ABSTRACT

Title of Dissertation / Thesis: STUDIES ON THE FORMATION, COMPOSITION AND DETECTION OF BIOFILMS OF FOOD-BORNE PATHOGENS

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Biofilms enable food-borne pathogens to resist removal from surfaces and survive disinfection. Biofilms of pathogens [e.g., *S. enterica* Serovar Typhimurium DT104 (STDT104)] formed on various surface types were probed with Calcofluor (β-D-glucan-specific) and lectins. All biofilms were detected after exposure to Calcofluor. Lectins bound to specific carbohydrates in bacterial exopolymeric substances (EPS). Results supported the *in vitro* use of Calcofluor and lectins as non-specific and genera-specific probes of biofilms. Next, the capsular polysaccharide (CP) of STDT104 was extracted and shown to contain glucose, mannose and trace amounts of galactose. Polyclonal antibodies against this extract were specific for a CP as demonstrated by western blots and immunoelectron microscopy. Confocal microscopy images revealed the following: thicker biofilms formed at 25°C, CP synthesis was not temperature-dependent and not produced by all cells. Synthesis of CP by STDT104 may represent an aggregative and protective substance in addition to curli and cellulose.

STUDIES ON THE FORMATION, COMPOSITION AND DETECTION OF BIOFILMS OF FOOD-BORNE PATHOGENS

By

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Dedication

I dedicate this thesis to Pamela M. Creed, my best friend. Thank you so much for

always helping me and being by my side.

Acknowledgements

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Chapter 1: **FOOD-BORNE PATHOGENS AND BIOFILMS**

The problem of food-borne pathogens

Food-borne diseases constitute a serious health threat as well as an economical burden. In the United States, food-borne pathogens may account for 6 million to 81 million cases of food-borne illness each year, resulting in up to 9,000 deaths (Mead *et al*., 1999). The accuracy of surveillance of food-borne illnesses has been compromised by the underreporting of mild cases of disease, the existence of alternative routes of transmission for pathogens (e.g., by water or contact between people) and the emergence of new undetected food pathogens (Mead *et al*., 1999). For example, in the last twenty years, the epidemiology of food-borne pathogens has changed as new organisms such as *Escherichia coli* O157:H7 and *Salmonella enterica* Serovar Typhimurium Definitive Type 104 (STDT104) have emerged as food contaminants (Meng and Doyle, 1997; Davis *et al*., 2002). Furthermore, other previously identified pathogens such as *Campylobacter jejuni* and *Listeria monocytogenes*, have been identified as a significant source of human disease associated with consumption of food (Mead *et al*., 1999).

National Food Safety Initiative

In response to this emerging problem, the federal government launched the National Food Safety Initiative (NFSI) in 1996 led by the Centers for Disease Control and Prevention (CDC), United States Department of Agriculture (USDA), and the Food and Drug Administration (FDA).

The primary objective of NFSI is to involve local, state, and federal public health agencies in a collaborative fashion to investigate food-borne outbreaks, develop strategies for fighting these pathogens and determine the impact of diseases on the population and economy. Furthermore, the following two food safety programs spurred from NFSI: Surveillance and Foodborne diseases Active Surveillance Network (PulseNet) and National Molecular Network for Foodborne Disease (FoodNet)(CDC, March 28, 1997).

PulseNet

The purpose of PulseNet is to help agencies identify and combat food related outbreaks via the use of a central database containing bacterial DNA "fingerprints", which are created by pulsed field gel electrophoresis (PFGE). Laboratories with PFGE capability generate and transmit DNA fingerprints to a database at the CDC for bacteria isolated from humans and from food products suspected of contamination. The database responds to these laboratories if similar or identical DNA patterns have been reported by others. This system can warn public health officials of a potential food-borne outbreak, and allows authorities to take all necessary preventive measures.

FoodNet

The CDC's FoodNet monitors and quantifies food related illnesses in the U.S. by collecting data on ten different food-borne pathogens. At inception, this surveillance system only monitored cases of infections caused by *Campylobacter*, *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella*, *Shigella*, *Vibrio* and *Yersinia enterocolitica* (CDC, April 19, 2002). The list has expanded to include

Cryptosporidium parvum, *Cyclospora cayatensis* and clinical cases of hemolytic

uraemic syndrome (HUS). The Food Safety Progress Report for the fiscal year 2000

indicated a nationwide reduction in the levels of the most common food pathogens

and an increase in the number of laboratories participating in the Pulsenet program.

Summary of the Pathogen Reduction and Hazard Analysis and Critical

Control Points (HACCP) Systems

The Food Safety and Inspection Service (FSIS) branch of USDA is responsible

for ensuring the safety of meat and poultry products for consumers. FSIS inspectors

monitor slaughter and processing plants to guarantee that only healthy animals are

processed and that facilities comply with federal sanitation guidelines. To address the

problem of bacterial contamination in slaughter and processing plants, FSIS proposed

in 1995 the implementation of the Pathogen Reduction and HACCP program (FSIS,

July 1996), which sets targets for the reduction of pathogens and demands routine

testing of surfaces for microbial contamination.

HACCP constitutes a process control system that must be adopted by all meat and

poultry plants that are federally inspected. The program is based on the following

seven principles:

1. hazard analysis

2. critical control point identification

3. establishment of critical limits

4. monitoring procedures

5. corrective actions

6. recordkeeping

7. verification procedures

Program efficacy is jointly verified by industry and FSIS inspectors. One method of verification includes testing carcasses for generic *E. coli* as an indicator of fecal contamination using methods published by the same agency. Levels of *E. coli* in a plant will be compared to nationwide baseline levels. FSIS will consider this information as one factor when deciding if a plant needs intervention.

Reduction performance standards for *Salmonella* have been also developed since this pathogen is present in many types of meat and poultry products and is responsible for high numbers of food-borne illnesses. For comparison, nationwide levels of *Salmonella* before the implementation of HACCP will be used as the baseline level.

Furthermore, FSIS will also require plants to adopt Standard Operating Procedures (SOP) since poor sanitation is a frequent reason for contamination of finished products. To ensure safety of food from "farm to table", the scope of the monitoring and intervention strategies must include pre-harvest environments and methods used for food distribution and preservation.

Significance of organisms included in this study

One of the objectives of this study was to develop general and specific fluorescent probes that would aid in detecting biofilms produced by important food-borne pathogens such as *Salmonella* spp., *E. coli*, *Aeromonas* and *Vibrio*. A brief review of the epidemiology of these pathogens is presented.

Escherichia coli

E. coli is the most common and important clinical isolate from the genus *Escherichia* and most commonly causes gastroenteritis, urinary tract infections septicemia, pneumonia *and E. coli*-associated meningitis (Joklik *et. al*, 1992). These infections occur as *E. coli* produces protective and adhesive organelles such as fimbriae, adhesions and a capsule (Joklik *et. al*, 1992). The polysyalic acid capsule protects the organism from the immune system by interfering with phagocytosis and this structure is present in 80% of isolates from neonates with meningitis (Joklik *et. al*, 1992). Fimbriae and adhesins permit *E. coli* to bind to a wide range of host tissues and are classified as mannose-sensitive or mannose-resistant. An example of mannose sensitive fimbriae is type I or common pili, which, although not usually associated with disease, allows the *E. coli* to colonize cells in the bladder, vaginal tract and large intestine (Joklik *et. al*, 1992). Mannose-resistant fimbriae are associated with this organism's ability to express other pathogenic traits (e.g., capsule). Examples of such fimbriae include the S-fimbriae, which aid in the colonization of endothelial cells and the P-fimbriae, which are associated with persistent urinary tract infections (Joklik *et al*, 1992).

Different strains of *E. coli* can also produce a variety of enterotoxins, which can be associated with human disease. For example, Enterotoxigenic *E. coli* (ETEC) secretes a plasmid encoded heat-labile toxin (LT) that upregulates the levels of cAMP (cyclic adenosine monophosphate) inside intestinal cells, resulting in the movement of sodium, chloride, potassium and water into the intestinal lumen (watery diarrhea) (Joklik *et a.l*, 1992). *E. coli* can produce a heat stable (ST) enterotoxin, which causes

diarrhea by preventing the absorption of electrolytes by the intestinal mucosa. This results in water being somatically driven from cells into the gut lumen. Humans infected with Enterohemorrhagic *E. coli* (EHEC) producing a Shiga-like toxin may develop serious hemorrhagic gastroenteritis or Hemolytic Uremic Syndrome (HUS), which may result in kidney failure. *E. coli* acquires the ability to produce this toxin after infection with a bacteriophage carrying the shiga-toxin gene. The most notorious serotype producing this toxin is *E. coli* O157:H7.

In 1992, hamburger patties contaminated with *E. coli* O157:H7 caused a multistate outbreak in the west coast of the United States (Bell *et al*., 1994). However, other foods such as unpasteurized apple cider and alfalfa sprouts have also been identified as sources of *E. coli* contamination (CDC, January 10 1997; CDC August 15, 1997). Fresh cheese curds from a dairy plant were recently identified as a source of contamination in an outbreak in Wisconsin (CDC, October 13, 2000).

The major reservoirs of *E. coli* O157:H7 include cattle and other ruminants and these organisms usually enter the food supply during the slaughter process (Meng and Doyle, 1997). A study performed by the USDA in 1992 indicated that 18 of 64 cattle herds tested positive for this bacterium (Meng and Doyle, 1997).

According to the CDC, 2000 and 2002, the estimated incidence of *E. coli* O157:H7 infections decreased 21% between 1996 and 2001; however, this organism still poses a serious threat to public health.

Salmonella

Salmonella is widely spread and environmental sources include water, soil, animal feces, raw meats, poultry products and seafood. *Salmonella* is a leading cause

of gastroenteritis worldwide and approximately 2 to 4 million cases of salmonellosis occur in the US annually (United States Department of Agriculture Food Safety and Inspection, 1998). Humans are usually infected with *Salmonella* by eating contaminated meat and poultry products. For example, grade "A" shell eggs are repeatedly implicated as the type of food responsible for human outbreaks of *S. enteritidis* (Guard-Petter, 1993). However, several outbreaks of *Salmonella* associated with other food sources such as raw ground beef, beef jerky and ice cream have been documented (CDC, December 15, 1995; CDC, October 27, 1995; CDC, September 16, 1994).

Once the organism is ingested it is able to invade and replicate inside the cells lining the lumen of the small intestine of a broad range of animal hosts. *Salmonella* produces invasin proteins that mediate the attachment of cells to microvilli.

From 1996 to 2001, the estimated incidence of *Salmonella* infections decreased 15%. More specifically, the rates for *S. typhimurium* and *S. enteritidis* decreased 24 and 22%, respectively (CDC, April 19, 2002).

Aeromonas

The genus *Aeromonas* consists of aquatic organisms that commonly cause disease in fish and cold-blooded vertebrates. Infection of humans usually occurs after the ingestion of contaminated food or water and the disease is usually limited to acute or chronic gastroenteritis. The disease can manifest itself as a watery (rice and water) diarrhea or as a dysenteric illness with the presence of loose stools containing blood and mucus (United States Department of Agriculture Food Safety and Inspection, 1998). The infection may become systemic in immunocompromised individuals. This

organism has been isolated from various food products such as seafood, raw milk, beef, pork, lamb and poultry (United States Department of Agriculture Food Safety and Inspection). The different species of *Aeromonas* possess a myriad of virulence factors such as endotoxins, enterotoxins, proteases, outer membrane proteins and lipopolysaccharide (LPS) (Gavin *et al.*, 2002). The frequency of infections in the U.S. caused by *Aeromonas* spp is not known since most cases have been sporadic rather than associated with large outbreaks (United States Department of Agriculture Food Safety and Inspection, 1998).

Vibrio

Vibrios are aquatic organisms. *V. parahaemolyticus* and *V. cholerae* serogroup Non-O1 are normal inhabitants of marine and estuarine environments in the U.S. (United States Department of Agriculture Food Safety and Inspection, 1998). Both organisms cause gastroenteritis although the disease caused by the latter is of greater severity and may lead to septicemia and death. *V. cholerae* Serogroup O1, which is the causative agent of epidemic cholera, is rare in the US and no outbreaks have yet been reported. Cholera is characterized by massive watery diarrhea, vomiting, muscle cramps, abdominal pain and severe dehydration (Ali *et al.*, 2002). This disease is caused by the cholera enterotoxin, a heat labile toxin that binds to receptors on cells in the intestine. The binding leads to an activation of adenyl cyclase and a subsequent accumulation of cAMP. This results in a secretion of sodium, chloride, potassium and water into the intestinal lumen. Interestingly, *V. cholerae* O139 (Bengal), which is a Non-O1 *Vibrio*, causes a disease that is indistinguishable from that caused by the O1 subtype.

Infections caused by *Vibrio* spp. are associated with the ingestion of contaminated water or food such as fish and shellfish. In 2001, 80 cases of *Vibrio parahaemolyticus* were diagnosed in the US, representing 83% higher incidence than in 1996 (CDC, April 19 2002).

An overview of biofilms

Capsular polysaccharides (CP)

Capsule-producing bacteria cause serious diseases in man such as septicaemia, meningitis, pneumonia and many others (Moxon and Kroll, 1990). Capsules, which are composed of polysaccharides, are commonly found on the surface of many prokaryotes, covering the O-antigens of the LPS. Capsular antigens are distinct from O-antigens. They are usually composed of one type of repeating polysaccharide subunit (Moxon and Kroll, 1990) and are highly hydrated structures (99% water). Monosaccharides from nucleotide precursors are polymerized into oligonucleotides that are further assembled into polysaccharides. This process takes place in the cytoplasm and is dependent on a cofactor such as undecaprenol pyrophosphate, which, in *Klebsiella* for example, mediates this process (Jann and Jann, 1990). The polysaccharides then are translocated through the periplasmic space and outer membrane by transferases, located in the cytoplasmic membrane (Jann and Jann, 1990).

