation at 20 × g for 25 min with an Orbit shaker (Lab-line Instruments Inc., Melrose Park, IL) followed by gently scraping the cultures off the agar plates. The obtained cell suspensions (10 ml) were centrifuged (Beckman GS 6-R centrifuge, Beckman Instruments Inc., Palo Alta, CA) at 2,500 × g for 25 min. The cell pellet of each culture was collected after the supernatant fluid was discarded. Three ml of acetic-nitric reagent (150 ml of 80% acetic acid and 15 ml of concentrated nitric acid) was added to the cell pellet of each culture in a glass centrifuge tube (17 × 118 mm; Fisher Scientific, Fair Lawn, NJ) and mixed for 5 s by vortexing. The centrifuge tubes were covered with aluminum foil and heated in a boiling water bath for 30 min. The contents in the tubes were re-centrifuged under the conditions described above. The cell pellet of each culture was collected after the supernatant fluid was discarded. The pellet was washed with 10 ml of distilled water after the centrifugation at 2,500 × g for 25 min. One ml of 67% sulfuric acid was then added and mixed with the cell pellet. The mixtures were allowed to stand for 1 h at room temperature. After dilution with 4 ml of distilled water, the mixtures in the centrifuge tubes were placed in an ice bath. Ten ml of anthrone (Sigma-Aldrich Inc., St. Louis, MO) reagent (0.2 g of anthrone in 100 ml of concentrated sulfuric acid) was added to each tube with intermittent mixing. The tubes were inverted gently and then heated in a boiling water bath for

16 min. The absorbance of each sample at 620 nm was recorded using the Novaspec II spectrophotometer (Pharmacia Biotech, Cambridge, UK). A standard curve of absorbance as a function of cellulose concentration was prepared. Cellulose produced by the cells of each STEC culture was expressed as μg per 10^{10} colony forming units (CFU) of STEC.

Quantification of the EPS-CA produced by STEC. STEC cultures were grown on minimal glucose agar (MGA) at 22°C for 48 h. The resulting cultures were harvested with 10 ml of 0.15 M NaCl. The amounts of the uronic acud (UA) in the suspensions were determined by a colorimetric assay with glucouronic acid as a standard (59). The quantities of the proteins (P) in the suspensions were determined using a BCA assay (Pierce, Rockford, IL, USA) to account for differences in quantities of bacterial growth. The UA/P ratio, defined as micrograms of UA per milligram of protein, was used to express the levels of the EPS-CA produced by different strains of STEC.

Serotyping. The STEC cultures described above were grown on brain heart infusion agar slants (Difco, Detroit, MI) at 37°C for 24 h. The O antigens on the STEC isolates were determined according to the procedures described by Orskov et al (34). The isolates were first tested against several pooled O anti-sera (Difco, Franklin Lakes, NJ). If an isolate tested positive against an antiserum pool, it was then examined against each single serum in that pool. The H antigens were determined based on restriction fragment length polymorphism (RFLP) analysis by amplifying the *fliC* gene using polymerase chain reaction and digesting the amplified products with restriction enzyme *HpaII* (29). The digested fragments were separated by agarose gel electrophoresis, and the generated profiles were compared against the RFLP profiles of 52 standard H antigens in order to designate an appropriate H antigen to the tested STEC cells.

PFGE. Genomic fingerprint profiles of 49 B, 49D, 19B and 19D were determined using

PFGE. Intact bacterial genomic DNA was digested with restriction enzyme *XbaI* (New England BioLabs, Beverley, MA). The PFGE was performed using a CHEF Mapper XA PFGE system (Bio-Rad, Hercules, CA). DNA fingerprint profiles were analyzed using Gel Doc 2000 and Molecular Analyst Fingerprinting Plus software, version 6.1 (Bio-Rad, Hercules, CA). The relatedness of the restriction profiles was generated using the unweighted pair group method with arithmetic average clusters based on Dice coefficients of each banding pattern (14).

Fluorescence microscopy. Cultures of 19B and 19D were grown on LBNS agar supplemented with calcofluor white stain which is specific to the EPS with either β-1,3- or β-1,4-glucosidic bonds (56). The resulting cultures were suspended in 10 ml of PBS buffer, respectively. A loop full of each cell suspension was placed on the surface of glass slides, and air dried. Cover slips were placed on the top of the slides, and sealed with Dabco-glycerol before examination with a BX60 epifluorescence microscope (Olympus America, Inc., Melville, NY). An appropriate filter with UV excitation wavelength at 330-385 nm, dichroic mirror at 400 nm and emission wavelength at 420 nm was used to observe the cells stained with calcofluor. Monochrome images of calcofluor-stained cells were captured with a Magnafire camera (Optronics, Goleta, CA) and Image Pro Plus software (Media Cybernetics, Silver Spring, MD).

SEM. SEM was used as a visual tool to compare the morphologic differences between the cellulose deficient mutants and cellulose proficient wild types of STEC cells used in the study. Cells of STEC were grown on LBNS agar plates at 28^{o}C for 96 h. The detailed sample preparation procedure for SEM was described elsewhere (21). Briefly, cells of STEC were 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 the STEC cells were fixed with 1% osmium tetroxide (OsO_4) at room temperature for 1 h.

The samples were then washed twice with SCB and dehydrated serially with 50, 70, 80, 90, and 100% ethanol, each for 15 min. The samples were stored at 4°C in 100% ethanol. The samples were dried at critical point temperature with liquid CO_2 using a critical point dryer (Samdri model 780-A, Tousimis, Rockville, MD). The samples were mounted on aluminum stubs and the cells were coated with gold in a sputter coater (Structure Probe, Inc., West Chester, PA). Samples were loaded and the cells were visualized at appropriate magnifications using a scanning electron microscope (LEO Electron Microscopy, Inc., Thornwood, NY).

TEM. The cultures of STEC were grown on LBNS agar plates at 28°C for 96 h. As the SEM samples, the cells were fixed with 2% glutaraldehyde in 0.1 M SCB at 4°C for 1 h. The fixed cells were washed 3 times with SCB, each for 10 min. Lipids on the STEC cells were fixed with 1% OsO_4 at 4°C for 1 h. The samples were then washed twice with deionized water and dehydrated once with 25, 50, 75, 85, and 95% ethanol, respectively and then three times with 100% ethanol. Each dehydration step took place at room temperature for approximately 10 min. Ethanol in the samples was then gradually replaced with acetone, first in 75% ethanol:25% acetone, then in 50% ethanol:50% acetone, 25% ethanol:75% acetone, and finally in 100% acetone. The samples were then embedded with 25% resin:75% acetone, 50% resin:50% acetone, 75% resin:25% acetone and 100% resin. Resin-embedded samples were polymerized in 60°C oven for 16 h. Polymerized samples were sectioned using the RMC MT-X ultramicrotome (Boeckeler Instruments, Inc., Tuscon, AZ). The sectioned samples were mounted on Formvar- and carbon-coated 300-mesh copper grids. To improve the contrast of TEM samples, copper grids were stained by one drop of 2% (w/v) uranyl acetate for 15 to 45 min in dark. After three times of rinsing with deionized water, the grids were stained for 1 min in dark with Reynold's lead citrate [2.66% (w/v) lead citrate and 3.52% (w/v) sodium citrate dissolved in CO_2-free

distilled water with pH 12.0] (37). At the following, the grids were washed once with 0.02 N NaOH, and three times with deionized water. The samples were then air dried for 1 h before being observed using the FEI Tecnai 20 (FEI Co., Eindhoven, Netherlands) at 200 kV.

PM. Selected phenotypic characteristics of 19B and 19D as well as 49B and 49D was determined using the PM technology as described by Bochner et al (7), with some modifications. All materials and equipment including the dye mix, PM plates, turbidometer, and incubation chamber were from Biolog, Inc. (Hayward, CA). Out of the 20 available PM microplates, PM9, PM10 and PM11 were selected because these three plates test the growth characteristics of the STEC cultures under arrays of optimal, suboptimal and stressful environmental conditions. According to the manufacturer's manual, the wells of PM 9, 10 and 11 contained osmolytes, acidulants and antibiotics, respectively.

Cells of STEC on the surface of LBNS agar were collected with a sterile cotton swabs, respectively, and suspended in 20 ml of diluted (5 folds) LBNS broth in 20 x 150 mm test tubes. The cell density was adjusted to 70% transmittance (approximately 5 x 10^6 CFU/ml) at 590 nm using a Biolog turbidometer. Two ml of the cell suspensions were mixed with 20 ml of 1% (v/v) dye mix A containing tetrazolium violet. One hundred microliters of each cell suspension and dye mixture were inoculated into each well of the PM microplates, and incubated at 37^{o}C in an OmniLog humidified incubator. The absorbance values of the broth cultures at 750 nm were measured hourly during a 24 h incubation period in order to document the development of a purple color in LBNS broth. The color development reflected the rate of cell respiration which was an indication of cell growth. The obtained data were averaged and analyzed using the OmniLog-PM software (ver. 1.20.02). The average absorbance values in each PM well during the course of incubation were used to express the growth characteristics of cellulose proficient

and cellulose deficient cultures under various conditions. Differences in the average absorbance between cellulose deficient and cellulose producing cultures were calculated by subtracting the absorbance values of cellulose producing cultures from those of cellulose deficient cultures. A resulting negative value indicated that the cellulose producing cultures grew better than the cellulose deficient cultures under a specific growth condition.

Each test was duplicated by following the design of the PM microplates. The tests included in each PM microplate were replicated for three times. Significant differences in the average absorbance values from the cellulose producing culture and cellulose deficient culture within a STEC pair were analyzed using the Student *t* test of the Statistical Analysis Software (SAS Institute Inc., 2003) at a 95% confidence interval. Absorbance values from the three replicate experiments were assembled into a single data list which was subsequently used to generate a kinetic plot. The plot demonstrated the variation as well as the reproducibility of the replicate experiments.

RESULTS

Selection of cellulose deficient mutants. Cultures of cellulose deficient cells, 19D and 49D as well as the wild type cellulose producing cells 19B and 49B are shown (Fig 4.1). The wrinkled and folded appearances (Fig. 4.1.A and 4.1.C) are the indication of cellulose production. Quantification results showed that cells of 49B and 19B produced an average of 8.46 ± 0.22 and 6.47 ± 0.08 μg of cellulose per 10^{10} CFU, respectively on LBNS agar (pH 6.0) supplemented with 2% ethanol under aerobic condition at 28°C. The average amounts of cellulose produced by 49D and 19D were 1.62 ± 0.85 and 1.36 ± 0.54 μg per 10^{10} CFU, respectively. The differences in the amounts of cellulose produced by the two members of the STEC pairs ranged from 4.77 -

5.23 folds. Strain 49B and 49D each produced a copious amount of the EPS-CA on MGA at 22°C, with an UA/P ratio of 9.70 and 10.64, respectively. The UA/P ratios of strain 19B and 19D were 1.28 and 3.48, respectively. The amounts of the EPS-CA produced by the 19B and 19D vs. 49B and 49D differed 4.28 folds.

Confirmation of cellulose expression by fluorescence microscopy. The extracellular material produced by the cells of 19B was able to bind to calcofluor (Fig. 4.2.A). Fluorescent amorphous areas were visible around the cells of 19B. In contrary, cell outlines of 19D were not visualized due to the lack of cellulose expression (Fig. 4.2.B).

Confirmation of STEC identities with serotyping and PFGE analysis. Serotyping revealed that 19B and 19D shared a common serotype which was O5:H-, while 49B and 49D were both O103:H2. Strain 19B shared the same PFGE pattern with 19D, and 49B shared the same pattern with 49D (Fig 4.3). Dendrogram analysis indicated that the two members within each pair of STEC shared 100% similarities.

SEM and TEM. Results of SEM showed that cells of 19B (Fig. 4.4.A) and 49B (Fig. 4.4.C) appeared to have significant morphological differences from those of 19D (Fig. 4.4.B) and 49D (Fig. 4.4.D), respectively. There was however, no significant morphological difference between 19B and 49B, even though the latter was capable of producing the EPS-CA. Cells of 19B and 49B were deeply trapped in densely packed matrices. On the other hand, 19D and 49D were loosely packed without the coverage by the same type of materials. Cells of STEC (Fig. 4.5) had irregular shapes depending on the location where the sectioning took place. Cells of 19B and 49B were surrounded by stained regions of tangled materials (Fig. 4.5.A and 4.5.C), however, cells of 19D and 49D did not have the stained materials around them (Fig. 4.5.B and 4.5.D).

PM. The overall results of PM indicated that the growths of 19B *vs.* 19D, and 49B *vs.* 49D were not significantly different on the osmolyte and acidulant panels (Table 4.1). However, the overall growths of 19B and 49B were significantly ($P < 0.05$) different from those of 19D and 49D, respectively on the antibiotic panel. Additionally, 49B and 49D grew better ($P < 0.05$) than 19B and 19D in all three PM microplates used in the study.

Among the 210 tested substances on the 3 PM microplates, only those that had significant ($P < 0.05$) influence on the growth of at least 1 pair of the STEC strains are listed in Table 4.2. On PM9, the osmolyte panel, strain 49D only had significantly ($P < 0.05$) poorer growth compared to 49B in LBNS broth supplemented with 6% urea or 60, 80 and 100 mM sodium nitrite, respectively. In contrast to 49D, 19D had similar growth to 19B under these conditions. On PM 10, the pH panel, significant differences ($P < 0.05$) in the growth of 19B and 19D were only observed in LBNS both with pH 4.5 and supplemented with L-histidine, hydroxyl-L-proline, L-ornithine or trimethylamine-N-oxide, and in LBNS broth with pH 9.5 supplemented with L-glutamine, L-methionine, hydroxyl-L-proline or anthranilic acid. Strain 49B grew significantly ($P < 0.05$) better compared to 49D in LBNS broth with pH 9.5 and supplemented with L-phenylalanine, L-serine, L-threonine, hydroxy-L-proline or L-homoserine. The cellulose producers in both the STEC pairs grew significantly ($P < 0.05$) better compared with their respective non-cellulose producing counterparts in LBNS broth supplemented with X-caprylate. On PM 11, the antibiotics panel, 49B and 49D had similar growth rates while 19D had poorer growths in the presence of all 24 tested antibiotics compared with 19B (Data not shown). However, significant differences ($P < 0.05$) in the growth of 19B and 19D were only observed in LBNS broth supplemented with lincomycin and ceftriaxone.

The average absorbance values resulted from the growth of each STEC culture during a 24 h incubation period in three replicate experiments are displayed, and overlapped in the plots (Fig. 4.6). The variations in the growth rates among the three replicate experiments are shown in the shaded areas of the plots. The limited appearance of the shaded areas in these plots indicated that the PM tests conducted in the present study were reproducible.

DISCUSSION

The biosynthesis of cellulose in several *G. xylinus* strains has been shown to be a rather unstable trait (11, 47, 52). Under certain culture conditions such as agitation, cellulose deficient mutants could evolve from cellulose producing cells (10). Spontaneous cellulose deficient mutants have been extensively studied with an attempt to enhance the yield of cellulose produced by acetic acid bacteria (2, 27), for cells converted to spontaneous mutants are not desirable during commercial cellulose production (28). Krystynowicz et al. (28) hypothesized that conversion to cellulose deficient mutants was the results of different levels of expression of the genes encoding the enzymes responsible for cellulose biosynthesis. The genes for cellulose biosynthesis (*bcs*) were first identified in *G. xylinus* by Wong et al (55). Cellulose synthesis by bacterial cells is catalyzed by four major enzymes, namely cellulose synthase, glucose-1-phosphate uridylyltransferase, phosphoglucomutase and glucokinase. Glucose-1-phosphate uridyltransferase and phosphoglucomutase were found missing from cellulose deficient mutant cells of *G. xylinus* (28).

Positive calcofluor fluorescence observed in the present study was only associated with the cells of 19B not with those of 19D, suggesting that calcofluor-binding EPS were produced only by cellulose producing cells. Although calcofluor is able to bind to the EPS with both β-

1,3- and β-1,4-glycosidic bonds, it has relatively higher affinity to the EPS with β-1,4-glycosidic bonds (56). In addition to cellulose, bacterial EPS with β-1,3- and β-1,4-glycosidic bonds also include curdlan and chitin (50, 56). Curdlan is a polysaccharide with β-1,3- glycosidic bonds, and is produced mainly by *Agrobacterium* sp. (46). Chitin is nevertheless, a polysaccharide with β-1,4-glycosidic bonds, and is composed of N-acetylglucosamine. It is mainly associated with the cell walls of yeast or fungi (53). There is no evidence that these EPS are produced by the cells of *E. coli*. Calcofluor is therefore, used in the present study to demonstrate the presence of cellulose around the cells of STEC.

The EPS-CA is loosely associated with bacterial cells, and the preparation procedures for SEM and TEM can separate it from its producing cells (5, 20). This explains why the EPS-CA produced by 49B and 49D was not visualized on either scanning or transmission electronic micrographs. SEM has been used to visualize the ultra structure of cellulose produced by *Salmonella* (21) and *G. xylinus* (19). Jain and Chen (21) used SEM to demonstrate the cellulose produced by *Salmonella* of poultry origin. Cellulose produced by the static culture of *G. xylinus* reportedly had similar structure to those observed in this study (60). TEM has been used to demonstrate the structure of cellulose produced by *G. xylinus* (19), *S*. Typhimurium (39) and *S*. Enteritidis (54). In the present study, TEM grids were stained with uranyl acetate followed by Reynold's lead citrate. Uranyl acetate reacts strongly with phosphate and amino groups of nucleic acids and phospholipids in cell membranes, and lead citrate is believed to bind to negatively charged molecules such as hydroxyl groups (15). Although lead has no known chemical affinity with cellulose, the glucose polymer could be stained by lead citrate through formation of lead-glucose complex through hydrogen bonding (37). Uranyl acetate and lead

citrate have been used to demonstrate cellulose microfibrils of plant (12) or bacteria (33) cells in thin sections (18).

Spontaneous cellulose deficient mutants and their respective cells had similar growth rates under optimal, and even some of the suboptimal culture conditions that did not have strong enough inhibition on their growths. Significant differences in growth between the two members of at least 1 set of the STEC strains were observed under only 19 out of a total of 210 growth conditions evaluated in this study (Table 4.2). The mutant cells appeared to be more susceptible to the high concentrations of urea and sodium nitrite, acidic and alkaline pH, and certain antibiotics such as lincomycin and ceftriaxone.

Strain 49D demonstrated similar growth rate to 49B on the osmolyte panel except in LBNS broth containing 6% urea or 60, 80 and 100 mM of sodium nitrite, respectively (Table 4.2). Urea is a strong protein denaturant that effectively disrupts the noncovalent bonds in proteins. In addition to protein denaturation, urea could increase the intracellular level of ammonia (NH_3), leading to a reduced intracellular proton concentration and rapid alkalinization of cell cytoplasm (35). Sodium nitrite has been used as a preservative to control *Clostridium botulinum* (13), but it is known to inhibit the growth of *E. coli* O157:H7 (51), *Enterobacter* (50), and *Listeria monocytogenes* (36) at 200 μg/ml, and of *Salmonella* (38) and enteropathogenic *E. coli* (9) at 400 μg/ml. The antibacterial activity of sodium nitrite against *E. coli* O157:H7 is attributed to the instantaneous inactivation of iron-sulfur containing enzymes of the respiratory chain (32). Sodium nitrite may inhibit active transport, oxygen uptake and oxidative phosphorylation during cellular respiration in *P. aeruginosa*. Inhibition on oxidative phosphorylation was accomplished by oxidizing ferrous iron of an electron carrier or binding to the heme iron of cytochorome oxidase (58).

On the pH panel, 19D and 49D only had poorer growth compared to 19B and 49B, respectively in LBNS broth with pH 4.5 and pH 9.5 supplemented with certain amino acids (Table 4.2). Previous studies have shown that the growths of *E. coli*, *Klebsiella pneumoniae*, *Enterococcus faecium*, *E. faecalis*, *E. casseliflavus* (48) and *S.* Typhimurium SL1344 were inhibited at pH 4.5 (16). Alkaline pH was also able to inhibit the growth of bacterial cells such as the growths of *S.* Enteritidis, *E. coli* O157:H7 (31), *Yersinia enterocolitica* (44) and *S.* Typhimurium (25). With the supplement of certain amino acids under acidic stress, *E. coli* produces alkaline amines in order to increase the intracellular pH by decarboxylation of amino acids, while under alkaline stress, it produces α–keto-acids to decrease the pH by deamination of amino acids (6, 30).

Data indicates that the cellulose producers in both STEC pairs grew significantly ($P < 0.05$) better compared with their respective non-cellulose producing counterparts in LBNS broth supplemented with X-caprylate (Table 4.2). Caprylic acid has been used for the treatment of infections caused by *E. coli* O157:H7 and enteropathogenic *E. coli* O103 (1, 42). It is a short chain fatty acid naturally present in human breast (23), and bovine milks (22). It penetrates cell membranes in the undissociated form and dissociates within the protoplasm causing intracellular acidification (17).

It was observed that 19D was most susceptible, among the tested STEC cultures tested to the antibiotics evaluated in the present study (Table 4.1). Strain 19B had increased resistance compared to 19D but was relatively less resistant to the evaluated antibiotics than the EPS-CA producing strain 49B and 49D (Table 4.1) The latter two strains had similar resistance to the antibiotics evaluated in the study, regardless of their abilities to produce cellulose . These results

suggest that the EPS-CA and cellulose work synergistically in protecting the cells of STEC against environmental stress.