Capsules serve several functions for microorganisms. Their chemistry helps cells acquire molecules required for metabolic functions (Moxon and Kroll, 1990). Capsules enhance the virulence potential of an organism by protecting cells from host

defense mechanisms such as the complement system and the activity of phagocytes (Jann and Jann, 1990). It has been suggested that the hydrophilic properties and negative charge of capsular polysaccharides are responsible for creating repulsion between phagocytic cells and the bacterium, making phagocytosis difficult (Moxon and Kroll, 1990).

Capsular polysaccharides are also poor immunogens although different capsules vary in ability to stimulate an immune response (Moxon and Kroll, 1990). One explanation for such an observation is that many capsular antigens of bacteria are homologous in composition and structure to sugar antigens on host cells. For example, the structure of the capsular polysaccharides of *E. coli* K1 is identical to the terminal sequence of neural cell adhesion molecules (N-CAM), which are present on the surface of neural cells (Jann and Jann, 1990).

Capsules may be covalently linked to other functional groups such as amino acids, fatty acids and other functional groups (Yu and Kochetkov, 1987). The capsular antigens of *E. coli,* also referred to as K antigens, have been extensively characterized and have been shown to be linked to different functional groups. These groups contribute to the chemical and immunogenic properties of capsules. For example, Capsular polysaccharides possessing phospho-diester or carboxylic acid groups are acidic (Jann and Jann, 1989). Furthermore, polysaccharides that are O-acetylated are more immunodominant than their deacetylated counterparts (Jann and Jann, 1989; Moxon and Kroll, 1990). O-acetyl groups have been reported to increase the immunogenicity of the capsular polysaccharides of *Streptococcus pneumoniae* type 1, *E. coli* K1, and *Neisseria meningitidis* group C (Moxon and Kroll, 1990). The

purpose of fatty acid substituents in some polysaccharides has been hypothesized to aid in the development and maintenance of the capsule (Jann and Jann, 1990). Therefore, capsular material is referred to as exopolymeric substances (EPS), unless it is known that it's composition is exclusively due to carbohydrates.

Capsules also promote the adherence of bacteria to surfaces such as inanimate objects or cells, which can lead to the formation of biofilms.

Exopolysaccharides (ES)

Exopolysaccharides (ES) constitute a second class of protective cell-synthesized polysaccharides and have been identified as one of the component of the matrix that encases cells. Similarly to CP, ES also varies in composition and charge. Most ES is polyanionic due to the presence of uronic acids or ketal-linked pyruvates although some can be polycationic or neutral (Sutherland, 2001). The structure of ES is described in terms of primary, secondary and tertiary conformations. The primary conformation refers to the individual monosaccharides constituting the ES. The secondary conformation relates to the geometric configuration adopted by the polymer as a function of its bonding pattern. For instance, an ES that contains predominantly 1,4-β- or 1,3-β- linkages will adopt a rigid and helical structure (Sutherland, 2001). Polymers with 1,2-β- or 1,6-β- linkages will yield more flexible polymers (Sutherland, 2001). The tertiary conformation refers to the interaction of the ES with other macromolecules such as proteins, lipids.

Evidence that supports the existence of species-specific ES is scarce and it has been suggested that polymers of ES and CP are similar or identical in composition (Sutherland, 2001). Furthermore, the relative amounts of a particular monosaccharide

may vary with the location and the age of the biofilm. The amount of ES in a biofilm

is dependent on levels of carbon substrates available to the bacteria and the lack of

nitrogen, potassium and phosphate can suppress the synthesis of the polymer

(Sutherland, 2001). The polysaccharide component of ES is capable of binding a

large number of molecules of water resulting in hydrated biofilms composed of up to

97% water (Sutherland, 2001).

Biofilms and their advantage to bacteria

Bacterial biofilms are composed of communities of microorganisms and their EPS

that are attached to a surface (O'Toole *et al.,* 2000). Extracellular polymers, which

may be composed of carbohydrates, proteins and lipids, that surround a bacterium are

also known as a glycocalix (Dr. Sam Joseph, personal communication). The biofilm

accretes as the organism secretes large amounts of EPS, chiefly composed of

polysaccharides (O'Toole *et al.,* 2000). The biofilms may provide cells with an

internal protective environment that possesses adequate physical and chemical

parameters that ensure survival of the cells. (e.g., pH, nutrients and osmotic

stress).This material also protects encased bacteria from chemical and physical

agents, making the organism more resistant to disinfectants (O'Toole *et al.* 2000).

Biofilms of *Listeria* have been shown to be more resistant to hypochlorite and heat

than free-swimming organisms (Lee and Frank, 1991). Studies have also shown that

biofilms of *S. typhimurium* and *L monocytogenes* were more resistant to trisodium

phosphate (TSP), an USDA-appoved antimicrobial treatment for poultry, than

planktonic cells (Somers *et al.,* 1994). *Vibrio cholerae* O1 has been shown to produce

an ES that provides the bacterium with resistance to osmotic stress and oxidation

(Wai *et al.*, 1998). *S. enterica* Serovar Typhimurium DT104 has been reported to shift to a rugose phenotype at room temperature. This phenotype was more resistant to oxidative stress than mutant cells without the ability to produce this morphology (Anriany *et al.*, 2001).

Another benefit of biofilms may be that nutrient availability is greater for attached bacteria than for suspended ones since organic and inorganic compounds will accumulate at solid-liquid interfaces (Rijnaarts *et al.* 1995). Cells may also exist in various physiological states in the biofilm matrix, rendering them less susceptible to antimicrobial agents (Korber *et al.,* 1997). Also, biofilms may create a symbiotic microhabitat, where different species of bacteria synergistically perform specific functions that ensure the survival of the multi-species microbial community. For example, the unique ability of one organism to degrade a complex molecule may help to provide other bacteria with by-products that can be used in their metabolism. Environmental signals such as temperature, osmolarity, pH, iron and oxygen may influence biofilm formation (O'Toole *et al.,* 2000). Such cues may explain why *V. cholerae* uses different type-IV pili to colonize the gut of animals as opposed to non-nutritive abiotic surfaces (O'Toole *et al.,* 2000). Culture media composition, whether rich or minimal, may dictate the ability of *E. coli* K-12 to form biofilms (O'Toole *et al.,* 2000).

Biofilm formation occurs in three main stages

Stage 1

Bacterial attachment and formation of a biofilm appears to occur in three stages. During the first stage, bacteria come into contact with a surface, either by

random Brownian motion (random motion of small particles suspended in gas or liquid), bacterial mobility or by the turbulent flow of the medium suspending the cells (Kumar and Anand, 1998). In natural environments, these surfaces are commonly coated with an organic film, referred to as a conditioning film (Wilson, 2001). In the food processing environment, this film may be composed of proteinaceous compounds from meat and milk products (Zotolla and Sasahara, 1994). The film provides the adhering bacteria with an environment rich in nutrients and changes the physico-chemical properties of the surface favoring attachment (e.g., hydrophobicity and electrostatic charges) (Kumar and Anand, 1998).

For the initial attachment of the organism to the substrata to be irreversible, the bacterium must overcome repulsive electrostatic forces between itself and the surface. The ability of the bacterium to overcome this hurdle is explained by the DLVO (Derjaguin, Landau, Verwey and Overbeek) theory. This theory states that bacterial cells can be treated as colloidal particles possessing a negative charge at neutral pH and surfaces such as glass and Teflon are also negatively charged (Rijnaarts *et al*, 1995). A bacterium experiences electrostatic repulsive forces and attractive van der Waals forces as it approaches a surface. At a distance of 10-20 nm from this surface, the cell experiences van der Waals attractive forces, which are countered by

electrostatic repulsive forces as the bacterium gets closer to the surface (Quintero, thesis 1994). These latter forces provide an energy barrier that prevents the organism from making contact with the surface. At this stage of biofilm formation, cells are reversibly bound to a contact surface and can be detached by shear fluid forces (Kumar and Anand, 1998). However, a bacterium can overcome or lower this electrostatic energy barrier by synthesizing extracellular structures such as flagella, fimbriae and ES (Kumar and Anand, 1998). These structures aid the bacterium in anchoring itself irreversibly to the surface, commencing the second stage of biofilm formation.

Stage 2

During this "colonization stage" the adherent bacteria begin synthesizing and secreting EPS. For example, *Pseudomonas aeruginosa* upregulates its expression of alginate after attachment and *Staphylococcus aureu*s produces a polysaccharide intercellular adhesin that binds cells into an interconnected cell matrix (Wilson, 2001). Concomitant with EPS secretion, the cell population grows with the multiplication of adherent bacteria and attachment of planktonic cells from the surrounding fluid environment. This leads to the formation of microcolonies, which develop into mature and dense biofilms as cells and EPS accumulate (Kumar and Anand, 1998; Wilson, 2001). Also, physiological processes of bacteria such as respiration rate, electron transport activity, growth rate and substrate uptake/breakdown may change after adhesion (Wilson, 2001).

Stage 3

Mature biofilms are well-anchored structures that resist the shear forces of fluids. However, sections from the top of the biofilm can be mechanically removed by environmental forces and colonize new areas.

Objectives of this research

The purpose of this study is to develop probes that can rapidly and accurately detect the presence of polysaccharides that are present in the biofilm matrix of biofilms of important food pathogens. To develop this study, the following four objectives will be utilized:

Objective 1) Explore the potential use of Calcofluor, which bind to β-D-Glucans, as a general fluorescent probe to detect biofilm material produced by food pathogens under laboratory conditions.

Objective 2) Evaluate the capacity of fluorescene isothiocyanate (FITC)-labeled lectins (sugar-binding proteins of non-immune origin) as species and genera-specific probes for biofilms.

Objective 3) To develop specific and sensitive antibody probes against the polysaccharide component of the EPS of STDT104 in order to study the process of biofilm formation by this organism *in vitro*.

Objective 4) Test above probes on samples collected from processing plants and production facilities or directly *in situ*.

Summary of procedures for all four objectives

The first objective will be accomplished by testing Calcolfuor against biofilm materials from five food pathogens (*Salmonella berta*, STDT104, *E. coli*, *Aeromonas hydrophila* and *Vibrio cholerae*) grown on four different types of contact surfaces (glass, polypropelene, Teflon™ and stainless steel). *Hyphomonas adhaerens* will be used as a positive control since this marine organism produces a copious polysaccharide biofilm that readily binds to Calcofluor.

For the second objective, the rationale for using lectins as a species-specific probe is based on literature reports showing that differences exist between the sugar compositions of biofilms amongst bacteria. A battery of different lectins, each with a unique specificity for a carbohydrate monomers or polymers (e.g., mannose, glucose, galactose), will be used to probe the same biofilms tested with Calcofluor. We expect to identify probes that are genera-specific, which could be used in a biofilm-detection protocol that offers higher specificity for inspectors of food.

For the third objective, the carbohydrate portion of the biofilm material of STDT104 will be purified, analyzed for composition and used for antibody production. These antibodies will be tested for specificity and then used to study how the maturation of this organism's biofilm progresses with the aid of fluorescent, confocal and electron microscopes.

The fourth objective will be accomplished by testing probes on naturally contaminated surfaces taken from commercial plants as well as testing all probes *in situ*.

Chapter 2: CALCOFLUOR AND LECTINS AS FLUORESCENT
PROBES TO DETECT BIOFILMS OF FOOD-BORNE
PATHOGENS

Rationale

Adequate sanitation is necessary to exclude pathogens in the food industry.

Preventive approaches to reduce contamination in production and processing facilities

include proper choices of surface materials and washes using effective detergents and

disinfectants (Kumar and Anand 1998; Tide *et al*., 1999). Hypochlorites and chill

tanks have been used to reduce bacterial populations in poultry processing plants

(Carr *et al*., 1999), while in broiler and layer farms, the reduction of available water

from the litter/manure has been shown to suppress the growth and spread of bacteria

such as *Salmonella* (Mallinson *et al*., 2000; Opara *et al*., 1992). Although such

approaches may effectively prevent the growth and spread of offending, free form

bacteria in the production area, they may not adequately disinfect bacterial biofilms.

A recent study has shown that current methods for decontamination and washing of

live-haul poultry containers and vehicles can be extremely inefficient in adequately

removing coliforms and *Salmonella* from surfaces (Carr *et al*., 1999).

Bacterial biofilms are communities of microorganisms surrounded by their

exopolymeric substances (EPS) that are attached to a surface (O'Toole *et al.,* 2000).

The biofilm accretes by the secretion of large amounts of EPS, chiefly composed of

capsular polysaccharides (CP) (Labare *et al*., 1989; O'Toole *et al.,* 2000). Encased in

this material, bacteria are protected from chemical and physical agents, resulting in

greater resistance to disinfectants (O'Toole *et al.,* 2000). Recent studies have shown that *Vibrio cholerae* O139, an important food-borne pathogen, synthesizes CP that provides increased resistance to chlorine, acids and osmotic stress (Ali *et al*., 2000). Similarly, S. *enterica* Serovar Typhimurium DT104 (STDT104) has been shown to exhibit a rugose phenotype that produces EPS found to protect against low pH and hydrogen peroxide (Anriany *et al*., 2001).

In addition to protection, the biofilm matrix can mask certain pathogens and render them less detectable by conventional protocols. Therefore, the development of technology to efficiently detect the polysaccharide component of bacterial biofilms in the environment will provide another important tool in assuring Good Management Practice on the farm and active oversight in processing plants.