Lincomycin, similar to macrolides, inhibits protein synthesis by binding to the 50S subunit of the bacterial ribosome, thereby inhibiting translocation of peptidyl tRNA (45). Macrolides have been reported to interfere with biofilm formation in *P. aeruginosa* (57). Although they did not reduce biofilm initiation, the antibiotics delayed the formation of biofilms (57). In addition, macrolides allow the access of other antibiotics to bacterial cells by increasing the permeability of biofilms (26). Ceftriaxone inhibits the synthesis of peptidoglycan of bacterial cell wall, and is therefore, particularly effective in inhibiting rapidly dividing bacterial cells (3).

Cells of STEC have several known cell surface structures. The interactions between these cell surface components are complicated. The precise protective mechanism of cellulose towards the cells of STEC is not understood. Since cellulose is a hydrophilic molecule, it might have something to do with changes in cell surface charge and electrostatic impulsion.

In conclusion, the spontaneous cellulose deficient mutant, and their respective parent cells shared the same serotypes and PFGE profiles, and grew at similar rates under optimal, and some of the suboptimal culture conditions which had no strong enough inhibitory effects on their growths. These strains could be useful in future studies designed to address the role of cellulose in protecting the cells of STEC to survive under extreme environmental conditions.

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Table 4.1. Statistical analysis and overall results of respiration rates of wild type cellulose producing cells (19B and 49B) and spontaneous cellulose deficient cells (19D and 49D) of STEC

		Respiratory activity (average values of A_{750nm})			
	N[a]	19B	19D	49B	49D
PM9[b]	90	95.5b[c]	103.1b	157.0a	153.6a
PM10	96	107.7b	97.8b	144.0a	130.7a
PM11C	24	106.1b	79.6c	110.4b	122.6a

[a]Number of tests performed in the PM plate.
[b]PM9 includes osmolytes; PM10 includes acidulants; PM11C includes antibiotics.
[c]Means in the same row followed by the same letter are not significantly different ($P > 0.05$).

Table 4.2. Differences in respiration rates between the cells of cellulose deficient mutant, and cellulose producing parent cells of STEC

	Test[a]	Difference[b]		Mode of action[c]
		19	49	
PM 9	Urea 6%	2[d]	-3[e]	Protein denaturant
	Sodium nitrite 60mM	14[d]	-32[e]	Toxicity, nitrite
	Sodium nitrite 80mM	13[d]	-27[e]	Toxicity, nitrite
	Sodium nitrite 100mM	1[d]	-31[e]	Toxicity, nitrite
PM10	pH 4.5 + L-Histidine	-13[e]	0[d]	Acidic pH, decarboxylase
	pH 4.5 + Hydroxy-L-Proline	-10[e]	3[d]	Acidic pH, decarboxylase
	pH 4.5 + L-Ornithine	-10[e]	0[d]	Acidic pH, decarboxylase
	pH 4.5 + Trimethylamine-N-Oxide	-9[e]	-2[d]	Acidic pH, decarboxylase
	pH 9.5 + L-Glutamine	-11[e]	-14[d]	Alkaline pH, deaminase
	pH 9.5 + L-Methionine	-64[e]	-9[d]	Alkaline pH, deaminase
	pH 9.5 + L-Phenylalanine	-4[d]	-24[e]	Alkaline pH, deaminase
	pH 9.5 + L-Serine	-47[d]	-21[e]	Alkaline pH, deaminase
	pH 9.5 + L-Threonine	-8[d]	-16[e]	Alkaline pH, deaminase
	pH 9.5 + Hydroxy-L-Proline	-11[e]	-20[e]	Alkaline pH, deaminase
	pH 9.5 + L-Homoserine	0[d]	-105[e]	Alkaline pH, deaminase
	pH 9.5 + Anthranilic Acid	-14[e]	-19[d]	Alkaline pH, deaminase
	X-Caprylate	-27[e]	-15[e]	Caprylate esterase
PM11	Lincomycin	-40[e]	12[d]	Lincosamide antibiotic
	Ceftriaxone	-49[e]	17[d]	Cephalosporin antibiotic

[a]Chemicals were tested in 96-well PMs.
[b]The OmniLog-PM software was used to generates time course curves for respiration, and calculate the differences in the growth rates of mutant and parent cells. Positive values indicate that the mutant cells had greater rates of respiration than the parent cells. Negative values indicate that the parent cells had greater rates of respiration than the mutant cells. The differences are the averages of three independent readings.
[c]Suggested by manufacturer's manual.
[d]Insignificant differences ($P > 0.05$)
[e]Significant differences ($P < 0.05$)

FIGURE LEGENDS

FIG. 4.1. STEC cultures of 19B (A), 19D (B), 49B (C) and 49D (D) grown on Luria Bertani no salt (LBNS) agar (pH 6.0) supplemented with 2% ethanol at 28^{o}C for 96 h.

FIG. 4.2. Epifluorescent microscopy of calcofluor stained STEC cells of 19B (A) and 19D (B). The cells were from the cultures grown on LBNS agar (pH 6.0) supplemented with 2% ethanol at 28^{o}C for 96 h, stained with calcofluor, and viewed under an epifluorescent microscopy with magnification of 40 times.

FIG. 4.3. Dendrogram analysis of DNA fingerprint profiles of the STEC strains used in the study. The dendrogram was generated using Gel Doc 2000 and Molecular Analyst Fingerprinting Plus software.

FIG. 4.4. Scanning electron micrographs of STEC cells of 19B (A), 19D (B), 49B (C) and 49D (D). All cultures were grown on LBNS agar (pH 6.0) supplemented with 2% ethanol at 28^{o}C for 96 h. Bars represent 1 μm.

FIG. 4.5. Transmission electron micrographs of STEC cells of 19B (A), 19D (B), 49B (C) and 49D (D). All cultures were grown on LBNS agar (pH 6.0) supplemented with 2% ethanol at 28^{o}C for 96 h. Bars represent 500 nm. Arrows indicate the presence of cellulose.

FIG. 4.6. Reproducibility of the phenotype microarray assay. Each graph shows the hourly changes in cell respiration rates during a 24 h incubation period. Data from three replicate experiments were overlapped in a single plot. The shaded areas in the graphs indicate the variations among three replicate experiments. The limited appearance of the shaded areas in the graph indicates the high reproducibility of the experiment. The letters indicate different STEC strains evaluated. A: 19B; B: 19D; C: 49B; and D: 49D. The numbers indicate the PM microplates used in the study. 1: PM9; 2: PM10; and 3: PM11C.

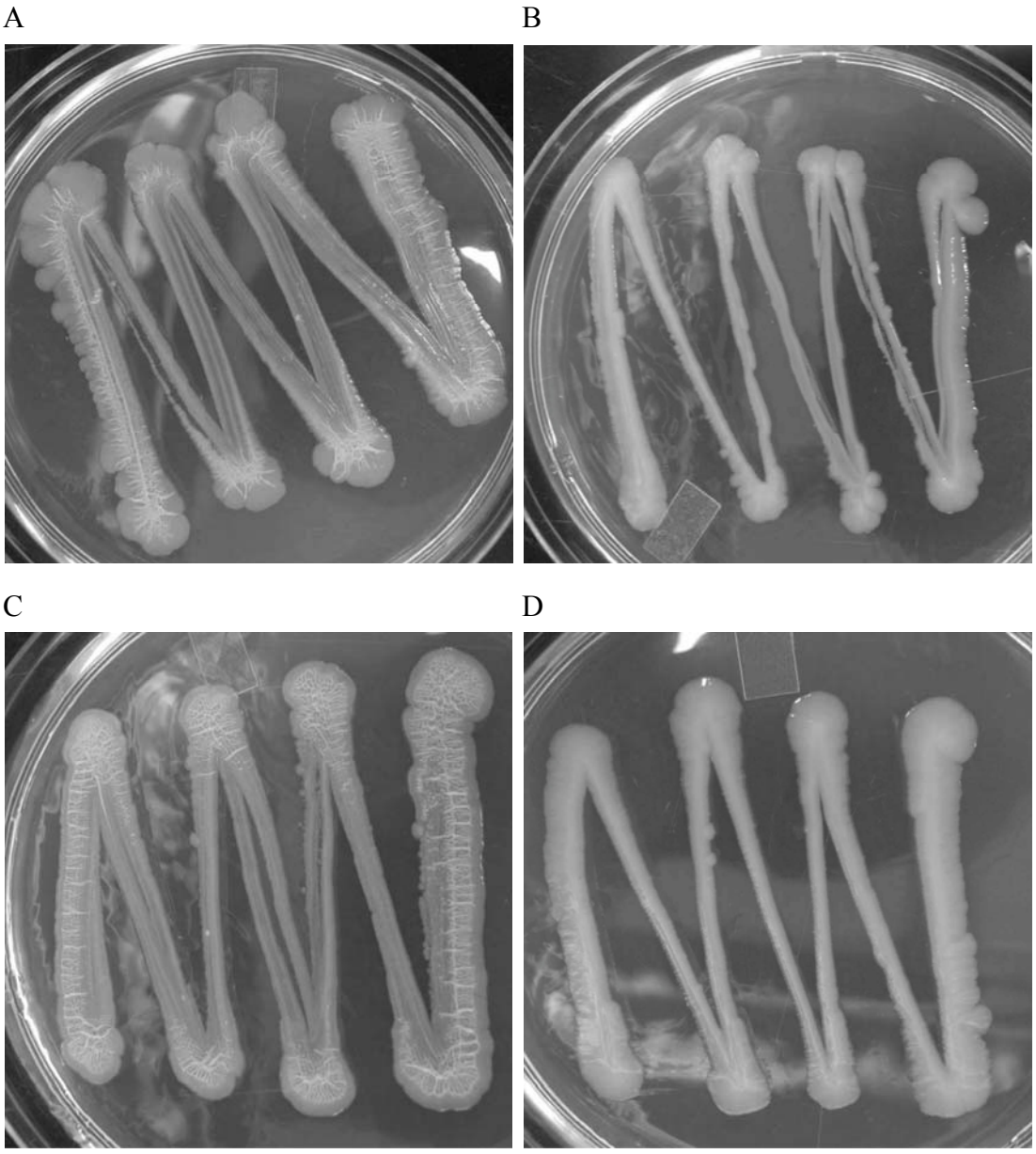

FIG. 4.1. Yoo et al.

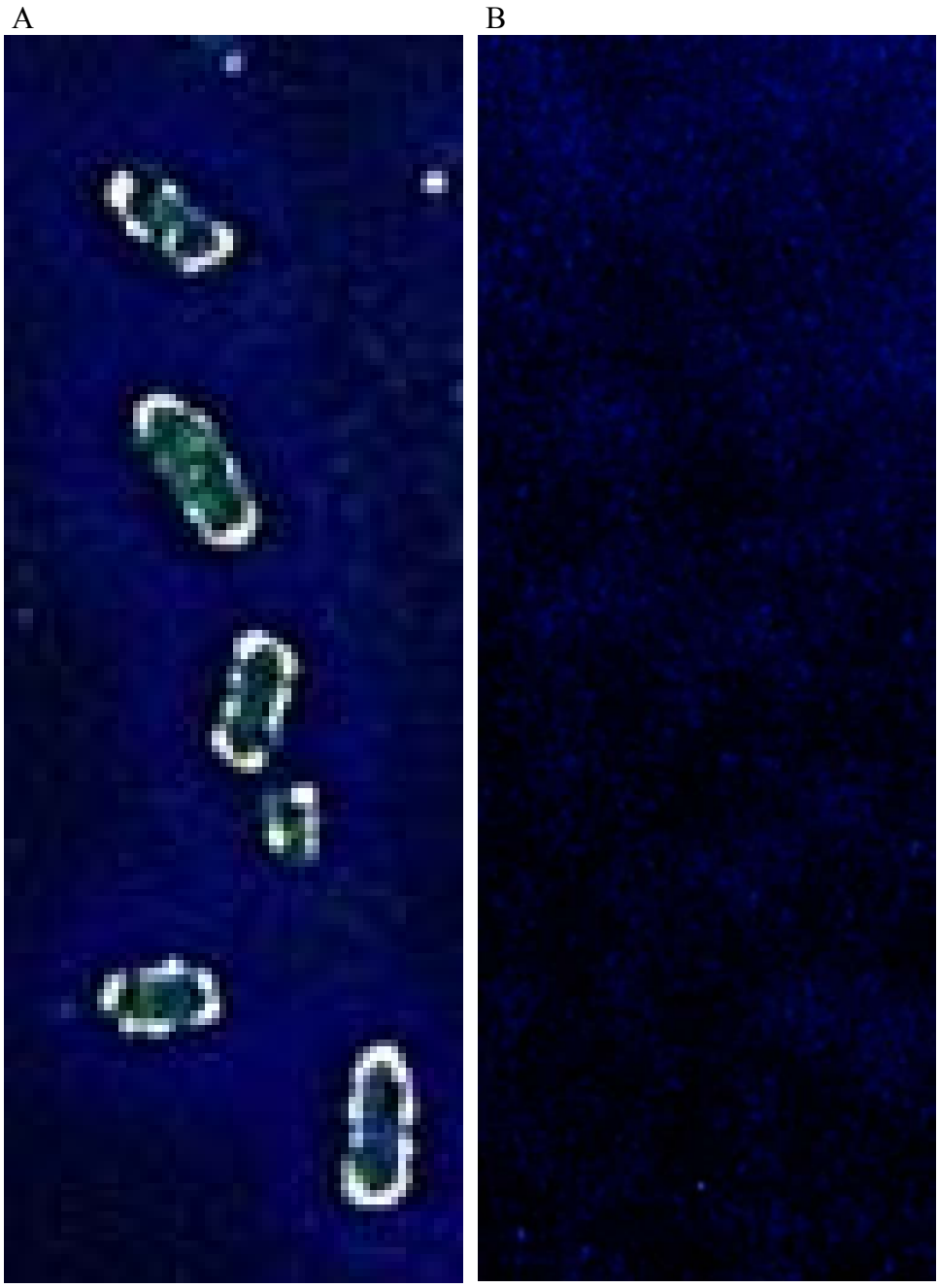

FIG. 4.2. Yoo et al.

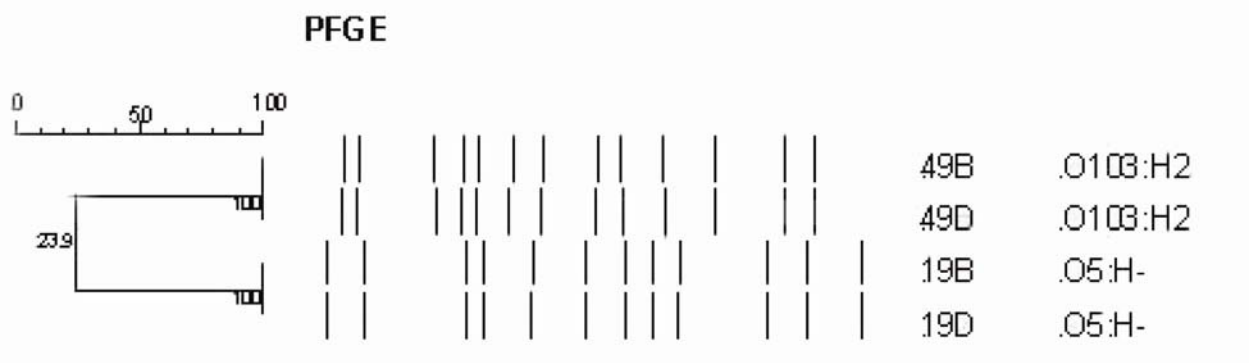

FIG. 4.3. Yoo et al.

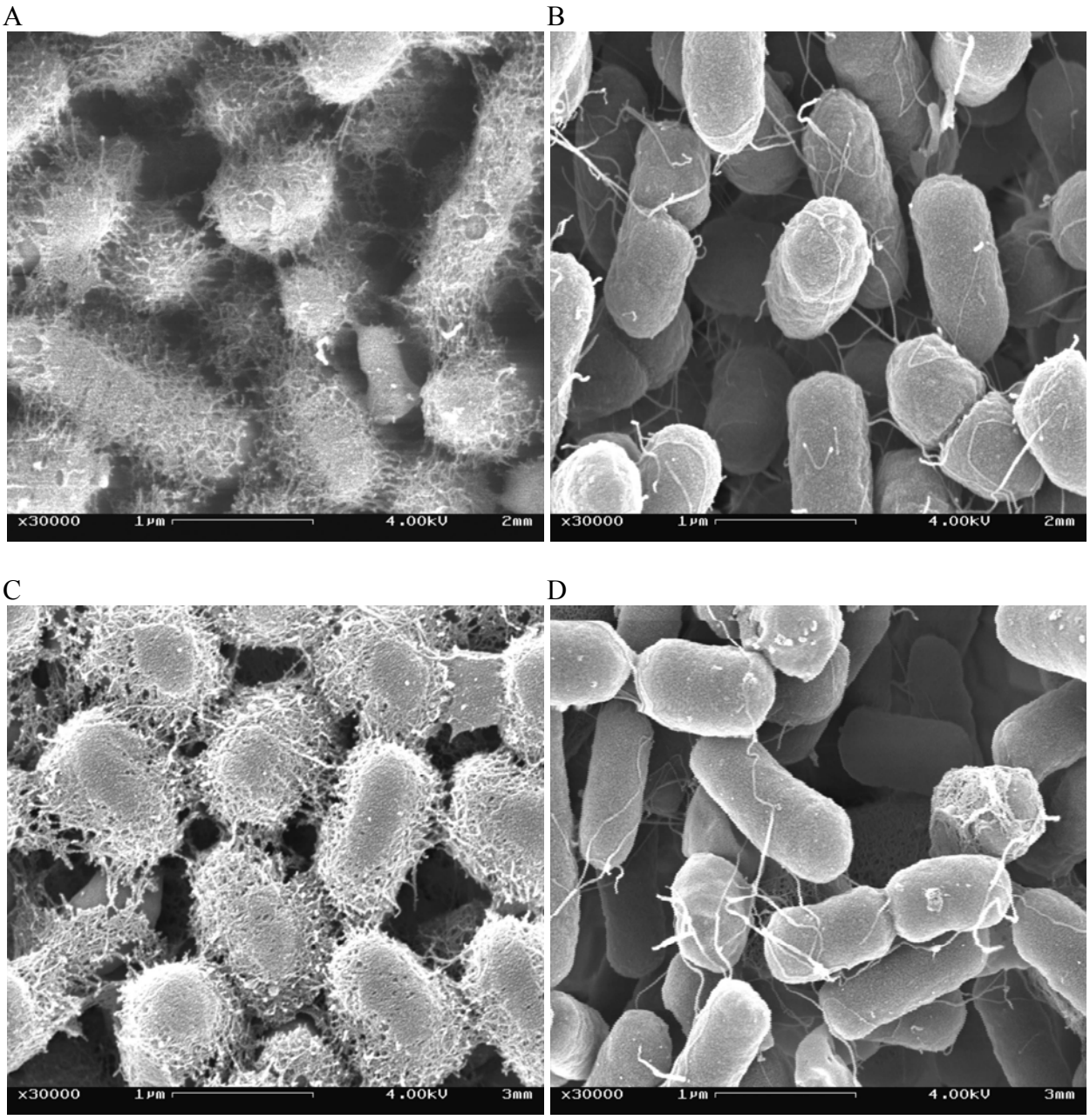

FIG. 4.4. Yoo et al.

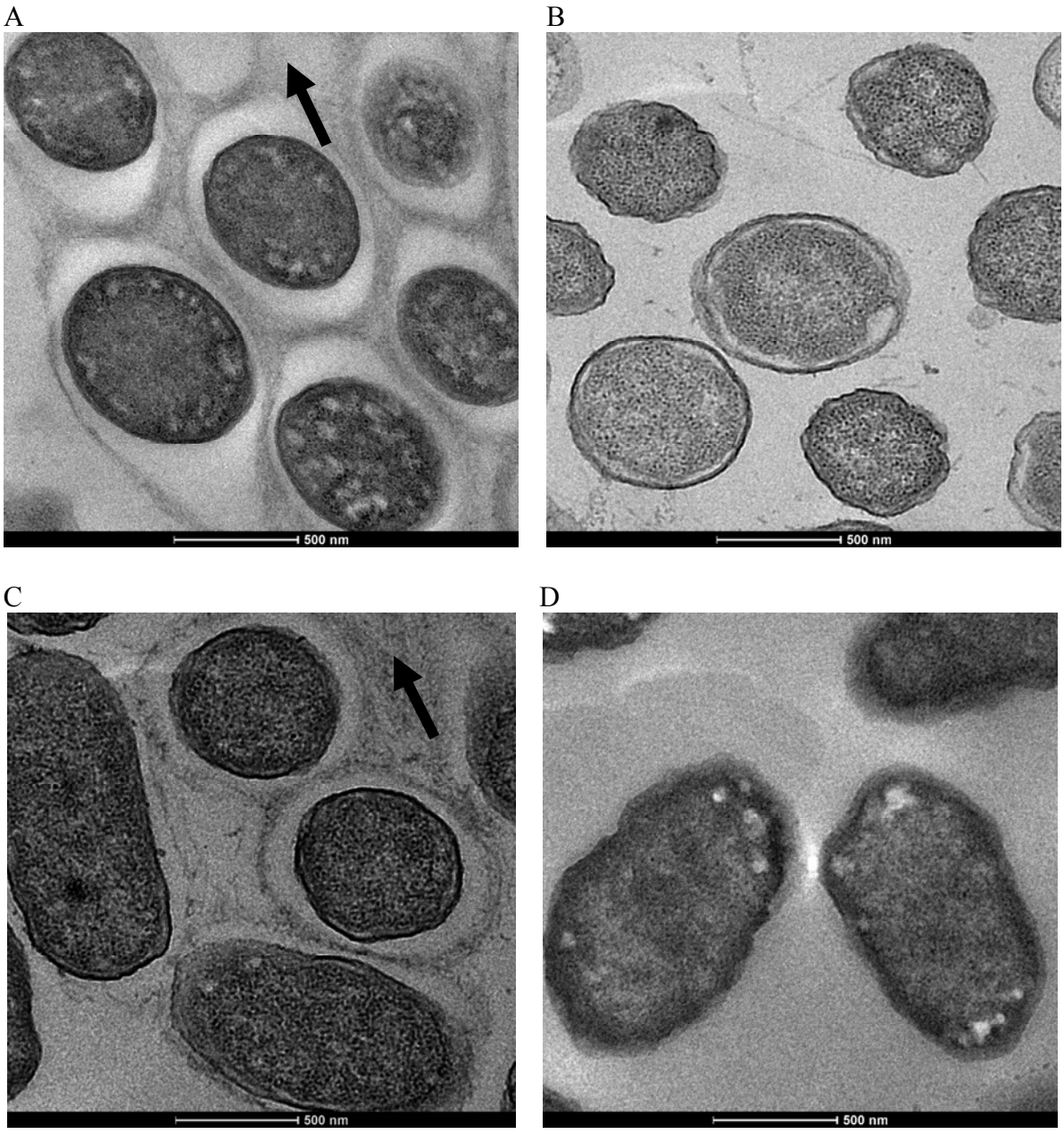

FIG. 4.5. Yoo et al.