This study evaluated the capacity of the fluorescent agents, Calcofluor and fluorescine isothiocyanate (FITC)-labeled lectins, as probes for bacterial biofilms. Calcofluor, which binds to (1-3) and (1-4) ß-linked D-glucans (Wood, 1980), and previously shown to bind to the EPS of *Hyphomonas adhaerens* (Quintero and Weiner, 1995; Weiner *et al*., 1999), was tested as a general presumptive probe for biofilms of four food-borne pathogens. Results showed that Calcofluor can detect biofilms produced by these pathogens, and can, indeed, be used as a non-specific test for biofilm contamination. FITC-labeled lectins (sugar-binding proteins of non-immune origin) (Goldstein *et al*., 1980) can be used as genera-specific probes for pathogens embedded in their biofilms.

Materials and Methods

Bacterial strains, media and culture conditions

Bacterial strains used in this study were *Aeromonas hydrophila*, S. *enterica* Serovar Berta (*S. berta*), ST DT104, *V. cholerae* O139 Bengal (gift from Dr. Judith Johnson, University of Maryland at Baltimore, Medical School), *Escherichia coli* UMD-5 and *Hyphomonas adhaerens* strain MHS-3 (MHS-3; ATCC 43965). Their EPS constituents are provided in Table 1. Frozen stocks were maintained at -80⁰C in Trypticase Soy Broth (TSB; Becton Dickinson, Sparks, MD) containing 10% glycerol. All strains were grown in TSB at 37⁰C in a rotary shaker incubator (100 rpm) (Psycrotherm Controlled Environment Incubator Shaker, New Brunswick Scientific CO., Inc. Edison, NJ), with the exception of STDT104, which was grown at 25°C. *H. adhaerens* was grown in Marine Broth 2216 (MB) (Becton Dickinson) at 37°C.

Calcofluor preparation and biofilm staining

A 10x stock solution of Calcofluor Fluorescent Brightener 28 (4,4'-bis[4-anilino-6-bis(2-hydroxyethyl)amino-s-triazine-2-ylamino]-2,2'-stilbenedisulfonic acid) (Sigma Chemical Co., St. Louis, MO) was prepared at a final concentration of 800μg/ml. The pH of the Calcofluor solution was adjusted to 8.5 and the solution was filter-sterilized (0.2μm pore membrane). The optimal concentration (i.e. – the least amount of Calcofluor that produced maximum excitation) of Calcofluor was determined by testing serial dilutions ranging from 0.1-1.0% concentrations on

TABLE 1. Reported capsular polysaccharides of bacteria used in this study.

Organism	EPS	candidate lectins	references
H. adhaerens	GalNAC β(1,4)-links	BPA, GSI, ABA Jacalin, MPA	Quintero *et al.* (1995)
S. berta	NR [b]		
STDT104	NR		
A. hydrophila	Glc, Man, Rham	GNA, ConA, LcH VFA, PSA	Martinez *et al.* (1995)
V. cholerae	Xylhex, Man GalA, Gal[d], GlcNAC	GSI, BPA, MPA, ABA, Jacalin, LcH, GNA, PSA UEA-I, ConA, VFA	Preston *et al*. (1995) Wai *et al.* (1998)
E. coli	Glc, Gal glucuronic acid	GSI, BPA, MPA, ABA Jacalin, LcH, GNA, PSA UEA-I, ConA, VFA	Jann and Jann (1990)

a) Selection of lectins was based on carbohydrate specificity and reported sugar composition of exopolymeric substances (EPS) of food pathogens.

b) NR: not reported.

c) Integral capsule.

d) Peripheral capsule.

control biofilms produced by *H. adhaerens* in a multi-well plate, using a fluorescent plate reader (Victor 2 Fluorescent Fluorometer, Wallac, Finland).

Labeling of biofilms and planktonic cells with Calcofluor

Cultures of each organism were grown to late logarithmic/early stationary phase as described above. Flasks were gently hand vortexed for 15 sec and 45 μl aliquots from each culture were incubated at either 25°C (*St* DT104) or 37°C (all other strains) with 5 μl of Calcofluor stock solution for 18 h (80 μg/ml final concentration of Calcofluor) under static conditions. Unbound Calcofluor was removed by centrifuging the suspension at 10,000 x g for 3 min and washing the pellet with Phosphate-Buffered Saline (pH 7) (PBS; 1.24 g of $Na_2HPO_4 \cdot H_20$, 0.18 g NaH_2PO_4, 8.5 g NaCl, 1000 ml dH_20) and resuspending it in 50 μl of PBS. Ten μl of each sample were examined using epifluorescence microscope (Axiophot Photomicroscope; Carl Zeiss, Inc., Thornwood, NY)(63x objective, numerical aperture 1.32. Zeiss filter set 3). Calcofluor absorbs light in the ultraviolet (UV) range (380 nm) and fluoresces in the blue range (440 to 450 nm).

Probing biofilm-covered surfaces with Calcofluor

Glass, Teflon™, polypropylene, stainless steel and aluminum coupons with surface areas of 18.75 cm^2 (2.5 cm X 7.5 cm) were used. Each test surface was acid-washed and placed inside a 500 ml Erlenmeyer flask containing 30 ml of broth. Flasks were inoculated and biofilms were allowed to accrue for 3 days at either 25°C (STDT104) or 37°C (all other strains) with broth replacement every 24 h. The fourth and last broth exchange contained 80μg/ml Calcofluor and cultures were re-incubated

for an additional 18 h. Control surfaces in sterile vessels were exposed to the same

broth exchange regimen and stained with Calcofluor. Slides were removed using

sterile forceps and rinsed with 50 ml of PBS eluted from a continuous stream from a

50 ml pipette to remove unattached cells and unbound dye. A hand-held UV-lamp

(Blak-Ray™ Lamp model UVL-21, Long wave UV-366 nm; UVP, Inc. San Gabriel,

CA, USA) was used to detect the presence of biofilm on each surface. The extent of

biofilm formation was evaluated based on the extent and intensity of fluorescence on

the surface. Biofilm formations on glass and polypropylene surfaces were confirmed

by examining slides under epifluorescence microscopy. Biofilm formations on

Teflon™, stainless steel and aluminum surfaces were detected as fluorescing areas.

The viability and purity of the adherent populations was confirmed by swabbing

surfaces and streaking onto McConkey agar (Difco Laboratories, Detroit MI, USA) in

the cases of *V. cholerae*, *A. hydrophila* and *E. coli* and on MM-agar (Mallinson *et al*.,

2000) in the case of *Salmonella* spp. Additionally, both contaminated and sterile

control surfaces were digitally photographed (BioRad Gel Doc 1000 chamber;

Rainbow TV Zoom Lens H6X8-II 8-48mm).

Labeling biofilms with lectins

Lectins labeled with FITC (EY Laboratories, Inc., San Mateo, CA, USA) were

selected based on reported polysaccharide composition of the capsules of the food-

borne pathogens used in this study (Table 1) (Appendix 1). The reported

predominant carbohydrate specificities of the lectins used in this study are:

Galanathus nivalis (GNA), α- Mannose (Man) and Man (α1,3); *Canavilis ensiformis*

(ConA), α-Man, α-Glucose (Glc), α-GlcNAc (N-acetyl-glucosamine) and branched

Man with α-Fucose (Fuc) as determinant; *Lens culinaris* (Lch), α-Man, α-Glc, α-GlcNAc and branched Man with α-Fuc as determinant; *Vicia faba* (VFA), α-Man, α-Glc, α-GlcNAc and branched Man with α-Fuc as determinant; *Pisum sativum* (PSA), α-Man, α-Glc, α-GlcNAc and branched Man with α-Fuc as determinant; *Griffonia simplicifolia* (GSI), α- Galactose (Gal), α- GalNac (N-acetyl-galactosamine); *Artocarpus integrifolia* (Jacalin), α- Gal, α- GalNac, and Gal(β1,3)GalNAc; *Maclura pomifera* (MPA), α-Gal, α-GalNac, and Gal(β1,3)GalNAc; *Agaricus bisporus* (ABA), β-Gal and Gal(β1,3)GalNAc; *Bauhinia purpurea* (BPA), α-GalNac and β-GalNAc; *Anguilla anguilla* (AAA), α-Fuc; *Lotus tetragonolobus* (Lotus A), α-Fuc and Fuc(α1,2)Gal(β1,4)[Fuc(1,3)GlcNAc; *Ulex europaeus* (UEA-I), α-Fuc and Fuc(α1,2)Gal(β1,4)GlcNAc.

Cultures were grown with aeration to early stationary phase and harvested. Aliquots of 100μl and small amounts of biofilm formed on walls of culture flasks were removed by scraping, mixed in PBS and centrifuged at 10,000 x *g* for 3 min. The pellet was washed and resuspended and 10μl aliquots were air dried on glass slides. Thirty μl of a 1:100 FITC-labeled lectin solution in dH$_2$0 were added to each sample and slides were incubated at 37°C for 30 min in a chamber pre-humidified with distilled water and protected from light. The biofilm-lectin mixtures were gently washed 3x with 25 ml of PBS for 10 min per rinse. Lectin binding was scored semi-quantitatively based on biofilm delineation and intensity of fluorescence under epifluorescence microscopy (63 x objectives, numerical aperture 1.32. Zeiss filter set 9).

Results and Discussion

Calcofluor was selected as a non-specific probe of biofilms because of its capacity to bind to a wide range of polysaccharides, especially those containing (1-3) and (1-4) β-D-glucan linkages (Wood, 1980). For example, Jann and Jann (1989). reported such linkages in the CP of several strains of *E. coli* . *V. cholerae* O139 has these bonds between Gal and GlcNAc residues (Preston *et al.,* 1995; Wai *et al.,* 1998). The capsule of *H. adhaerens*, a copious biofilm-forming bacterium and used as a control in this study, has been shown to form complexes with Calcofluor and to bind lectins with high affinity for Gal (1-3) GalNAc moeties (Quintero and Weiner, 1995). The optimal concentration of Calcofluor to obtain maximum excitation of *H. adhaerens* was 80 μg/ml (data not shown).

As theoretically anticipated, Calcofluor (80 μg/ml conc.) strongly bound to the biofilms *of H. adhaerens*, *V. cholerae*, *S. berta* (Figure 1) and STDT104 (Figure 2).

Additionally, Calcofluor was clearly visible around the planktonic cells of *H. adhaerens*, *V. cholerae* and STDT104, which suggests that these bacteria may produce integral capsules. Calcofluor also bound to biofilms of *A. hydrophila,* and *E. coli*; however, the biofilm-dye complexes fluoresced with a lower intensity under ultraviolet light. These results predicted that Calcofluor could be a useful non-specific probe for polysaccharide capsules and biofilms of some food-borne pathogens.

The suitability of Calcofluor as a biofilm probe was tested more extensively using 3-day-old biofilms grown on five different types of surfaces (glass, polypropylene, Teflon™, stainless steel, aluminum). Calcofluor detected biofilms produced by each
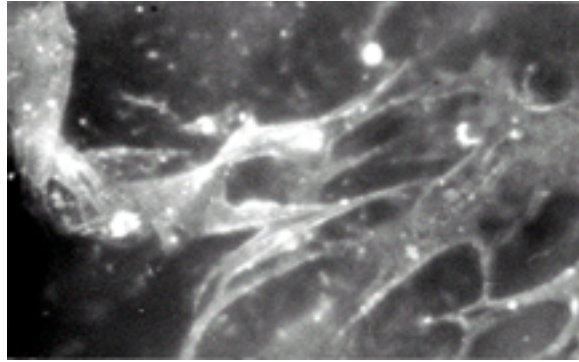
FIGURE 1. Biofilm of *Salmonella enterica* serovar Berta stained with Calcofluor.
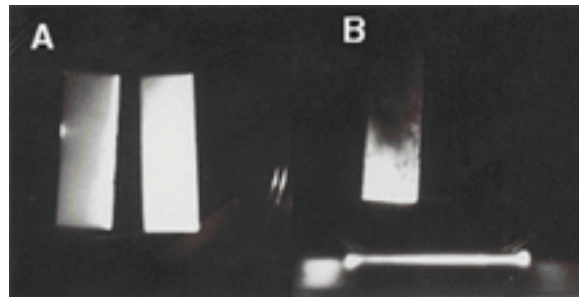
FIGURE 2. Detection of *Salmonella enterica* serovar Typhimutium DT104 biofilm on Teflon™ surface using Calcofluor as a probe. Biofilm-contaminated coupons (A left and B left) and non-contaminated coupons (A right and B right) were illuminated with white light (A) and ultraviolet light (B), respectively. Note that only the coupon contaminated with the biofilm fluoresced under UV exposure when treated with Calcofluor.

of the tested bacteria (Table 2); however, it is important to note that the amount of fluorescence is a function of the affinity of Calcofluor for the CP and the quantity of adherent CP. Thus a negative or weak result could denote minimal biofilm formation, which is exactly the intention in using the probe for detection.

Calcofluor was an extremely effective probe of *Salmonella* and *Vibrio* biofilms on all tested surfaces (Figures 1, 2 and Table 2). The identification of biofilms of *Salmonella* on stainless steel is especially important since this durable corrosion-resistant metal is frequently used for food contact surfaces (Tide *et al*., 1999). Furthermore, the ability of *Salmonella* and other food-borne pathogens to form biofilms creates a serious problem with presumably post-washed and disinfected poultry transport containers and vehicles (Carr *et al*., 1999).

Significantly, clean surfaces (sterile) and surfaces contaminated with STDT104 biofilms appeared identical when viewed under visible light (Figure 2A). However, only the surfaces contaminated with *Salmonella* fluoresced under UV light after staining with Calcofluor (Figure 2B).

Even on relatively lightly colonized surfaces (e.g.- STDT104 on polypropylene, *V. cholerae* on glass and *A. hydrophila* on stainless steel and aluminum), Calcofluor was sensitive enough to detect those biofilms. The ability of Calcofluor to detect trace amounts of biofilm in such areas is relevant because biofilms are likely to exist particularly in the sharp angles and corners of food contact surfaces (Tide *et al*., 1999).