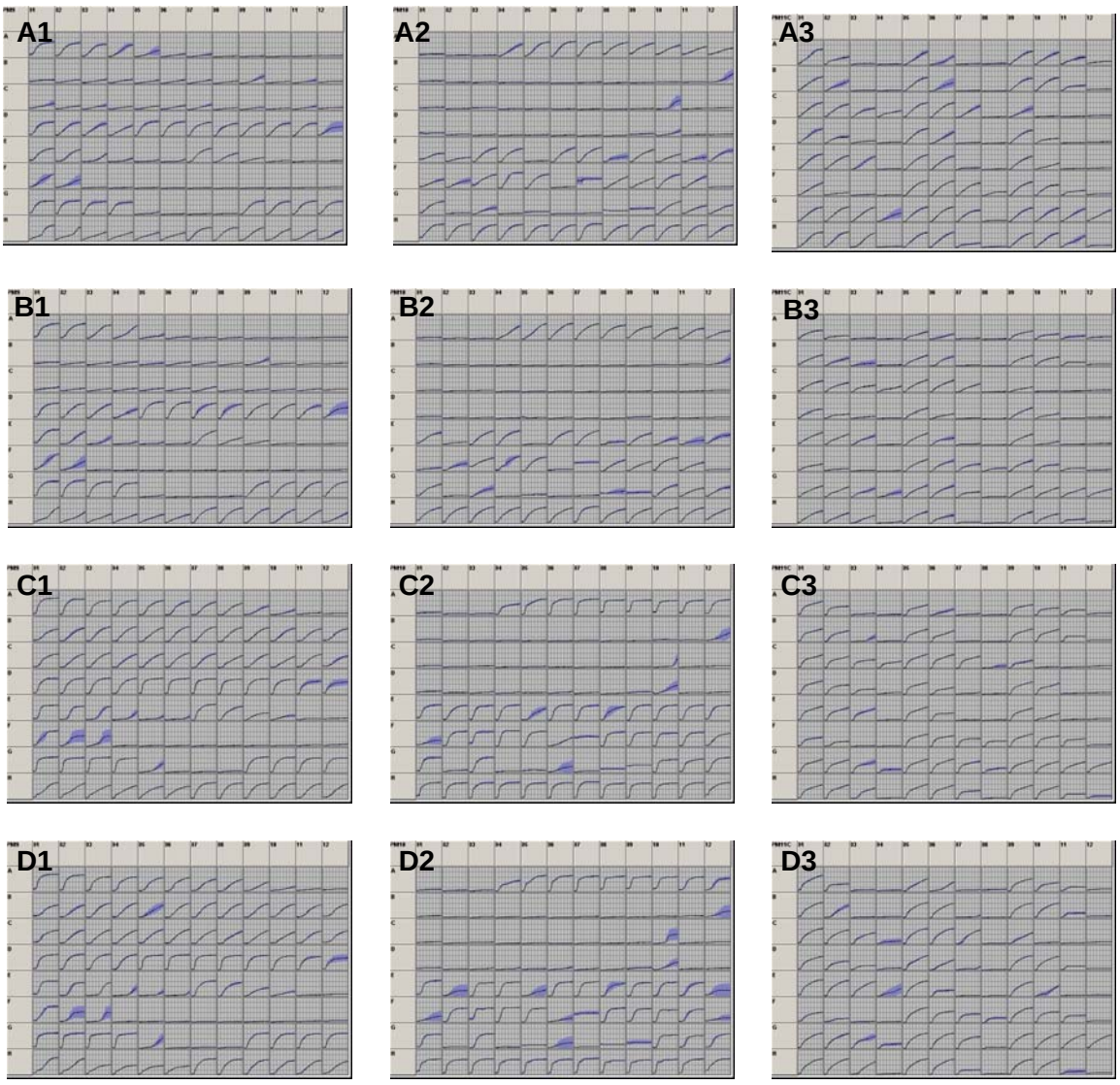

FIG. 4.6. Yoo et al.

CHAPTER 5

ROLE OF EXOPOLYSACCHARIDES IN PROTECTING THE CELLS OF SHIGA TOXIN PRODUCING *ESCHERICHIA COLI* AGAINST STRESS [1]

[1] B.Yoo and J. Chen. To be submitted to *International Journal of Food Microbiology*

ABSTRACT

Cellulose and/or the exopolysaccharide (EPS) comprised of colanic acid (CA) (EPS-CA) are produced by some strains of Shiga toxin producing *Escherichia coli* (STEC). Previous studies have shown that cells of STEC producing the EPS-CA have greater tolerance to adverse environmental conditions. To define the role of cellulose, and cellulose in conjunction with the EPS-CA in protecting the cells of STEC against environmental stresses, STEC cells, producing cellulose (Cel+CA-) or cellulose and the EPS-CA (Cel+CA+), and their cellulose deficient mutants (Cel-CA- or Cel-CA+) were subjected to oxidative (10, 20 and 30 mM H_2O_2), osmotic (1, 2 and 3 M NaCl) and acidic (pH 3.0, 3.5 and 4.5) stress as well as chlorine treatment (25, 50 and 100 µg/ml NaOCl). Approximately 4.25, 2.46 or 1.74 log CFU/ml of the Cel+CA- cells survived a 2.5-h treatments with 10, 20 and 30 mM H_2O_2, respectively, while none of the Cel-CA- cells (8.02 logs) survived the same treatments. The population of Cel-CA+ decreased from 7.70 to <0.6 log CFU/ml, whereas the population of Cel+CA+ decreased from 7.70 to 2.47 log CFU/ml by a 2-h treatment with 30 mM H_2O_2. Approximately 2.63 log CFU/ml of the Cel+CA- cells survived at pH 3.0 for 1 h and 4.75 log CFU/ml of the Cel+CA+ cells survived at pH 3.0 for 1.5 h, but the Cel-CA- and Cel-CA+ cells did not survive these treatments. Approximately 4.95, 4.28 or 3.81 log CFU/ml of the Cel+CA- cells survived a 5-min treatment with 25, 50 and 100 µg/ml of NaOCl, respectively, and none of the Cel-CA- cells (8.03 logs) survived these treatments. The population of Cel-CA+ decreased from 7.98 to 0.47 log CFU/ml, while the population of Cel+CA+ decreased from 7.98 to 2.62 log CFU/ml by a 10-min treatment with 100 µg/ml of NaOCl. Cells of EPS-CA producing STEC (Cel-CA+ and Cel+CA+) were significantly more persistent ($P < 0.05$) to 1 h treatments with 30 mM H_2O_2, a 1.5 h treatment at pH 3.0, and at all sampling intervals during treatments with 25 µg/ml of NaOCl in comparison to

the cells of EPS-CA deficient STEC (Cel+CA- and Cel-CA-). The results of the study indicated that cellulose and cellulose in conjunction with the EPS-CA protected STEC cells against oxidative treatments with 10-30 mM of H_2O_2, chlorine treatments with 50-100 μg/ml of NaOCl and acidic treatments at pH 3.0-3.5. The protections of the EPS against osmotic stress were also observed, but not without exceptions.

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is one of the most important groups of bacterial pathogens in the United States as well as many other countries in the world. The pathogen causes severe gastroenteritis that may progress to hemorrhagic colitis and hemolytic uremic syndrome in individuals with compromised immune systems.

As many other bacteria, STEC can live as individual, free floating cells in suspensions (planktonic) or attach to solid surfaces forming biofilms (sessile) (Chmielewski and Frank 2003). The biofilms are a great concern of the food industry because they offer physical, mechanical, and biological protection to bacterial cells. Exopolysaccharides (EPS) have been shown to play a structural role in the formation of biofilms (Branda et al. 2005). Cells of *E. coli* have diverse composition of EPS. Among these, only a few including cellulose and the polysaccharide comprised of colanic acid (CA) (EPS-CA), are better studied. The EPS-CA is a negatively charged polymer of glucose, galactose, fucose and glucuronic acid Cellulose is nevertheless, a long chain glucose polymer with β-1, 4 glycosidic bonds. Previous studies have shown that cells producing the EPS-CA have a greater tolerance to adverse environmental conditions such as oxidative and osmotic stresses (Chen et al. 2004).

Cellulose and cellulose in conjunction with the EPS-CA were hypothesized in this study to play a role in protecting the cells of STEC against oxidative, osmotic and acid stress as well as chlorine treatment. To test this hypothesis, the survival of STEC cells, which are capable of producing cellulose or both cellulose and the EPS-CA, was compared to the survival of their respective cellulose deficient strains under various adverse environmental conditions.

2. Materials and methods

2.1. STEC strains and their cellulose deficient mutants

The STEC strains used in the study were from our laboratory culture collections. Strain 49B and 19B were wild type cellulose producers, while strain 49D and 19D were the spontaneous cellulose deficient derivates selected from their respective parent cells (Yoo and Chen, Unpublished). Strain 49B and 49D produced the EPS-CA on minimal glucose agar (MGA) at 22°C whereas strain 19B and 19D did not produce the EPS. The designations of the four STEC strains were as follows: 19B (Cel+CA-), 19D (Cel-CA-), 49B (Cel+CA+) and 49D (Cel-CA+).