The presumptive biofilm-Calcofluor screen can yield false-positive results if the surface is unusually marred or if one does not perform the washing steps properly. In

TABLE 2. Detection (using Calcofluor and UV light) of relative biofilm

formation by food-borne pathogens on different test surfaces.

Organism	Test Surface				
	Glass	Polyprop	Teflon	S. Steel	Aluminum
S. berta	3[b]	2	3	3	3
STDT104	2[b]	1	2	3	3
A. hydrophila	-[b]	-	2	1	1
V. cholerae	1	3	3	2	2
E. coli	-	1	3	1[c]	1[c]
H. adhaerens	3	3	3	3	3

a)   Calcofluor bound to individual cells as well as the entire biofilm matrix.
b)   +++ reaction: fluorescence visible over a large area of test surface; ++
     reaction: patches of fluorescence visible on test surfaces: + reaction: only
     specs of fluorescence visible on test surfaces; - reaction: no visible
     fluorescence detected.
c)   False-positives. Although fluorescing specs were detected on rough and
     uneven areas of the surface, *E. coli* was not confirmed by plating
     procedure.

this study false-positives were occasionally observed on Teflon™, stainless steel and

aluminum surfaces as minute fluorescing specks in the imperfections (i.e.,- scratches)

on the surface of the tested coupon. They were confirmed as false-positives by plating

the apparent biofilm onto MaConkey agar, a medium that readily supports the growth

of the suspect species. As a presumptive screen, rare Calcofluor false-positive tests

are balanced by its sensitivity and the almost non-occurrence of false-negatives

associated with its use. For example, Calcofluor aided in the detection of marginal

biofilm contamination of *A. hydrophila* on stainless steel and aluminum surfaces that

was not apparent to the naked eye.

These results show that Calcofluor could be used as a presumptive probe to detect

biofilm contamination on food production and preparation surfaces. It binds to many

different polysaccharides rendering them readily detectable under UV light. With

these promising results a system that uses Calcofluor dye and a field UV-lamp is

being developed to detect biofilm contamination *in situ* (i.e., transport containers and

conveyor belts).

Lectins have more specificity for capsular material than Calcofluor so they were

tested as presumptive, species-identifying, rapid probes of food-borne pathogens.

Lectins have been used to detect biofilms in river samples and to quantify

accumulation of biofilm in response to nutrient limitation (Mohammed *et al*., 1998;

Neu and Lawrence, 1999). Additionally, lectins have aided in the visualization of

ultrastructures of bacteria such as capsules and holdfasts (Bayer, 1990; Quintero and

Weiner, 1995). The specific lectins that were selected for use in this study were

chosen on the basis of their theoretical affinities to the capsular polysaccharides reported for selected food pathogens (Table 1).

The lectins are grouped according to carbohydrate-binding specificity. The selectivity and sensitivity of each lectin to each biofilm was scored visually (Table 3). This study identified the following lectins as having the highest specificity to test biofilms: GNA to *H. adhaerens,* ConA and Lotus A to *St* DT104, Con A to *S. berta*, GNA to *E. coli*, GSI to *A. hydrophila* and MPA and ABA to *V. cholerae* (Table 3). A "three plus" reaction was assigned when a biofilm was well delineated by the lectin and fluoresced with intensity (Figure 3). The use of a fluorometer should further support the results obtained and more objectively quantify the specificity of each lectin for a particular biofilm.

The specificity and relative avidity of each lectin is contingent on how it is applied. For example, although BPA (50μg/ml) was previously identified as highly specific for the capsule of *H. adhaerens* (Quintero and Weiner, 1995), this study used a five-fold greater dilution of lectin (10μg/ml) and a longer rinsing procedure to increase the specificity of the test. Under these conditions the EPS of *H. adhaerens* fluoresced more strongly when stained with GNA than BPA, suggesting the presence of high quantities of mannose. Mannose has been identified as a component of the capsular polysaccharide of other adherent prosthecate bacteria such as *Caulobacter crescentus* (Ravenscroft *et al.*, 1991). Mannose moieties also were implicated as an important component of the capsular exopolysaccharides of *Hyphomonas rosenbergii* and in its

TABLE 3. Sensitivities and Specificities of 13 FITC-Labeled Lectins[a] to biofilms[b] produced by tested organisms.

	man, branched man, glc [c]					fuc, branched fuc- [c]			gal, galNAc [c]				
	GNA	ConA	LcH	VFA	PSA	AAA	LotusA	UEA-I	GSI	BPA	Jacalin	MPA	ABA
STDT104	3	3[d]	2	1	1	1	3[d]	2	-	-	-	1	1
S. berta	3[d]	3[d]	1	1	1	1	2	1	2[d]	2[d]	1[d]	1	1[d]
E. coli	2[d]	1	1	-	-	1	-	1	1	1	1	-	-
A. hydrophila	1	1[d]	-	-	1	1	2[d]	2[d]	2[d]	2[d]	1	2[d]	2[d]
V. cholerae	1[d]	-	-	-[d]	-	-	-	-	-[d]	1[d]	2[d]	3[d]	3[d]
H. adhaerens	3	2	1	1	2	-	1	-	-	-	-	-	-

a) Galanthus nivalis (GNA): α-Man, Man (α1,3)Man); Canavalis ensiformis (ConA): α-Man, α-Glc, α-GlcNAc and branched Man; Lens culinaris (LcH): α-Man, α-Glc, α-GlcNAc and branched Man with α-Fucose as a determinant; Vicia faba (VFA): α-Man, α-Glc, α-GlcNAc and branched Man with α-Fucose as a determinant; Pisum Sativum (PSA): α-Man, α-Glc, α-GlcNAc and branched Man with α-Fucose as a determinant; Anguilla anguilla (AAA): α-Fucose; Lotus tetragonolobus (Lotus A): α-Fucose and Fuc(α 1,2)Gal (β1,4)[Fuc(α1,2)]GlcNAc;Ulex europaeus (UEA-I): α-Fucose and Fuc(α1,2)Gal(β1,4)-GlcNAc; Griffonia simplicifolia I (GSI): α-Gal and α-GalNAc; Bauhinia purpurea (BPA), α-GalNAc and β-GalNAc; Artocarpus integrifolia (Jacalin): α-Gal, αGalNAc and Gal(β1,3)GalNAc; Maclura pomifera (MPA): α-Gal, α-GalNAc and Gal(β1,3)GalNAc; Agarus bisporus (ABA): b-Gal and Gal(β1,3)GalNAc.

b) **"3"reaction**: biofilms of all sizes fluoresced with high intensity and architecture was very well defined; "**2"reaction**: biofilms of all sizes fluoresced with moderate/high intensity and with well defined architecture; "**1"reaction**: large and small biofilms fluoresced with moderate or weak intensities. Some biofilm architecture was usually visible for large biofilms but not for smaller ones; "**-" reaction**: wide variety of reactions were observed. Large biofilms fluoresced with a weak intensity and appeared fuzzy, which allowed only for their localization. Small biofilms fluoresced weakly revealing only the edges. In some cases they were undetectable.

c) EY Laboratories, Inc., San Mateo, CA 94401 U.S.A.

d) Lectin delineates individual cells.
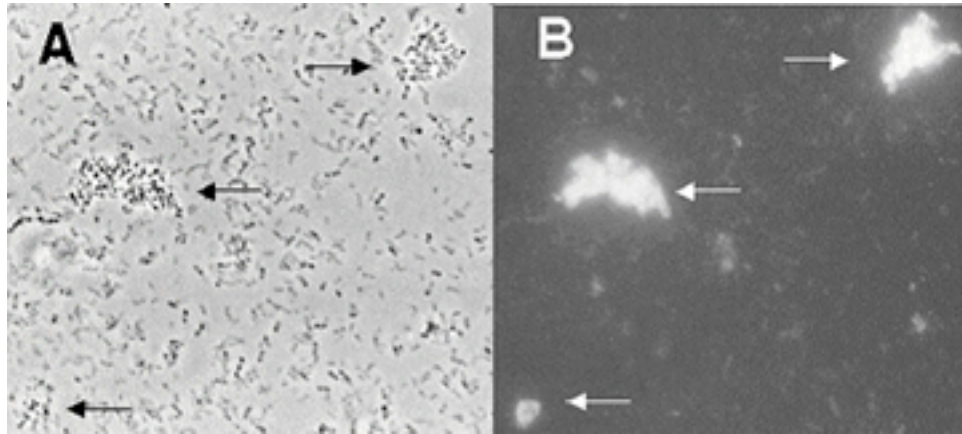
FIGURE 3. *Vibrio cholerae* biofilms stained with *Agaricus bisporus* (ABA) lectin. Two day old biofilms of *Vibrio cholerae* cultured in Tryptic Soy Broth at 37°C viewed under phase contrast microscopy. Same ABA-stained biofilms viewed under epifluorescence microscopy (representing a typical 3+ reaction). Arrows denote microcolonies in (A) phase micrograph and (B) in corresponding epifluorescent micrograph.

ability to adhere to glass surfaces (Langille *et al.,* 2000). Because there are small but notable gradations of differences between reactions of the various organisms, the tests must be performed in a careful, standardized fashion as described. .

GNA (specific for mannose and branched mannoses) and ConA (specific for mannoses, branched mannoses, glucose and N-acetyl glucosamine) bound more strongly to the biofilms of *S. berta*, STDT104 and *E. coli* than to the biofilms of *A. hydrophila* and *V. cholerae.* Furthemore, LcH, VFA and PSA, which have the same monosaccharide specificity as GNA and ConA in addition to possessing additional affinity for branched mannoses with α-fucose as a determinant, also bound to the enteric biofilms; however, their reactions were weaker than those with GNA and Con A (Table 3). GNA also bound to planktonic cells of each of the studied bacteria. Thus, it can be developed into a relatively non-specific probe of the planktonic (as opposed to adherent) population of enterics removed by washing.

Jacalin, MPA and ABA, specific for Gal(β1,3)GalNAc, each reacted more strongly to biofilms produced by *V. cholerae* and *A. hydrophila* than to biofilms of other bacteria. *V. cholerae* polysaccharide has been reported to contain two β-Gal(1,3) residues per repeating subunit of the capsular polysaccharide (Preston *et al.*, 1995). Each of these lectins labeled the biofilm matrix of *V. cholerae*, with MPA and ABA binding and delineating its architecture with superior sharpness and fluorescence. Thus, these would be good candidates as presumptive *V. cholerae* biofilm probes. All FITC-labeled lectins specific for galactose moieties (GSI, BPA, Jacalin, MPA, ABA) bound to individual planktonic *V. cholerae.*

The positive reaction of Jacalin, MPA and ABA for *A. hydrophila* is more complex. Galactose has not been identified as a component of the capsular polysaccharide but it is present in the core-oligosaccharide of *A. hydrophila* (Michon *et al.,* 1984). Arguably, this is why galactose-specific lectins bind individual cells of *A. hydrophila* but not as well to the biofilm matrix. Furthermore, in contrast to AAA (specific for α-fucose only), which did not bind to *A. hydrophila* cells, LotusA and UEA-I, which have the same specificity as AAA with additional binding sites for oligosaccharides containing Gal(β1,4) and GlcNAc groups, also bound to individual cells of *A. hydrophila*.

Results from the experiments using Calcofluor support its use as a general and presumptive probe for biofilm contamination. Its characteristic fluorescence under UV illumination is currently being explored as an *in situ* probe to aid in the detection of biofilms in the production environment. There are groups of lectins that appear to be promising for development as presumptive probes for specific genera or species of food-borne pathogens. It is possible that a battery of specific lectins may be developed to identify pathogens forming a biofilm. For example, ConA is a potential probe for the identification of biofilms of *Salmonella* spp. whereas MPA and ABA fluoresced noticeably more strongly on *V. cholerae* biofilms and to a lesser extent on biofilms produced by *A. hydrophila*. The specificity of lectins reported in this study could be even further delineated by quantifying the number of cells in a biofilm and the intensity of their fluorescence (e.g., using a fluorometer), adding sensitivity to the current protocol. Current studies are also testing the use of lectins as an aid to identify specific bacteria in multiple species biofilms, which better represents biofilms in

nature. Thus, Calcofluor and lectins can provide producers and inspectors with a

potent set of probes to detect bacterial contamination of surfaces associated with the

production and processing of produce, meat and poultry.

Chapter 3: A CAPSULAR POLYSACCHARIDE SURROUNDS SMOOTH AND RUGOSE VARIANT CELLS OF *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM DT104

Rationale

Salmonella enterica Serovar Typhimurium DT104 (STDT104) is a food-borne pathogen that emerged in high numbers during the 1980's, particularly in cattle (Zhao *et al*., 2002) and is now recognized as an important pathogen of humans and animals (Poppe *et al*., 2002). Infection of humans results in enterocolitis with diarrhea and abdominal pain between 8 to 72 hr after ingestion of large numbers of this organism. In contrast to *S. enteritidis*, which is commonly isolated from layer hens and eggs, STDT104 has a wider animal reservoir that includes cattle, pigs and humans (van Duijkeren *et al*., 2002). Furthermore, STDT104 also carries chromosomally-integrated resistance genes to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclines (ACSSuT) (CDC, April 11, 1997), causing this organism to present a great risk to public health.