2.2. Culture growth and preparation

The STEC cultures were grown on Luria Bertani no salt (LBNS) agar (pH 6) supplemented with 2% ethanol at 28^{o}C for 96 h under aerobic condition. These conditions enhance cellulose production by selected STEC strains in a previous study conducted by our laboratory (Yoo and Chen, unpublished). The ingredients of the LBNS agar included 1% (w/v) Bacto tryptone (Beckton, Dickinson and Co., Sparks, MD), 0.5% (w/v) yeast extract (Beckton, Dickinson and Co., Sparks, MD) and 1.5% (w/v) agar (Acros Organics, Morris Plains, NJ). Each of the STEC cultures on the LBNS agar with 2% ethanol was harvested with 10 ml of phosphate buffered saline (PBS, pH 7.4) by gentle agitation at 20 × *g* for 25 min at room temperature using an Orbit shaker (Lab-line Instruments Inc., Melrose Park, IL) followed by gently scraping the cultures off the agar plates. The cell suspensions were collected, and the populations of cells in the suspensions were estimated by determining the plate counts so that approximately equal numbers of each type of cells would be used in the experiments.

2.3. Survival under oxidative stress

Hydrogen peroxide solutions were prepared by adding 0, 11.5, 23, and 34.5 µl of 30% H_2O_2 (Fisher Scientific, Fair Lawn, NJ) into 10 ml of PBS, respectively, in order to obtain a final H_2O_2 concentration of 0, 10, 20 or 30 mM. Cells of strain 19B, 19D, 49B and 49D were inoculated separately into each of the prepared H_2O_2 solutions (10 ml) at a final cell concentration of ca. 10^8 CFU/ml, and mixed thoroughly. The treatments were conducted at 22°C for 3 h. Samples were drawn in a 30-min interval. At each sampling point, 1 ml of each cell suspension was taken, and serially diluted in PBS. Three appropriate dilutions (0.1 ml) of each tested sample as well as the undiluted aliquots (0.25 ml), when appropriate, were plated in duplicate on tryptic soy agar plates using the Autoplate® 4000 (Spiral Biotech, Norwood, MA). The inoculated plates were incubated for 48 h at 37°C before colonies were enumerated.

2.4. Survival under osmotic stress

Sodium chloride solutions were prepared by dissolving 0, 0.58, 1.17 and 1.75 g of NaCl (Fisher Scientific) in 10 ml of PBS, respectively in order to obtain a final salt concentration of 0, 1, 2 or 3 M. Cells of strain 19B, 19D, 49B and 49D were inoculated separately into each of the prepared NaCl solutions as described above. The experiments were conducted at 22°C for a period of 48 h. Samples were drawn at 3, 6, 9, 12, 24, 36 and 48 h. The populations of 19B, 19D, 49B and 49D were determined using the methodology described above.

2.5. Survival under acidic stress

LBNS broth was acidified to pH 4.5, 3.5 or 3.0 using glacial acetic acid (99.5-100%; J. T. Baker, Phillipsburg, NJ). The same broth with pH 7.4 was used as a control. The acidified LBNS broth was sterilized using 0.45-µm Corning filter units (Corning, Corning, NY). Cells of strain 19B, 19D, 49B and 49D were inoculated into the acidified LBNS broth as described above.

The inoculated broth with pH 4.5 and 3.5 was incubated at 22°C for 48 h, and the broth with pH 3.0 was incubated at the same temperature for 3 h. Samples were drawn at 3, 6, 9, 12, 24 and 48 h from the LBNS broth with pH 4.5 and 3.5, whereas those in the LBNS broth with pH 3.0 were drawn in a 30-min interval. Populations of 19B, 19D, 49B and 49D were determined using the methodology described above.

2.6. Survival under treatment with sodium hypochlorite

Chlorine solutions were prepared by diluting 5% sodium hypochlorite (Fisher Scientific) with 10 ml of saline solution to obtain a final chlorine concentration of 0, 25, 50 or 100 μg/ml. The pH of the solutions was adjusted to 6.8 using 1 N NaOH. Cells of 19B, 19D, 49B and 49D were inoculated separately into each of the prepared chlorine solutions using the protocol outlined above. The treatments were conducted at 22°C for 10 min. At 1, 3, 5 and 10 min treatment intervals, 2 ml of the treated STEC cultures were drawn and added to 2 ml of Dey-Engley neutralizing broth (Fisher Scientific). The populations of 19B, 19D, 49B and 49D were determined using the aforementioned methodology.

2.7. Statistical analyses

Each experiment conducted in the study was repeated for three times. Data collected from the study were analyzed using the Student *t* test of the Statistical Analysis Software (SAS Institute Inc., Cary, NC). Significance of differences in survival between each pair of the STEC strains under various treatments was determined based on a 95% confidence interval.

3. Results and discussions

3.1. Survival of STEC under oxidative stress

The populations of Cel+CA+ and Cel+CA- were either significantly ($P < 0.05$) or insignificantly ($P > 0.05$) higher than those of Cel-CA+ and Cel-CA-, respectively under the treatments with all three concentrations of H_2O_2 except for the samples treated with 10 mM H_2O_2 for 0.5 h (Table 5.1). When treated with 30 mM H_2O_2 for 0.5-1.5 h, the population of Cel+CA+ was significantly higher than the population of other three cultures tested in this study ($P < 0.05$). In comparison, the populations of Cel-CA- was the lowest among the four STEC cultures tested in the study starting from this sampling point till 2.5 h treatment with 10 and 20 mM of H_2O_2. From the 1.5 to 3.0 h sampling point, the average populations of the four STEC strains was in the order of Cel+CA+, Cel+CA-, Cel-CA+ and Cel-CA-. The population of Cel-CA- dropped to the undetectable level (<4 CFU/ml) after a 2.5 h treatment with 10 mM H_2O_2. In comparison, an approximate of 4.25 log CFU/ml of the Cel+CA- cells survived at the end of the same treatment.

The populations of Cel-CA- dropped to the undetectable level while an approximate of 4.63 log CFU/ml of Cel+CA- survived a 1.5 h treatment with 20 mM H_2O. Cellulose deficient cells were relatively more susceptible to the treatments with an increasing concentration of H_2O_2. A 0.5 h treatment with 30 mM H_2O_2 reduced the population of Cel-CA- by 5.73 logs compared to a 2.70 log reduction in the population of Cel+CA-. Extending the treatment with 30 mM H_2O_2 to 1 h reduced the population of Cel-CA- to the undetectable level. The Cel+CA- survived a 3 h treatment with 30 mM H_2O_2. Population of Cel+CA+ was 1.36 log CFU/ml after the 3 h exposure to 30 mM H_2O_2.

Similar to the cellulose producing cells, the EPS-CA producing cells were relatively more persistent ($P < 0.05$) to the oxidative stress than those that did not produce the EPS. An average

of 5.21, 5.28 and 3.63 log CFU/ml of the EPS-CA deficient cells (Cel+CA- and Cel-CA-) survived a 1 h treatment with 10 mM of H_2O_2 and 0.5 h treatment with 20 mM and 30 mM of H_2O_2, respectively. At the same sampling points, an average of 6.03, 6.20 and 5.78 log CFU/ml of the EPS-CA producing cells (Cel+CA+ and Cel-CA+) survived the respective treatments. Longer treatments with all the three concentrations of H_2O_2 significantly improved the rates of inactivation against all four STEC cultures used in the study.

The H_2O_2 and superoxide anions are bactericidal reactive oxygen intermediates which inactivates bacterial cells by reducing the availability of oxygen during electron transport in aerobic metabolism (Gonzalez-Flecha and Demple 1995). The reactive oxygen intermediates also cause damages to cellular DNA as well as proteins on bacterial cell membranes (Farr and Kogoma 1991). To our best knowledge, there have been no studies addressing the role of cellulose in protecting bacterial cells against oxidative stresses. However, a similar feature of the EPS-CA has been reported. The EPS-CA producing cells of *E. coli* O157:H7 were found to be more resistant to treatments with H_2O_2 than were the EPS deficient cells (Chen et al. 2004). It was hypothesized that the EPS-CA, as a negatively charged polyanion functions as a buffered shield to interrupt the accumulation of reactive oxygen molecules on the surface of *E. coli* cells (Hanna et al. 2003). Cellulose could be nevertheless, oxidized to certain degrees by oxygen or hydrogen peroxide to yield a short chain cellulose which has a lower degree of polymerization compared to a long chain cellulose molecule (Belasheva et al. 1973). To oxidize each glucose unit in the cellulose molecule, 1-1.5 unit(s) of oxygen atoms is/are consumed (Nevell 1985b). With this consumption, the amounts of reactive oxygen species available for inactivating the cells of STEC would be significantly reduced.

3.2. Survival of STEC under osmotic stress

The populations of the Cel-CA- decreased as the osmotic stress treatments progressed (Table 5.2). Significant ($P < 0.05$) differences in the populations of Cel-CA- and Cel+CA- were observed at the 24, 36 and 48 h sampling points under treatments with 1 M NaCl, at the 9-48 h sampling points under treatments with 2 M NaCl, and at the 9 h sampling point under 3 M NaCl. It was observed that 4.89, 3.27, and 4.08 log CFU/ml of the Cel-CA- cells survived the 48 h treatments with 1, 2 and 3 M NaCl, respectively. The population of the Cel+CA- also decreased as treatment time increased, and 6.24, 5.26 and 4.61 log CFU/ml of the Cel+CA- cells survived the 48 h treatment with 1, 2 and 3 M NaCl, respectively. The differences between the populations of Cel-CA- and those of Cel+CA- treated by 1 and 2 M of NaCl at the 48 h sampling point were significant ($P < 0.05$). The populations of the two types of cells resulted from the treatment with 3 M NaCl were not significantly different ($P > 0.05$). Significant ($P < 0.05$) differences between the population of Cel+CA+ and that of Cel-CA+ were observed occasionally throughout the treatments, i.e. at sampling point of 9, 36 and 48 h under treatments with 2 M NaCl, and of 3, 12, 36 and 48 h under treatments with 3 M NaCl.

Production of the EPS-CA did not seem to significantly alter the fates of STEC cells under treatments with NaCl. Even after a 48 h treatment, the populations of EPS-CA proficient cells were not significantly different ($P > 0.05$) from those of EPS-CA deficient cells, with the differences between the two types of cells being 0.27 logs with treatment by 1 M NaCl, 0.19 logs by 2 M NaCl, and 0.28 logs by 3 M NaCl, respectively. Longer treatment time with 2 and 3 M NaCl significantly ($P < 0.05$) improve the rates of inactivation. Under treatment with 1 M NaCl however, only the inactivation rates of Cel-CA- and Cel-CA+ were significantly ($P < 0.05$) improved by the prolonged treatments.

Elevated osmotic pressure in the environment leads to dehydration of bacterial cells due to osmotic gradients (Beney et al. 2004). As a result, cell metabolic activities are inhibited because of the migration of water from cell interior to the exterior environment. Cell membranes can be damaged, and eventually ruptured due to mechanical limitations caused by shrinkage of cell volume (Beney et al. 2004). Microbial cellulose is able to hold up water more than 100 times its weight (Ross et al. 1991). Therefore, it could reserve water for bacterial cells in the high osmotic environment. In addition, cellulose may increase the viscosity of free water by slowing down water movement across cell membrane and stimulate water absorption of bacterial cells (Takahashi et al. 2005).