A strain of STDT104 that forms a temperature-regulated, protective morphotype, when grown in a nutrient rich environment has been described (Anriany *et al*., 2001). This strain, which formed wrinkled (rugose) colonies on Trypticase Soy agar (TSA) plates at 25°C (Rv/25) was more resistant to low pH and hydrogen peroxide than a stable, spontaneous, smooth variant (Stv). The characteristics associated with rugosity have been also described for other foodborne or waterborne organisms such as *Vibrio cholerae* O1 El Tor, *V. cholerae* O139 Bengal, *Salmonella enteritidis* and *S.*

typhimurium (non DT104) (Yildiz and Schoolnik, 1999; Wai *et al.*, 1998; Ali *et al.*, 2000; Allen-Vercoe *et al.*, 1997; Romling and Rohde, 1999). In order to better understand the rugose phenomenon and to develop methods of decontamination that are effective against these organisms, it is important to determine the nature of the composition of the extracellular matrix that mediates attachment to food preparation areas and protects the organism from decontamination.

Cell-secreted EPS may be composed of several different types of macromolecules such as lipids, polysaccharides and proteins (Sutherland, 2001). *E. coli* and *Salmonella* synthesize curli and thin aggregative fimbriae, proteinaceous components that allow these organisms to form a rigid and adherent network of cells under laboratory conditions (Zogaj *et al.*, 2001). *S. typhimurium* forms a pellicle in liquid media at ambient temperature that is dependent on the expression of AgfA, which encodes for the subunit of the aggregative fimbriae (Romling *et al.*, 1998). Furthermore, other proteinacious extracellular structures have been reported to play significant roles in the adhesion of bacteria to abiotic substrata. For example, flagella and type I pili were shown to be required for *E. coli* to form normal biofilms in broth and on abiotic surfaces (Pratt and Kolter, 1998).

Exopolysaccharides are important for aggregative behavior as well. *H. adhaerens,* a marine bacterium, synthesizes an CP associated with formation of cell floccules in broth and thick biofilms on surfaces (**Quintero *et al.*, 1998**). *Staphylococcus epidermidis* secretes polysaccharide intercellular adhesin (PID) with *N*-acetylglucosamine as a major component that causes cells to form clusters (Heilmann, 1996). Cellulose was identified as an additional component of the extracellular matrix

of *S. typhimurium* and *E. coli* that provides rigidity to multicellular structures produced by these organisms (Zogaj *et al.*, 2001). This polymer combined with curli appears to be responsible for the rugose appearance of some *Salmonella* strains observed after incubation at 25°C. There now appears to be an additional exopolysaccharide associated with these two factors during rugose production (White *et al.*, 2003).

In the course of our studies on a rugose producing *S. typhimurium*, an exopolysaccharide appearing as a capsular component of the organism was recognized in a capsular stain on microscopic examination, which led us to examine the material to determine if it was yet another component of rugose formation. The monosaccharide composition of the surface exopolysaccharide synthesized by Rv at 37°C (Rv/37) was isolated and characterized. Using polyclonal antibodies raised against this exopolysaccharide and electron microscopy, it was learned that this capsular material was produced by Rv/25, Rv/37 and Stv at ambient temperature and at 37°C. Scanning confocal laser microscopy (SCLM) demonstrated that the CP is not required for surface colonization, is not produced in temperature-sensitive fashion (thus, is not involved in rugose formation) and is more predominant in the outer layers of biofilms.

Materials and Methods

Bacterial strains, media and culture conditions

Frozen stocks of *Salmonella enterica* Serovar Typhimurium DT104 strain 11601 (STDT104) and *E. coli* DH5α were maintained at -80°C in Trypticase Soy Broth (TSB) containing 10% glycerol. Stocks of *H. adhaerens* were maintained and grown

in Marine Broth (MB) at 37°C. STDT104 and *E. coli* were cultured on Trypticase soy

agar (TSA) and Luria-Bertani broth (LB) (Difco) at 25°C and 37°C for the formation

of rugose (Rv/25) and smooth (Rv/37) colonies, respectively (Anriany *et al.*, 2001).

A stable smooth variant (Stv) of the same organism was cultured identically and

formed smooth colonies at both temperatures.

Screening Rv and Stv for production of EPS

Of all the EPS produced by STDT104, the ability of Rv and Stv to produce a CP

containing (1-3)-β and (1-4)-β-glucopyranosyl units was tested. Five μl of overnight

cultures grown at 37°C in LB broth were plated in triplicate onto LB agar plates

without salt and supplemented with 200 μgml^{-1} of Calcofluor (fluorescent brightner

28; Sigma). Plates were incubated at 25 and 37°C for 4 and 2 days, respectively.

Colony fluorescence was inspected using a hand held UV light source and

photographed in a GelDoc (BioRad 100 chamber) equipped with a digital camera

(Rainbow TV Zoom Lens X6X8-II; 8-48 mm).

LB agar plates without salt and supplemented with Congo Red (CR; 40μg ml^{-1})

and Coomassie brilliant blue (20μg ml^{-1}) were inoculated and incubated as described

above to test for production of CR-binding fibrillar extracellular organelles by Rv and

Stv (Romling *et al.*, 1998). Both dyes bind proteins.

Adhesion assay

Sterile 12 mm round microscope cover slips (Fisher Scientific) were aseptically

placed inside tissue culture plate wells of 22 mm diameter (Corning, New York).

Each well was filled with 4 ml of LB broth and inoculated with 10 μl of an overnight

culture of Rv and Stv grown at 37°C. Tissue culture plates were incubated at 25°C under rotation (100 rpm) for 24h. Coverslips were aseptically transferred to a Petri dish using forceps and triple washed with 2 ml of dH_2O for 1 min. This step was performed to remove planktonic cells. For improved visualization, cells attached to the glass surface were stained with a 1% acridine orange solution for 5 min and washed with dH_2O. Coverslips were inverted onto a glass slide and viewed under an epifluorescent microscope (Zeiss Axiophot microscope) at 630 magnification. Cells attached to 0.016 mm^2 areas (determined by microscope grid) were counted. Seven separate areas were empirically counted on three different coverslips per experiment for a sample size of 21.

A second adhesion experiment was performed to compare the amounts of biofilm formed by Rv/25, Rv/37 and Stv after extended incubation. Polystyrene centrifuge tubes containing 5 ml of LB broth were inoculated in triplicate with 5μl of overnight cultures of each organism grown in the same broth at 37°C. Tubes were incubated at 25 and 37°C without shaking for nine days. The spent medium was discarded, tubes were rinsed twice with Phosphate Buffered Solution (PBS) and biofilms on tube walls were stained with a 0.4% solution of Crystal Violet (CV) in dH_2O for 5 min. The dye solution was discarded, the tubes rinsed with PBS and the CV bound to biofilms was solubilized with a ethanol-acetone solution (80:20). Absorbance readings were measured for each tube at 570nm.

Purification of CP

Two liters of TSB each were inoculated with an overnight culture of Rv and incubated at 37°C in a rotary shaker (100rpm) for 5 days. The spent medium was

exchanged for sterile TSB after 48h. Cells and flocks of biofilm adhering to the walls of flasks were harvested by scraping and centrifugation (7,000 x g) for 20 min. The supernatant was discarded after not yielding a precipitate by extraction with 2-propanol , indicating the absence of significant quantities of soluble polysaccharides.

The pellet obtained was blended in a Waring blender (Dynamics Corporation of America, Hartford, CT, USA) with a solution of 10 mM EDTA and 3% NaCl in distilled water for 1 min (1:10 sample to EDTA-NaCl solution) to shear off outer material (EPS) including the integral CP from the cells. The resulting suspension was centrifuged (7,000 x g) for 20 min. The supernatant was precipitated with ice-cold 2-propanol (4 parts of propanol to 1 part of supernatant) and then centrifuged (16,000 x g) for 20 min (Langille et al., 2000).

The exopolysaccharide was suspended in a minimal volume of dH_2O and dialyzed exhaustively against dH_2O for 3 days to remove ions and low molecular weight contaminants using a Spectrapor 1 membrane with a 6,000-8,000 molecular weight cut off (Spectrum Medical Industries, Los Angeles, CA, USA) and lyophilized. The resulting solute was suspended in a minimal volume of 0.1M $MgCl_2$, incubated for 4 h at 37°C with a 0.1mg/ml solution of Dnase and Rnase (Sigma Chemical Company, St. Louis, MO, USA) (Read and Costerton, 1987; Weiner et al., 1999). This was followed by an overnight treatment in 0.1mg/ml of proteinase K (Sigma) at 37°C.

The enzyme-treated exopolysaccharide was subjected to a hot phenol-chloroform extraction to further purify the sample from protein and LPS contamination. Briefly, the partially purified carbohydrate was heated to 70°C in a water bath for 10 min and then added to an equal volume of 90% phenol preheated to the same temperature. The

mixture was vigorously stirred for 15 min, cooled in ice and the emulsion centrifuged (30,000 x g) for 30 min (Methods for General and Molecular Bacteriology, 1994).

The aqueous layer underwent a methanol-chloroform extraction (Bligh and Dyer, 1959). Briefly, 20 ml of the aqueous layer from the phenol extraction was mixed with 60 ml of a chloroform-methanol solution (1:2) and the mixture was vigorously vortexed for 1 min. An additional 20 ml of chloroform was added and the mixture was vortexed for 30 sec. Lastly, 20 ml of dH_2O were added and the suspension was vortexed for 30 sec. The chloroform and the methanol layers were allowed to separate and the methanol layer was exhaustively dialyzed against dH_2O and lyophilized. The final purified CP was white and had a fluffy consistency.

Measurement of carbohydrate content

The hot phenol-sulfuric acid assay was performed to quantify the sugar content of the purified CP (Dubois *et al.*, 1956). Briefly, 0.5 ml of a glucose standard (10-100μg/ml) and a 1mg/ml solution of the purified CP were mixed with an equal volume of phenol. Next, 2.5 ml of sulfuric acid were added to each carbohydrate solution and the mixture was vortexed. Tubes were incubated in the dark at room temperature for 18 h. Absorbance was measured at 490 nm for each solution and the percentage of carbohydrate in the purified CP was estimated.

Measurement of protein content

The amount of protein in the final CP extract was measured using the Pierce BCA Protein Assay (Pierce, Rockford, Il, USA) according to the manufacture's instructions. Briefly, 0.1 ml aliquots of the CP solution (1mg/1ml) and a Bovine

Serum Albumin (BSA) standard solution (10-100 μg/ml) were mixed with 2 ml of the BCA reagent in glass tubes. Samples were incubated at 37°C for 30 min and their absorbance measured at 562 nm. The protein content in the purified CP was estimated.

Measurement of Lipopolysaccharide (LPS) content

The Limulus Amebocyte Lysate assay (LAL; Haemochrom Diagnostica) was performed according to the manufacture's instructions to determine the amount of LPS contaminant in the CP extract. Briefly, 5 ml of the LAL reagent was added to 0.5μg of *E. coli* endotoxin standard. The mixture was allowed to stand at room temperature for 1 h, and then vortexed for 1 min every 10 min. A serial dilution of control standard endotoxin (CSE) and of sample (1mg /ml) in LAL water was performed. Tubes were left at room temperature for 1 h and then gently inverted. Detection of endotoxin was recorded if the mixture in the tube solidified. The endotoxin contamination in the CP extract was determined.

FACE monosaccharide composition analysis of CP

For sugar composition analysis, the extracted CP was hydrolyzed according to the protocol provided by Glyco Inc. Briefly, neutral sugar hydrolysis was performed by incubating 50μl of a 1:2.5 solution of CP extract in dH$_2$O with 50μl Trifluoroacetic acid (TFA; 4N) at 100°C for 5 h. Amine sugar hydrolysis was performed with hydrochloric acid (HCL; 8N) at 100°C for 1 h. After hydrolysis, samples were dried in a Speedvac and the sample that underwent amine hydrolysis was re-acetylated according to instructions provided. The hydrolyzed CP was incubated with MONO

labeling dye overnight at 37°C, dried in a Speedvac evaporator and resuspended in labeling solvent. The sample was mixed in loading buffer and 6μl volumes were loaded onto a polyacrylamide gel for electrophoresis and finally photographed in a GelDoc (BioRad 100 chamber) equipped with a digital camera (Rainbow TV Zoom Lens X6X8-II; 8-48 mm).

Production of anti-CP polyclonal antibodies

A small amount of the purified CP (1.3 mg) was sent to Sigma Genosys (Sigma Genosys, The Woodlands, TX, USA) for production of polyclonal antibodies in rabbits.

SDS-PAGE gel electrophoresis of CP

Samples of cell lysates and CP were subjected to polyacrylamide electrophoresis with sodium dodecyl sulfate (SDS-PAGE) on a 12% Tris-Glycine gel (iGels™, Gradipore Ltd., Australia) in Tris Glycine buffer (Biod-Rad; 25mM Tris, 192nM Glycine and 20% (v/v) methanol, pH 8.3). Briefly, CP extracts of Rv/37 and exopolysaccharide extract of *H. adhaerens* (negative control; Quintero *et al*., 2001) were dissolved in Pipes buffer to a final concentration of 4 mg/ml. Whole cell lysates were prepared from late logarithmic cultures of *E. coli* grown at 37°C, Stv grown at 25°C, Rv grown at either 25°C or 37°C adjusted to an OD_{600} of 1.0. Cells were harvested by centrifugation (5 min at 10 x g), washed in Pipes and resuspended in the same buffer. Aliquots of 100μl were stored at -70°C.

Polysaccharide and cell lysate samples were diluted in Laemmli Sample Buffer (Bio-Rad) (1:2) containing beta-mercaptoethanol (βME; 1:20), heated for 5 min at

44

95°C and allowed to cool to room temperature. Some samples were treated with a proteinase K solution (20mg/ml) at 37°C for 18 h before loading. Wells were loaded with 35µl and 20 µl of polysaccharide and cell lysate samples, respectively. Kaleidoscope Prestained Standard (Bio-Rad) (protein standard) was included (5µl). Gels were run for 2 h at constant voltage (200V).