Previous studies have shown that the EPS-CA affected the survival of *E. coli* O157:H7 under desiccation (Obadia et al. 2007) and osmotic stress (Chen et al. 2004). However, production of the EPS did not seem to significantly enhance the survival of EPS-CA proficient cells under osmotic stress in the present study. The differences in these findings could be caused by the types of bacterial cultures used in these studies. The study by Chen et al. (2004) used a wild type *E. coli* O157:H7 strain and its knock off mutant which was deficient in EPS-CA production. The difference in EPS-CA production in the present study on the other hand, were basically between the two original wild type STEC strains 49 and 19.

3.3. Survival of STEC under acidic stress

The populations of cellulose deficient and cellulose proficient cells were not significantly ($P > 0.05$) different at pH 4.5, except at the 6 and 48 h sampling points (Table 5.3). At this particular pH, the surviving populations of all tested STEC cultures were between 6.77 and 7.10 logs after a 48 h treatment. At pH 3.5, the cell populations of all tested STEC cultures decreased as treatment time increased, and the difference between the populations of cellulose deficient

cells and cellulose proficient cells became significant ($P < 0.05$) after a 3 h exposure, and this trend persisted till the 24 h sampling point. The cell population of Cel-CA- decreased to the undetectable level after a 24 h treatment, but the Cel+CA- population was 3.13 log CFU/ml at the same sampling interval.

The population of the Cel+CA- was significantly higher ($P < 0.05$) than that of the Cel-CA- after a 0.5-1.0 h treatment at pH 3.0. The population of the Cel+CA+ was significantly higher ($P < 0.05$) than that of Cel-CA+ after a 0.5-2.5 h treatment at the same pH (Table 5.4). The population of 2.63 log CFU/ml of the Cel+CA- cells survived the 1 h treatment at pH 3.0, while the Cel-CA- cells did not survive the same treatment. Similarly, 4.75 log CFU/ml of the Cel+CA+ cells survived the 1.5 h treatment at pH 3.0, but Cel-CA+ cells became undetectable by the same treatment. The cells of Cel+CA+ and Cel-CA+ were relatively more resistant than those of Cel+CA- and Cel-CA-, respectively at 0.5-2.0 h sampling points at pH 3.0. Approximately 3.10 log CFU/ml of the Cel+CA+ cells survived the 2.5 h treatment, while the other types of cells were undetectable after the same treatment.

The antimicrobial effect of organic acids is dependent on the dissociation constant (pKa) which determines the strength of an organic acid and the proportion of the undissociated (hydrophobic) form of the acid at a particular pH. Although pH 4.5 is lower than the pKa value of acetic acid, 4.75, the pH did not cause a significant reduction in cell populations of cellulose producing, vs. cellulose deficient strains of STEC. At lower pH levels such as 3.5 and 3.0, large portions of the acetic acid molecules are present in the undissociated form, the inhibitory effect of the acid to STEC cells as well as the protective role of cellulose were therefore, observed. Although the glycosidic linkage in cellulose molecule is sensitive to acid-catalyzed hydrolysis, but such a reaction only occurs in the presence of strong inorganic acid (Nevell 1985a). The

mechanisms of action of cellulose in protecting the cells of STEC are not fully understood. Since cellulose has both hydrophilic and hydrophobic moieties, it may cause changes in cell surface charge through electrostatic impulsion or hydrophobic repulsion.

Different from cellulose, the negatively charged EPS-CA could neutralize the protons in the acidic environment (Hanna et al. 2003). Furthermore, the EPS could make the surface of a STEC cell more hydrophilic. This may hinder the hydrophobic association of the undissociated acetic acid molecules with the phospholipid bilayer of *E. coli* cell membranes (Barua et al. 2002; Srividhya et al. 2006; Kumar et al. 2008). At pH under its pKa value, acetic acid is in the undissociated form and thus hydrophobic. Without hydrophilic hindrance such as that of the EPS-CA, the undissociated acetic acid molecule can easily penetrate cell membranes, and dissociates into ionic forms in the cytoplasm of bacteria. With the interference of EPS-CA on the surface of STEC cells, the access of undissociated acetic acid across cell membranes would be greatly reduced.

3.4. Survival of STEC under treatment with sodium hypochlorite

Cellulose deficient cells were significantly more vulnerable to chlorine treatment ($P < 0.05$) than their cellulose producing counterparts with only one exception, i.e., after 1 min treatment with 50 µg/ml sodium chlorite (Table 5.5). The Cel+CA- cells were significantly more resistant ($P < 0.05$) to the chlorine treatments than the Cel-CA- cells at all sampling intervals. The difference in the populations of Cel+CA- and Cel-CA- cells was 1.39 logs at 1 min after the treatment with 25 µg/ml sodium hypochlorite, and the difference increased to 3.55 logs when the treatment prolonged to 10 min. The greatest difference between populations of the two types of cells was 4.13 logs, which was observed in samples taken after a 3 min treatment with 50 µg/ml sodium hypochlorite. Similarly, the Cel+CA+ cells were significantly more resistant ($P < 0.05$)

than the Cel-CA+ cells to a 10 min treatment with 25 μg/ml of sodium hypochlorite, a 5 min treatment with 50 μg/ml of sodium hypochlorite, and a 3 min treatment with 100 μg/ml of sodium hypochlorite. The populations of Cel+CA+ and Cel-CA+ cells in samples taken before these intervals were not significantly different ($P > 0.05$). The greatest difference between the populations of Cel+CA+ and Cel-CA+ cells, 2.50 logs, was observed in samples taken after a 5 min treatment with 100 μg/ml of sodium hypochlorite.

The EPS-CA producing cells were significantly less vulnerable ($P < 0.05$) than the EPS-CA deficient cells to treatments with 25 μg/ml of sodium hypochlorite at all sampling intervals, 50 μg/ml of sodium hypochlorite after the 3 and 5 min sampling intervals, and 100 μg/ml of sodium hypochlorite after the 3 min sampling interval. The populations of the cells producing the EPS-CA were not significantly ($P > 0.05$) different from those of the cells deficient in the production of the EPS-CA at 1 and 10 min treatments with 50 μg/ml and 100 μg/ml of sodium hypochlorite, respectively.

In the present study, the Cel-CA- cells were inactivated by a shorter than 5-min treatment with more than or equal to 25 μg/ml of sodium hypochlorite. In comparison, 3.25 log CFU/ml of Cel+CA- cells survived a 10-min treatment with 100 μg/ml of sodium hypochlorite. White et al. (2006) found that the populations of wild type, cellulose producing *S*. Typhimurium strains were 4 logs higher than those of the *S.* Typhimurium strains with a *bcsA* deletion after a 20-min exposure to 60 μg/ml of sodium hypochlorite. Solano et al. (2002) reported that cellulose producing *S.* Enteritidis strains were more resistant than cellulose deficient mutants to a 20-min treatment with 30 μg/ml of chlorine. Scher et al. (2005) suggested that cellulose formed by *S.* Typhimurium could react with sodium hypochlorite, and neutralize the available chlorine. Epstein and Lewin (1962) indicated that the undissociated hypochlorous acid and dissociated

hypochlorite ions might directly react with cellulose by oxidizing the carbonyl groups on a cellulose molecule to form oxidized cellulose, which could reduce the amount of active chlorines for bacterial inactivation

It has been reported that sodium hypochlorite inactivates bacterial cells through depleting ATP by modification of the proton-translocating ATP synthase (Barrette et al. 1987), inhibiting *oriC* mediated DNA replication (Rosen et al. 1998) and sulfhydryl enzymes involved in glycolysis (Knox et al. 1948), as well as destroying bacterial membranes (Nakajima et al. 2004; Hurst et al. 1991). To avoid the damaging effects of sodium hypochlorite to cell membrane and metabolism, the polyanionic EPS-CA limits the penetration of active chlorine to bacterial cells by slowing down diffusion or through chemical reaction between the EPS-CA and reactive hypochlorite ions (De Beer et al. 1994; Chen and Stewart 1996; Stewart et al. 2001)

The overall results of the present study indicate that cellulose and cellulose in conjunction with the EPS-CA protected STEC cells against oxidative stress with 10, 20 and 30 mM of H_2O_2, chlorine treatments with 50 and 100 μg/ml of NaOCl and acidic treatments at pH lower than or equal to 3.5. The protections exerted by cellulose or cellulose in conjunction with the EPS-CA to osmotic stress were also observed, but not without exceptions. These protective effects explain the extraordinary surviving abilities of STEC under adverse environmental conditions.

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Table 5.1. Average (n = 3) cell populations (log CFU/ml) of STEC after treatments with various concentrations of H_2O_2

Conc. of H_2O_2	Strains of STEC	Populations of STEC (log CFU/ml) at Different Sampling Point (h)					
		0.5	1	1.5	2	2.5	3
0 mM	Cel+CA-	6.92ab[a]A[b]	6.88abA	6.76aA	6.91aA	6.84aA	6.93aA
	Cel-CA-	7.27aA	7.28aA	7.19aA	7.37aA	7.29aA	7.32aA
	Cel+CA+	6.97abA	6.73abAB	6.84aAB	6.61aB	6.52aB	6.63aAB
	Cel-CA+	7.05abA	6.90abA	6.80aA	7.07aA	7.00aA	7.14aA
10 mM	Cel+CA-	6.25cdA	5.76cdAB	5.22bBC	4.64bcCD	4.25bcDE	3.63bcE
	Cel-CA-	6.59bcA	4.65eB	1.23deC	1.15ghC	<[c]0.60fC	<0.60eC
	Cel+CA+	6.54bcA	6.26bcA	5.47bB	5.24bB	4.72bBC	4.36bC
	Cel-CA+	6.62bcA	5.79cdA	4.64bB	3.67cdBC	3.14cdC	2.65cdC
20 mM	Cel+CA-	6.13cdA	5.24deAB	4.63bAB	3.44deBC	2.46deC	2.42cdC
	Cel-CA-	4.43fA	1.30gB	<0.60eB	<0.60hB	<0.60fB	<0.60eB
	Cel+CA+	6.65bcA	5.92cdA	4.84bB	4.08cdB	3.03cdeC	2.39cdC
	Cel-CA+	5.75dA	3.21fB	2.65cB	2.44efB	2.21deB	1.74deB
30 mM	Cel+CA-	4.96efA	3.50fB	1.63dC	1.76fgC	1.74efC	<0.60eC
	Cel-CA-	2.29gA	<0.60gB	<0.60eB	<0.60hB	<0.60fB	<0.60eB
	Cel+CA+	6.51bcA	4.95eB	2.84cC	2.47efC	1.73efD	1.36deD
	Cel-CA+	5.04eA	3.70fB	0.63eC	<0.60hC	<0.60fC	<0.60eC

[a]Means followed by the same lowercase letter in the same column are not significantly different ($P > 0.05$).
[b]Means followed by the same uppercase letter in the row are not significantly different ($P > 0.05$).
[c]Detection limit is 0.60 log CFU/ml.