Electroblotting, protein staining and eastern blot

Samples were electrotransferred onto an Immobilon-P membrane (Millipore Corp., Bedford, Mass.) in a Tris-glycine-methanol buffer (0.025 M Tris, 0.19 M glycine, 20% methanol) at a constant voltage of 100V for 1 h. After transfer, membranes were submerged in methanol for 1 min, allowed to air dry and stained for proteins using SYPRO Ruby Protein Blot Stain (Molecular Probes) according to protocol provided by the manufacturer.

Briefly, membranes were floated on a solution of 7% acetic acid and 20% methanol for 15 min at room temperature with gentle rocking. Membranes were rinsed four times in dH$_2$O for 5 min per rinse and incubated in 25 ml of SYPRO Ruby Blot Stain for 15 min at room temperature. After staining, membranes were double rinsed in dH$_2$O, allowed to air dry and visualized and photographed as described previously.

Membranes were destained in methanol and blocked in 5% PBS-Tween for 18 h at 4°C. Membranes were triple washed in PBS-Tween (0.1%) and incubated for 1 h at room temperature with 50 ml of anti-CP polyclonal antibodies in PBS-Tween containing 2% skim milk (1:500). Blots were washed 3x with PBS-Tween and

incubated for 1 h with horseradish peroxidase (HPR) labeled goat anti-rabbit IgG

secondary antibodies (1:5000) (Poti-4CN Detection Kit; Bio-Rad). Blots were washed

2x in PBS-Tween and developed.

Immunoelectron microscopy

Cultures of Rv/25, Rv/37 and Stv/25 in late logarithmic phase grown in LB were

washed three times in Pipes buffer to remove medium and resuspended in 1 ml of the

same buffer. Drops of each culture were applied to a small piece of parafilm. Cells

were attached to Nickel Formvar-coated copper grids (200 mesh) by floating grids on

drops of sample for 1 min. Grids were blotted dry, blocked with 1% skim milk for 15

min and serially washed in three successive drops of Pipes buffer. Grids were then

inverted on a drop of 1:100 anti-CP polyclonal antibody suspension in Pipes buffer

containing 0.1% of skim milk for 30 min at room temperature. Grids were blotted

dry, successively rinsed in three drops of Pipes and inverted on a drop of 1:100 goat

anti-rabbit secondary antibodies conjugated to 12nm colloidal gold for 20 min. Grids

were rinsed by inverting on three drops of dH$_2$0 and observed with a Zeiss EM10 CA

(Leon Electron Microscope, Thornwood, NY, USA).

Lectin-gold labeling of cells

Gold-labeled *Concavalin A* (Con A-Au; 20-nm-diameter gold particles) and *Helix*

Pomatia (HP-Au; 20-nm-diameter gold particles) lectins were used to label capsular

and peripheral polysaccharides of Rv/25. Briefly, Rv/25 cells from a culture grown to

late logarithmic phase were allowed to attach to formvar-coated copper grids for 1

min and the grid was blocked as previously described. Grids were incubated in a 1:10

dilution of each lectin in Pipes buffer containing 0.1% of skim milk for 50 min at room temperature and triple rinsed with dH$_2$0. Grids were observed with a Zeiss EM10 CA (Leon Electron Microscope).

Scanning Confocal Laser Microscopy (SCLM)

SCLM analysis of biofilms attached to coverslips was performed using a Zeiss inverted microscope and a dual laser-scanning confocal imaging system equipped with a 100mW argon ion laser and a 5 mW krypton-argon laser. Biofilms of Rv/25, Rv/37 and Stv were allowed to accrue for 45 h on 12-mm round glass coverslips as previously described (see adhesion assay). Planktonic cells were gently removed by submerging glass slides in PBS three times. Preliminary trials showed that this protocol removed most planktonic cells (data not shown).

Biofilms were observed using probes as follows: Adherent bacteria were stained with SYTO 9, a fluorescent nucleic acid stain that is part of the Live-Dead staining kit (Molecular Probes Inc., Eugene, OR, USA) that excites at 488 nm and emits at 522 nm. One hundred μl of SYTO 9 (1 part stain to 3 parts water) was gently applied to the biofilm and incubated at room temperature in the dark for 15 min. The coverslip was rinsed for 1 min twice with PBS. Biofilms were then stained with anti-DT104 CP polyclonal antibodies (1:200) in PBS for 30 min at room temperature, triple washed and then incubated with Alexa Fluor 546 goat anti-rabbit IgG (excitation = 556 nm; emission = 573) (Molecular Probes) in PBS (1:3000) for 20 min at room temperature. Biofilms were washed twice in distilled water. Mounted on a glass slide and analyzed with a scanning confocal laser microscope (SCLM). Analysis of digital thin sections

of images was used to determine the thickness of biofilms, bacterial cell area and localization of CP producing organisms within the biofilm.

Results

Screening for CP production by Rv/25, Rv/37 and Stv

Rv and Stv were inoculated on LB agar plates without salt supplemented with Calcofluor and CR and tested for their ability to bind each dye. At ambient temperature, Rv bound significantly more CR and Calcofluor than Stv (Figure 4). Rv formed rugose and red colonies whereas Stv formed smooth colonies with a light red coloration (Figure 4). At 37°C, both Rv and Stv showed a reduced ability to bind CR with no observable dye pigmentation in the outer periphery of each colony. Interestingly, some discrete regions of the Rv colony bound CR with similar intensity as observed at ambient temperature. These regions were also associated with intense fluorescence when cells were grown on Calcofluor-supplented plates (data not shown).

On LB plates supplemented with Calcofluor, Rv colonies fluoresced with stronger intensity than Stv colonies during exposure to an UV light source (Fig. 6). At 37°C, Rv colonies fluoresced with a slight lower intensity than at ambient temperature. Stv demonstrated much weaker affinity for the dye, which was mainly visualized as a concentric circle in the center of the colony. These results suggested that Rv produces a CP that readily binds CR and Calcofluor at ambient temperature but mainly Calcofluor at 37°C. These observations agree with findings that

Salmonella typhimurium (non DT104) produces thin aggregative fimbriae and cellulose preferably at ambient temperature (Zogaj *et al*., 2001).

Adherence assay

Anriany *et al.* (2001) showed that Rv formed a more copious biofilm than Stv on the inner surface of glass flasks. In this study, cells of Rv and Stv adhering to glass coverslips after incubation at ambient temperature were visually counted under a Zeiss Aziophot microscope at 630x magnification. Ten fold more Rv cells bound to glass surfaces than Stv (Figure 6). This difference was conservative considering that in various areas Rv biofilms had developed into multi-layered structures where cell numbers could not be accurately counted. A second adherence study quantified the number of attached cells of Rv and Stv on the inner surface of polyvinyl test tubes incubated for 9 days at ambient temperature and 37°C (Figure 7). Visual inspection of test tubes after biofilms accrued at ambient temperature showed an indistinguishable

binding intensity between Rv and Stv after staining with CV. This delayed adherence by Stv was probably due to a defective pathway in its ability to produce and assemble the components of the biofilm. However, the quantification of CV released after treatment with ethanol-acetone solution showed that the Rv biofilm bound higher amounts of the dye.
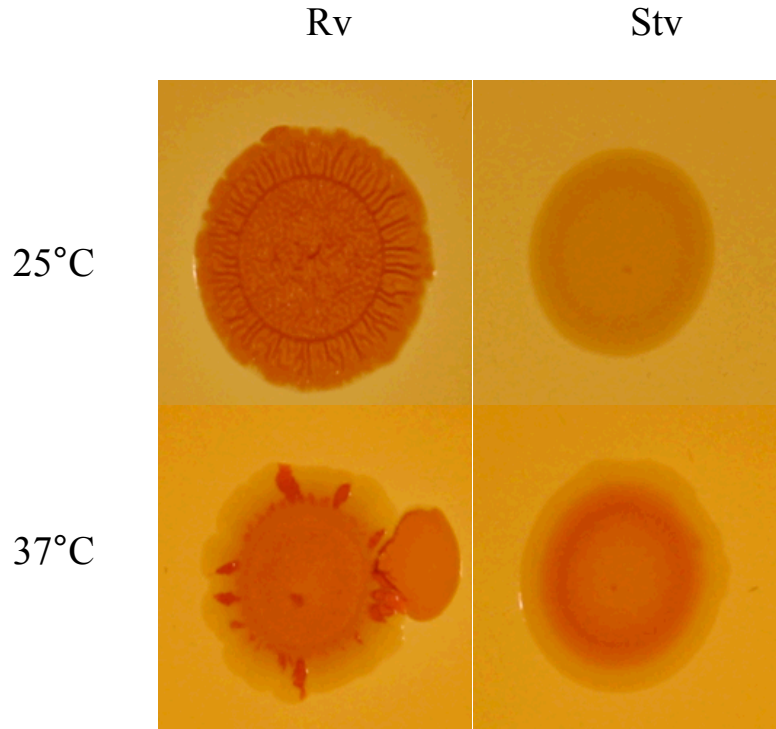
FIGURE 4. Morphotypes of colonies of *Salmonella enterica* serovar Typhimurium
DT104 rugose (Rv) and smooth variants (Stv) on Luria-Bertani agar plates (LB)
without salt supplemented with Congo red (CR; 60μgml^{-1}) and Coomassie Blue (CB;
20μgml^{-1}). Plates were incubated with 5μl of and overnight culture of Rv and Stv in
LB broth incubated at 37°C. LB agar plates were incubated at 25 and 37°C for 4 days
and 2 days, respectively.
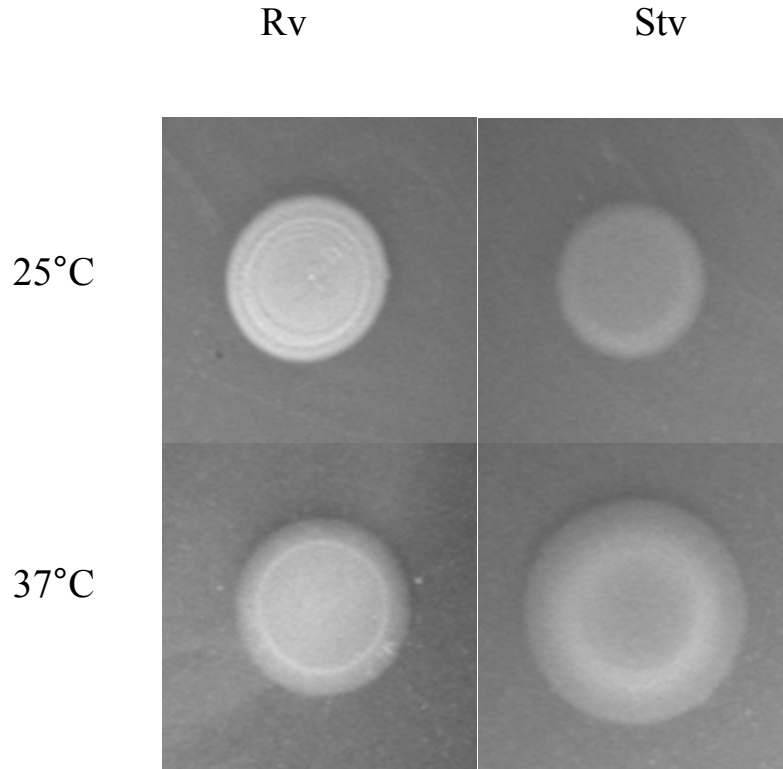
FIGURE 5. Colonies of *Salmonella enterica* serovar Typhimurium DT104 rugose (Rv) and smooth variants (Stv) grown on Luria-Bertrani agar plates (LB) without salt supplemented with Calcofluor dye (200μgml^{-1}). Plates were incubated with 5μl of and overnight culture of Rv and Stv in LB broth incubated at 25 and 37°C for two and four days, respectively.
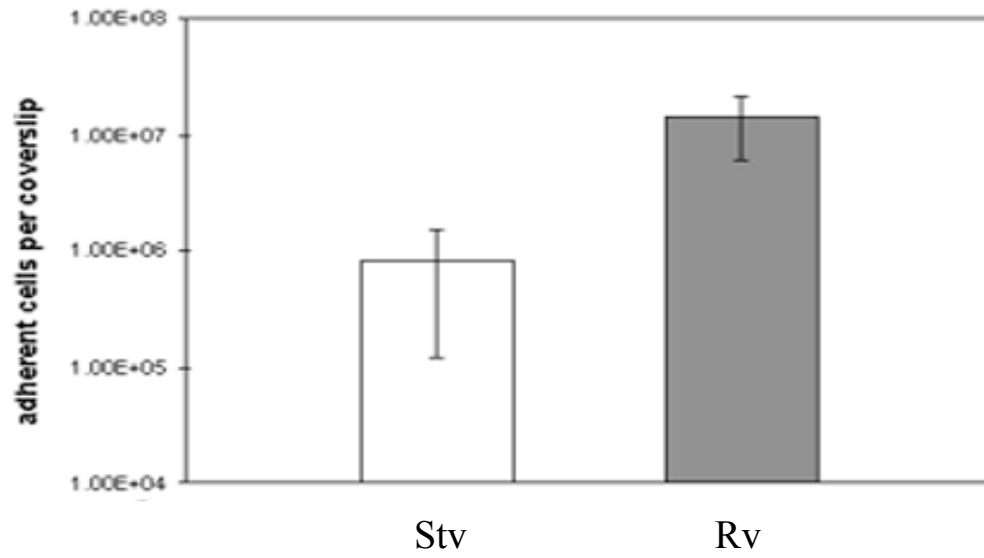
FIGURE 6. Adhesion of *S. enterica* serovar Typhimurium DT104 smooth (Stv) and rugose variants (Rv) on glass coverslips after 24 h of growth in Luria-Bertani broth (LB) at 25°C. Cells were triple washed with distilled water and stained with acridine orange (1% solution for 5 min). Seven separate areas of 0.016 mm^2 were quantified on two coverslips using a Zeiss Axiophot microscope at 630 x magnification and the total number of attached cells to coverslips was averaged and calculated.
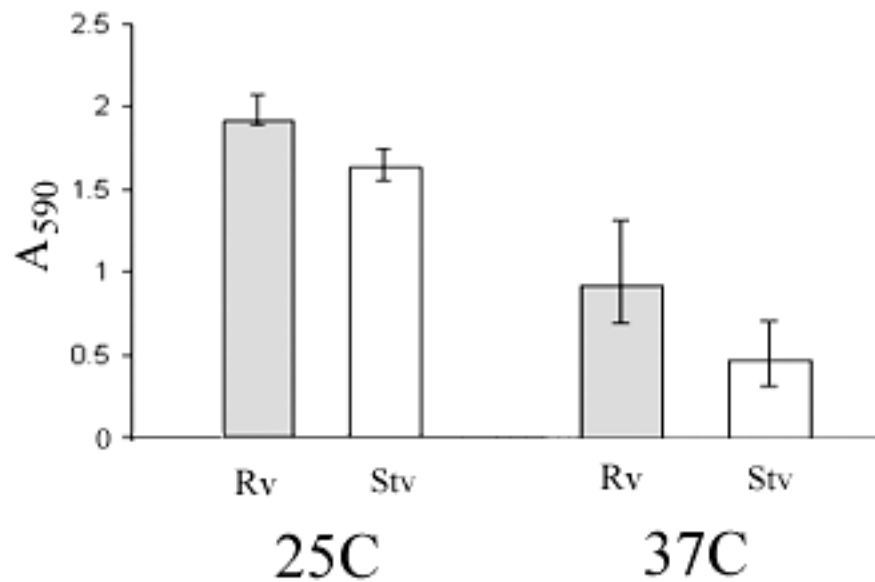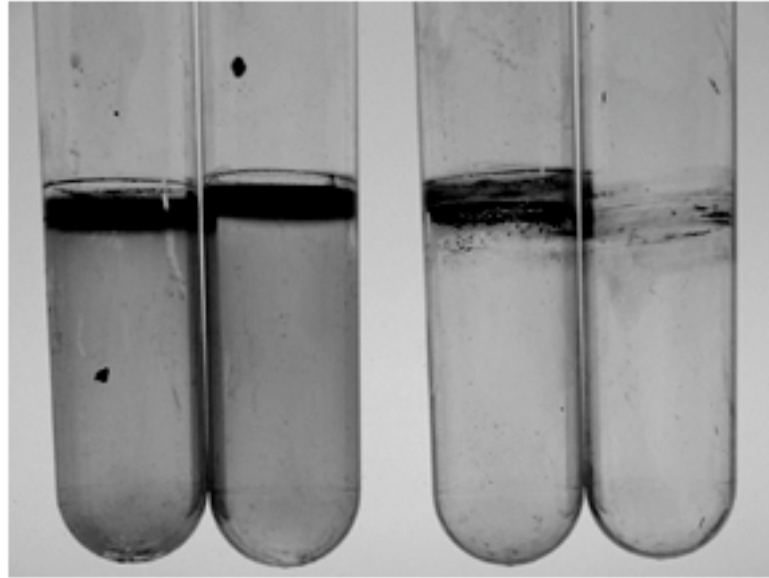
FIGURE 7. Adherence to plastic test tubes by *Salmonella enterica* serovar Typhimurium DT104 rugose variant (Rv) and smooth mutant (Stv) after growth in Luria-Bertani broth (LB) at 25°C and 37°C for 9 days without shaking. Tubes were inoculated in triplicates. After incubation, the spent medium was discarded and tubes were rinsed with Phosphate Buffered solution (PBS). Adherent cells were stained with a 0.4% crystal violet (CV) solution and rinsed with PBS and the dye was made soluble with a 20:80 acetone-ethanol solution. The amount of CV staining the cells was quantified using a spectrophotometer at 590 nm.