Table 5.2. Average (n = 3) populations (log CFU/ml) of STEC after treatment with various concentrations of NaCl

Conc. of NaCl	Strains of STEC	Populations of STEC (log CFU/ml) at Different Sampling Point (h)						
		3	6	9	12	24	36	48
0 M	Cel+CA-	6.63bcd[a]BC[b]	6.50bcdC	6.52abcC	6.59abcdeBC	6.81aABC	6.87abAB	6.99aA
	Cel-CA-	7.25aA	7.18aA	7.08aA	7.13abA	7.12aA	7.15abA	7.24aA
	Cel+CA+	6.82abcdA	6.94abA	6.77abA	6.87abcA	6.73abA	6.94abA	6.92abA
	Cel-CA+	7.03abA	7.02abA	7.09aA	7.30aA	7.16aA	7.40aA	7.47aA
1 M	Cel+CA-	6.58bcdeAB	6.55bcAB	6.64abcAB	6.75abcdA	6.62abAB	6.53bcAB	6.24bcB
	Cel-CA-	6.67bcdA	6.75abcA	6.38bcdAB	6.51bcdeAB	5.79cBC	5.23efCD	4.89deD
	Cel+CA+	6.66bcdA	6.62abcA	6.63abcA	6.40bcdefA	6.61abA	6.41bcA	6.15cA
	Cel-CA+	6.52bcdeA	6.54bcA	6.91abA	6.06defghAB	6.01bcAB	6.00cdAB	5.52cdB
2 M	Cel+CA-	6.88abcA	5.90defB	6.11cdeB	5.90efghB	5.56cdBC	5.28defC	5.26deC
	Cel-CA-	6.40cdeA	5.53fAB	5.34fAB	5.09ijB	4.51fBC	3.82iCD	3.27gD
	Cel+CA+	6.94abcA	5.80efBCD	6.69abAB	6.20cdefgABC	5.68cdBCD	5.52deCD	4.93deD
	Cel-CA+	6.79abcdA	6.15cdeAB	5.93deB	5.69fghiBC	4.98defCD	4.61fghDE	3.99fgE
3 M	Cel+CA-	6.30defA	5.87efAB	5.91deAB	5.46hiBC	5.32cdeBC	4.94efgCD	4.61efD
	Cel-CA-	5.81fA	5.66efA	5.24fA	5.47ghiA	5.28cdefA	4.20ghiB	4.08fB
	Cel+CA+	6.80abcdA	5.93defB	5.95deB	6.01defghB	5.53cdeBC	5.15efC	5.15deC
	Cel-CA+	6.04efA	5.79efA	5.64efAB	4.45jC	4.77efBC	4.00hiC	4.10fC

[a]Means followed by the same lowercase letter in the same column are not significantly different ($P > 0.05$).
[b]Means followed by the same uppercase letter in the row are not significantly different ($P > 0.05$).

Table 5.3. Average (n = 3) populations (log CFU/ml) of STEC after treatment at various pH levels

pH	Strains of STEC	Populations of STEC (log CFU/ml) at Different Sampling Point (h)					
		3	6	9	12	24	48
7.4	Cel+CA-	7.09bcd[a]BC[b]	7.00cdC	7.24abA	7.27abA	7.21abAB	7.23abAB
	Cel-CA-	7.34aAB	7.38aA	7.39aA	7.35aAB	7.26aB	7.38aA
	Cel+CA+	6.97deB	7.18bA	7.03bcdAB	7.11abAB	7.21abA	7.21abA
	Cel-CA+	7.18abcdAB	7.02cdB	7.25abA	7.19abAB	7.25aA	7.29aA
4.5	Cel+CA-	7.21abcA	6.89dBC	6.94cdBC	6.97abB	6.86cBC	6.77dC
	Cel-CA-	7.28abA	7.14bcAB	7.12bcAB	7.10abB	7.05abcB	7.10bcB
	Cel+CA+	7.00cdeA	6.92dAB	6.86dBC	6.89bAB	6.82cBC	6.78dC
	Cel-CA+	7.19abcdA	6.96dAB	7.03bcdAB	6.98abAB	6.99bcAB	6.95cdB
3.5	Cel+CA-	5.96gA	5.46gB	4.95fC	4.36dD	3.13dE	<[c]0.60eF
	Cel-CA-	6.84eA	5.83fB	4.66gC	2.14fD	<0.60eE	<0.60eE
	Cel+CA+	6.81eA	6.06eB	5.61eC	5.14cD	3.25dE	<0.60eF
	Cel-CA+	6.56fA	5.69fB	4.23hC	2.78eD	0.63eE	<0.60eE

[a]Means followed by the same lowercase letter in the same column are not significantly different ($P > 0.05$).
[b]Means followed by the same uppercase letter in the row are not significantly different ($P > 0.05$).
[c]Detection limit is 0.60 log CFU/ml.

Table 5.4. Average (n = 3) populations (log CFU/ml) of STEC after treatment at pH 3

Strains of STEC	Populations of STEC (log CFU/ml) at Different Sampling Point (h)					
	0.5	1	1.5	2	2.5	3
Cel+CA-	4.13c[a]A[b]	2.63bB	<[c]0.60bC	<0.60bC	<0.60bC	<0.60aC
Cel-CA-	2.83dA	<0.60cB	<0.60bB	<0.60bB	<0.60bB	<0.60aB
Cel+CA+	6.86aA	5.58aB	4.75aBC	3.69aCD	3.10aD	<0.60aE
Cel-CA+	5.63bA	1.84bcB	<0.60bC	<0.60bC	<0.60bC	<0.60aC

[a]Means followed by the same lowercase letter in the same column are not significantly different ($P > 0.05$).
[b]Means followed by the same uppercase letter in the row are not significantly different ($P > 0.05$).
[c]Detection limit is 0.60 log CFU/ml.

Table 5.5. Average (n = 3) cell populations (log CFU/ml) of STEC after treatments with various concentrations of sodium hypochlorite

Conc. of NaOCl	Strains of STEC	Populations of STEC (log CFU/ml) at Different Sampling Point (min)			
		1	3	5	10
25 µg/ml	Cel+CA-	5.24bB	4.81bB	4.95bB	3.85cC
	Cel-CA-	3.85dfB	1.47fC	<[c]0.30gD	<0.30gD
	Cel+CA+	6.91aB	6.90aB	6.89aB	6.89aB
	Cel-CA+	6.81aB	6.60aB	6.65aB	6.22bB
50 µg/ml	Cel+CA-	4.60cB	4.43bcC	4.28cC	3.42cdD
	Cel-CA-	3.89deB	<0.30gC	<0.30gC	<0.30gC
	Cel+CA+	3.51eB	3.94cdB	4.17cB	3.52cdB
	Cel-CA+	4.18cdB	3.73deBC	3.24eC	1.88fD
100 µg/ml	Cel+CA-	4.45cB	3.98cdC	3.81cdC	3.25dD
	Cel-CA-	2.89fB	<0.30gC	<0.30gC	<0.30gC
	Cel+CA+	4.30cdB	4.01cdB	3.44deC	2.62eD
	Cel-CA+	3.80deB	3.20eB	0.94fC	0.47gC

[a]Means followed by the same lowercase letter in the same column are not significantly different ($P > 0.05$).
[b]Means followed by the same uppercase letter in the row are not significantly different ($P > 0.05$).
[c]Detection limit is 0.30 log CFU/ml.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The following is a summary of results and conclusions drawn from the research reported in Chapter 3-5 of this dissertation:

1. The influence of culture conditions and medium composition on the production of cellulose by selected Shiga toxin producing *E. coli* (STEC) strains was determined. Incubation temperatures (15, 22, 28 and 37^{o}C), incubation atmospheres (aerobic, microaerophilic and anaerobic), medium compositions (ethanol and fructose), medium pH (5.0, 6.0 and 7.0) and medium water activities (0.96, 0.97, 0.98 and 0.99) were evaluated. The culture conditions that favored the production of cellulose by the selected STEC included a 28^{o}C incubation temperature, aerobic atmosphere, and presence of 2% of ethanol in LBNS agar with a pH value of 6.0 and a water activity of 0.99. These findings will assist in formulating microbiological media useful for cellulose and biofilm research.

2. Spontaneous cellulose deficient mutants of STEC, 19D and 49D were isolated, and the isolated mutants and their respective parents, 19B and 49B were characterized using serotyping, scanning and transmission electron microscopy as well as pulsed field gel electrophoresis. The growth characteristics of the STEC strains were studied using the phenotypic microarray (PM) technology. The cellulose deficient mutants and their respective parents shared the same

serotypes and similar PFGE profiles. Profound morphological differences were however, found between the two types of cells. The overall results of PM indicated that 49B and 49D grew better ($P < 0.05$) than 19B and 19D in all three PM panels used in the study, probably due to the production of another polysaccharide by the former two strains. The growth of 19B *vs.* 19D and 49B *vs.* 49D were only significantly ($P < 0.05$) different in the presence of two antibiotics, lincomycin and ceftriaxone on the antibiotic panel. Among 90 tested substances on the osmolyte panel, strain 49D only had poorer ($P < 0.05$) growth than 49B in LBNS broth with 6% urea or 60, 80 and 100 mM sodium nitrite, respectively. In contrast to 49D, 19D had similar growth to 19B under these conditions. Among 96 tested acidulants on the pH panel, significant differences in the growth of 19B and 19D were only observed in LBNS broth with pH 4.5 and supplemented with three different amino acids or trimethylamine-N-oxide, and with pH 9.5 and supplemented with four different amino acids. Strain 49B grew significantly ($P < 0.05$) better than 49D only in LBNS broth with pH 9.5 and supplemented with five different amino acids. Additionally, 19B and 49B grew significantly ($P < 0.05$) better than 19D and 49D, respectively in LBNS broth supplemented with X-caprylate. These results suggest that the two members of each STEC pair shared similar growth characteristics except under extreme stress. These strains could be useful in investigating the role of cellulose in protecting the cells of STEC under adverse environmental conditions.

3. The roles of cellulose and cellulose in conjunction with the EPS-CA in protecting the cells of STEC against environmental stresses were investigated. STEC cells, producing cellulose (Cel+CA-) or cellulose and the EPS-CA (Cel+CA+), and their cellulose deficient mutants (Cel-CA- or Cel-CA+) were subjected to oxidative (10, 20 and 30 mM H_2O_2), osmotic (1, 2 and 3 M

NaCl) and acidic (pH 3.0, 3.5 and 4.5) stress as well as chlorine treatment (25, 50 and 100 µg/ml NaOCl). Cellulose and cellulose in conjunction with the EPS-CA protected STEC cells against oxidative stress with 10, 20 and 30 mM of H_2O_2, chlorine treatments with 50 and 100 µg/ml of NaOCl and acidic treatments at pH lower than or equal to 3.5. The protections exerted by cellulose or cellulose in conjunction with the EPS-CA to osmotic stress were also observed, but not without exceptions. These protective effects explain the extraordinary surviving ability of STEC under adverse environmental conditions.