At 37°C, Rv and Stv cells were not able to form confluent biofilms and strong binding to the walls of the test tube was predominantly observed at the area of air-medium interphase. The binding of Rv was more intense than that observed for Stv, which was confirmed by the quantification of CV dye. At 25°C, Rv bound more dye than Stv; however, both strains bound significantly more dye than at 37°C.

Purification and analysis of STDT104 CP

The final CP extract was white and had a fluffy consistency. According to the carbohydrate, BCA and limulus assays, the final extract was composed of approximately 63% carbohydrates, 10% proteins and 2% LPS, respectively. The sugars contained in the CP-extract were determined using FACE monosaccharide composition analysis. STDT104-CP was hydrolyzed to individual sugars by treatment with TFA and HCL for neutral and amine sugar analysis, respectively.

Face analysis determined that the STDT104 CP extract contained mannose, glucose and galactose with glucose occurring as the predominant sugar (figure 8). There was no difference in the band intensity between analysis of neutral and amine sugars (Figure 8; lanes 1 and 2, respectively).
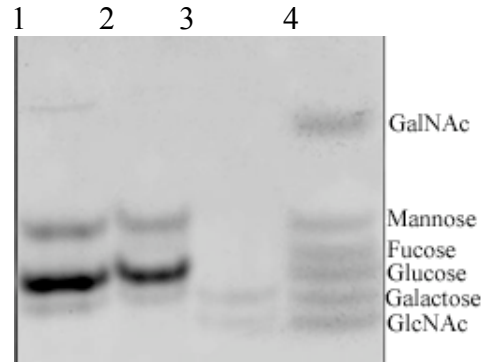
FIGURE 8. FACE monosaccharide composition analysis of capsular polysaccharides (CP) extracted from *Salmonella enterica* serovar Typhimurium DT104 (STDT104) rugose variant (Rv) after growth in Trypticase Soy broth (TSB) at 37°C for 5 days. Lanes: 1, Rv CP extract; 2, Rv CP extract analyzed for acetylated sugars; 3, MONO reacetylation control; 4, MONO control ladder standard (100 pmol of each monosaccharide).

SDS-PAGE analysis of STDT104 CP extract

Total proteins in cell lysate and STDT104-CP samples were detected on PVDF membranes using SYPRO Ruby protein blot stain (sensitivity of 2-8 ng of protein per band) (Figure 9A). The location of protein bands for cell lysate samples of Rv/25, Rv/37, Rv/25w (grown for 1 week at 25°C), Stv were identical; however, the band strengths obtained for Rv/25 (Figure 9, lane 4) were stronger than those for Rv/37 (Figure 9, lane 5). Proteins were not detected in the STDT104-CP sample (Figure 9, lane 7).

The specificity of polyclonal antibodies raised against the STDT104-CP extract was tested against the exopolysaccharide of *H. adhaerens* and cell lysates of Rv/25, Rv/37, Stv, *E. coli*. Polyclonal antibodies did not bind the EPS of *H. adhaerens* (data not shown), which has been described to have high affinity for lectins specific for *N*-acetyl-D-galactosamine (Quintero and Weiner, 1995). Unabsorbed antibodies reacted with STDT104-CP transferred to PVDF membranes with strong affinity resulting in a diffuse region running from above the 216kD molecular weight marker band to the 78kD band (Figures 9B and 9C; lane 7). On some blots, a ladder-like sequence of bands was observed immediately under the 78kD molecular weight mark (data not shown). Pre-immune serum did not react with the CP-extract nor with cell lysate samples. This observation was confirmed using immunoelectron microscopy, where colloidal gold-conjugated secondary antibodies did not label cells of Rv/37that had been incubated with pre-immune serum (data not shown).   Immunoblots of cell lysates of Rv/25, Rv/25w, Rv/37 and *E. coli* revealed that polyclonal antibodies also
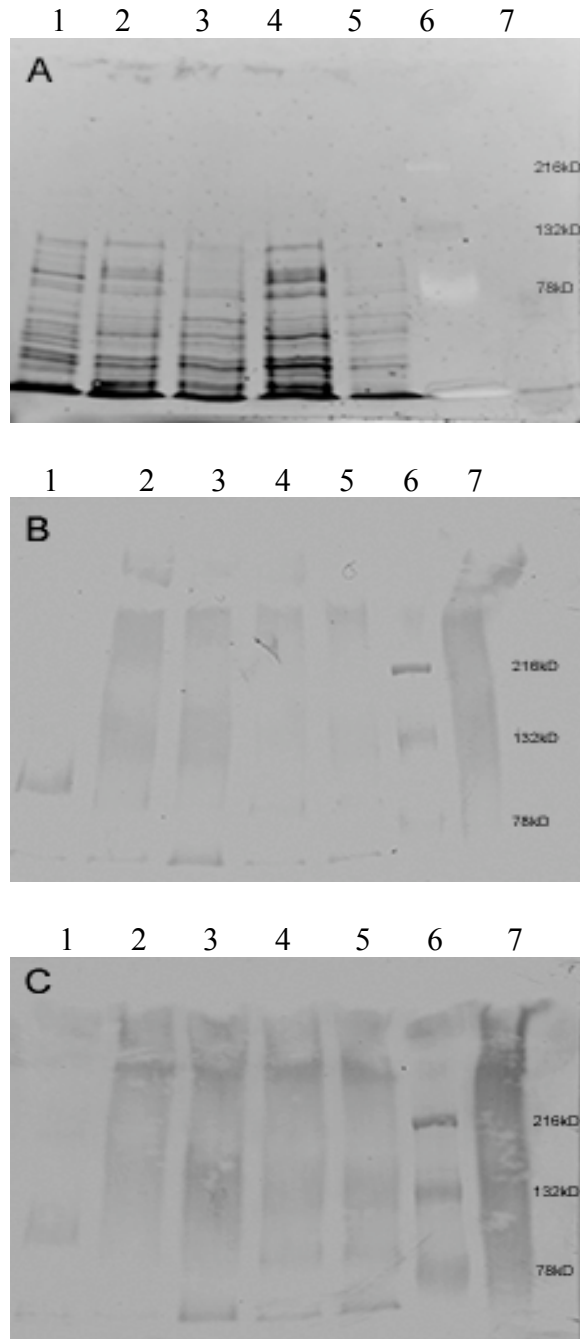
FIGURES 9A, B and C. SYPRO Orange protein gel stain analysis (A) and electroblots (B and C) of *Escherichia coli* DH5α grown at 37°C (1), *Salmonella enterica* serovar Typhimurium DT104 (STDT104) smooth variant (Stv) grown at 25°C (2), STDT104 rugose variant (Rv) grown at 25°C for 1 week (3), STDT104 Rv grown at 25°C for 24h (4), STDT104 Rv grown at 37°C for 24h (5), kaleidoscope prestained standards (6) and STDT104 Rv capsular polysaccharide extract (7).
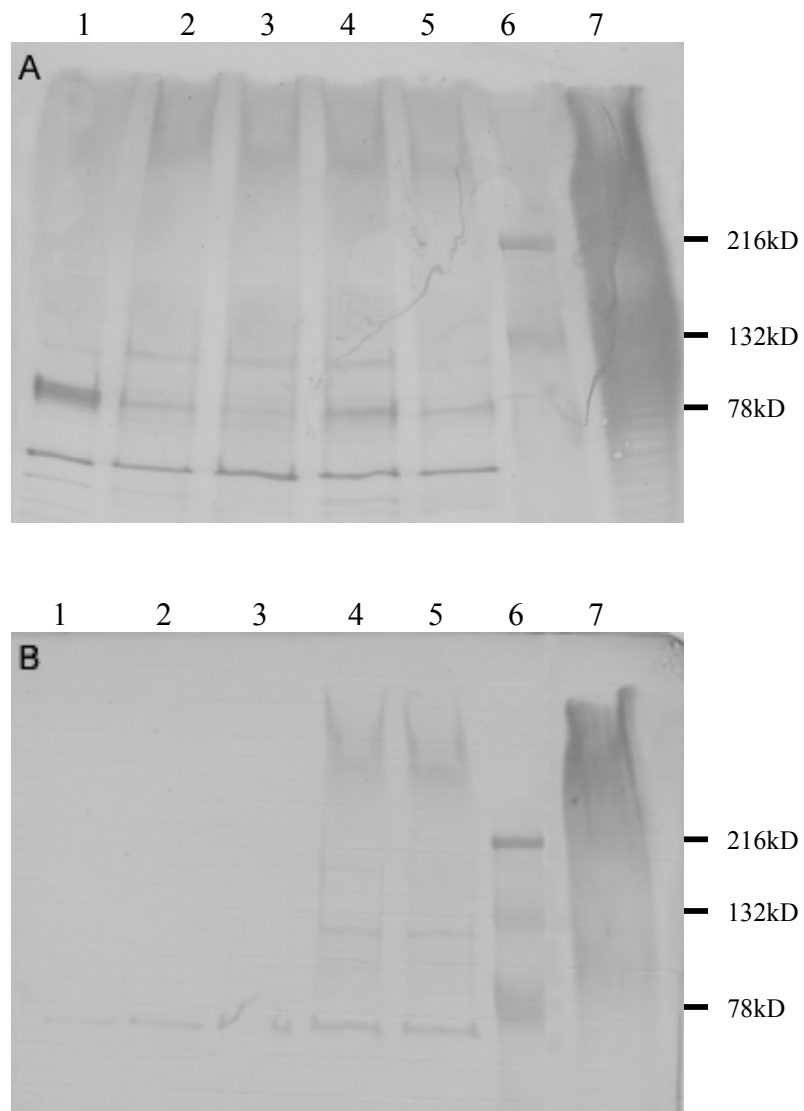
FIGURES 10 A and B. Electroblots of *Escherichia coli* DH5α (1), *Salmonella enterica* serovar Typhimurium DT104 (STDT104) smooth mutant (Stv) grown at 25°C (2), STDT104 rugose variant (Rv) grown at 25°C for 1 week (3), STDT104 Rv grown at 25°C for 24h (4), STDT104 Rv grown at 37°C for 24h (5), kaleidoscope prestained standards (6) and STDT104 Rv capsular polysaccharide (CP) extract (7) using anti STDT104 CP antibodies (A) and anti-CP antibodies absorbed nine times with *E. coli* acetone powder (B).

bound two distinct bands present in all cell lysates located immediately above and below the 78kD marker (Figure 10A, lane 1). These bands disappeared when cell lysates were treated with proteinase K for 18 h at 37°C before loading onto gels (data not shown). The signal for the heavier of the two proteins was especially strong for *E. coli*. The strength of this signal was significantly reduced after adsorbing out cross-reacting antibodies using *E. coli* acetone powder (five times, 20 min each time) (data not shown). The band became invisible after increasing the number of adsorption steps from five to nine (Figure 10B, lane 1). Although the binding intensity of the antibodies to the STDT104-CP using penta-adsorbed antibodies was similar to that obtained with non-adsorbed serum, the signal strength to the STDT104-CP was reduced when using antibodies that had been adsorbed against *E. coli* acetone powder nine times (Figure 10A vs. 10B, lane 7).

Immunoelectron microscopy

The reactivity of the anti-STDT104-CP polyclonal antibodies was tested with cells of Rv/25, Rv/37, Rv/25w, Stv, *E. coli* and *H. adhaerens*. The colloidal gold particles conjugated to secondary antibodies were seen in regions of the outer membrane of STDT104, consistent with the presence of a capsular material (Figures 11, 12, 13). Binding was less intense for Rv/25 (Figure 12). Cells not labeled with colloidal gold were observed in all sample preparations. In fact, cells that did not react with antibodies were seen immediately adjacent to cells that were strongly labeled by antibodies (Figure 14). This confirmed observations obtained in preliminary trials using fluorescence microscopy, which revealed that only a portion of all cells subjected to antibody labeling fluoresced upon UV excitation (data not
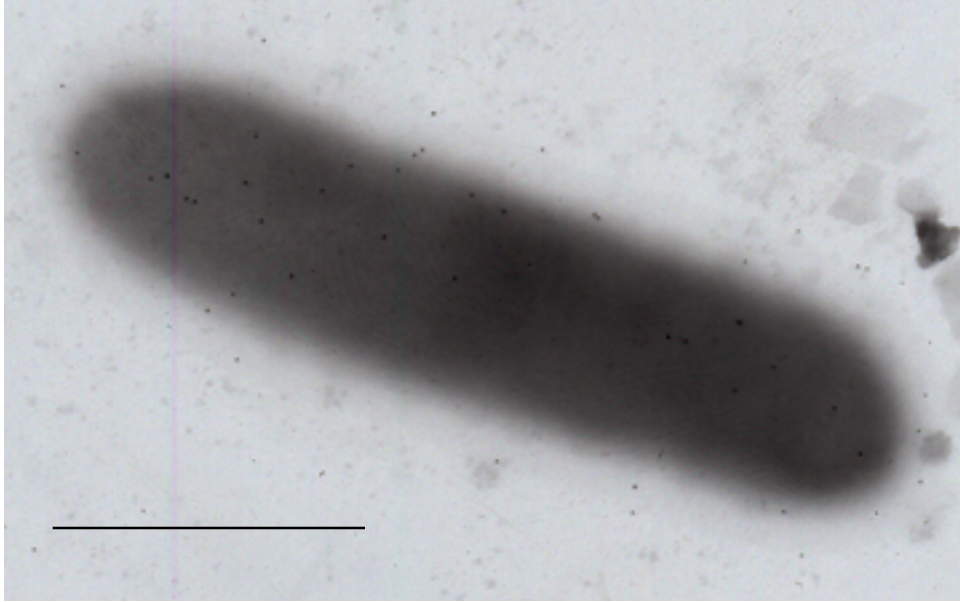
FIGURE 11. Immunoelectron micrograph of *Salmonella enterica* Serovar Typhymurium DT104 rugose variant (Rv) grown at 37°C and probed with anti-CP polyclonal antibodies. Magnification: 25,000x. Scale bar = 1µm.
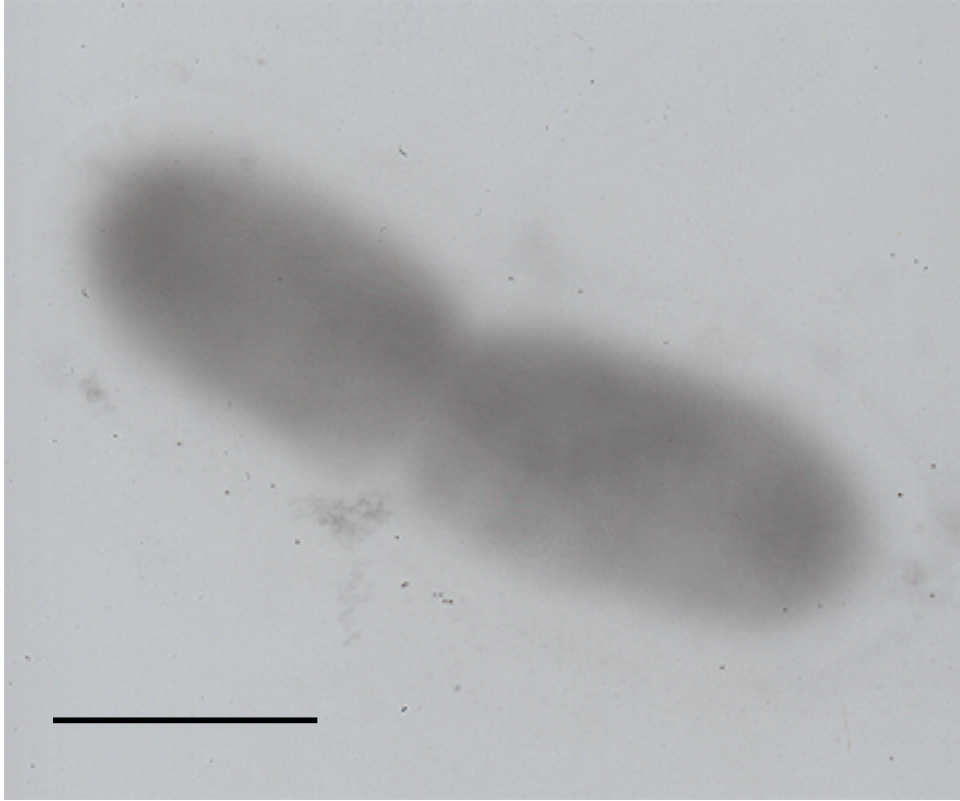
FIGURE 12. Immunoelectron micrograph of *Salmonella enterica* Serovar Typhimurium DT104 rugose variant (Rv) grown at 25°C and probed with anti-CP polyclonal antibodies. Magnification: 25,000x. Scale bar = 1μm.
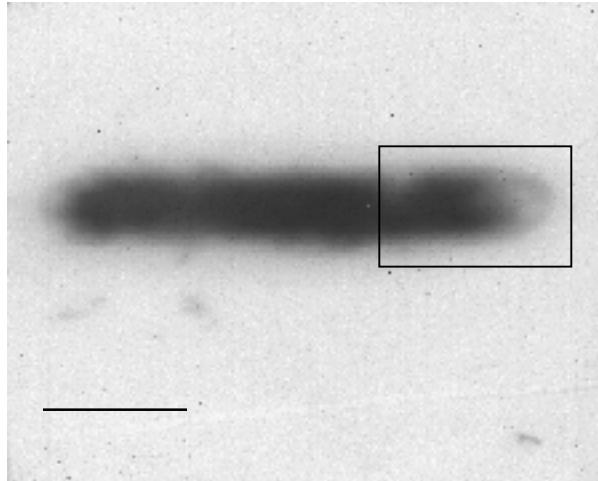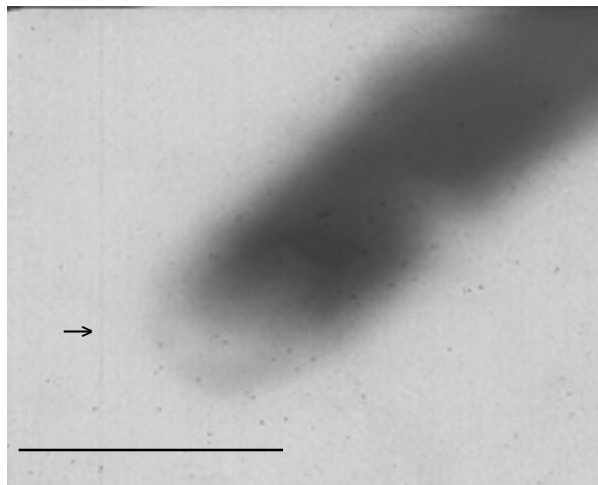
FIGURES 13A and B. *Salmonella enterica* serovar Typhimurium DT104 rugose variant (Rv) cells grown for 1 week at 37°C. Cells were labeled with anti-CP antibodies followed with secondary antibodies conjugated to 12 nm colloidal gold (A). Arrow shows the affinity of the antibodies to the organism (B). Magnifications of A and B are 20,000 and 40,000 x, respectively. Scale bar = 1µm.

FIGURE 14. *Salmonella enterica* serovar Typhimurium DT104 rugose variant (Rv) cells grown for 1 week at 37°C exhibiting aggregative behavior. Cells were labeled with anti-CP antibodies followed with secondary antibodies conjugated to 12 nm colloidal gold. Arrow (→) shows a cell, to which antibodies bind with greater affinity than others. Magnification = 20,000 x. Scale bar = 1 μm.

shown). Controls using cells of Rv/37 incubated with pre-immune serum or secondary antibodies only were consistently negative when examined with colloidal gold particles (Figures 15A and B).

Adsorbed antibodies bound cells of *E. coli*, although with less frequency and intensity (Figures 16A and B) and did not label cells of *H. adhaerens* (data not shown).

Lectin-gold labeling of Rv/25

A preliminary study using a battery of 13 different lectins with specificities for several carbohydrates revealed that biofilms of STDT104 probed with Fluoroisothiocyanate (FITC) labeled lectins specific for mannose and glucose fluoresced with strong intensity. Conversely, biofilms stained with lectins specific for galactose fluoresced poorly. These observations were confirmed using gold labeled ConA and HP. The former has a reported specificity for glucose and mannose residues whereas the latter is specific for terminal galactose amine residues. ConA bound to cells of Rv/25 with much greater intensity than HP and was observed surrounding the outer membrane of Rv cells and bound to an extracellular matrix located in the periphery of cells (Figures 17A and B); however, cells that were poorly labeled were observed (data not shown). The lectin HP bound to cells of Rv/25 with a comparatively lower affinity than ConA (Figure 18).
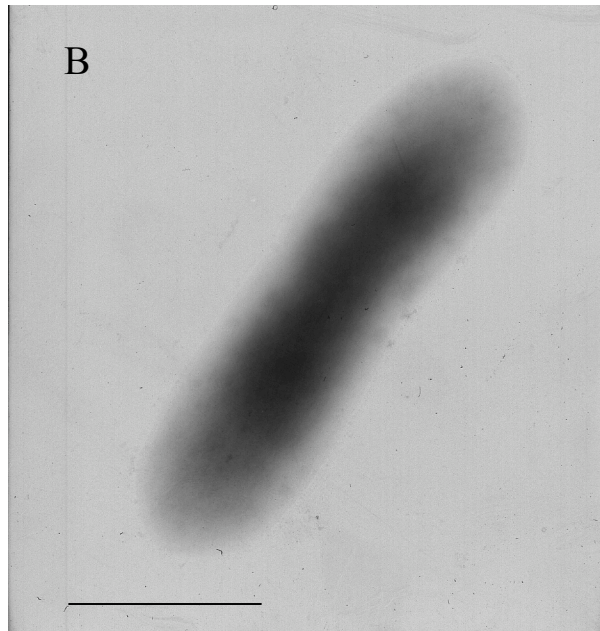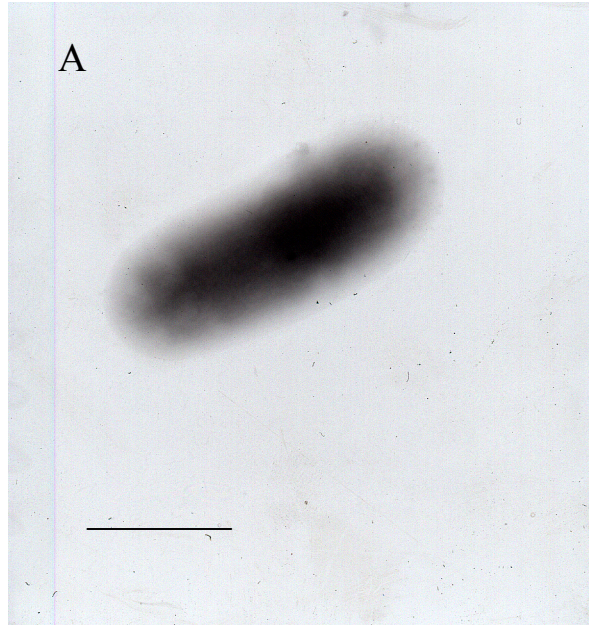
FIGURES 15A and B. *Salmonella enterica* serovar Typhimurium DT104 rugose variant (Rv) grown for 1 week at 37°C. Control cells were probed with pre-immune antibodies followed by secondary antibodies conjugated to 12 nm colloidal gold (A) or secondary antibodies only (B). Note that no binding was observed for either controls. Magnification = 25,000 x. Scale bar = 1 μm.
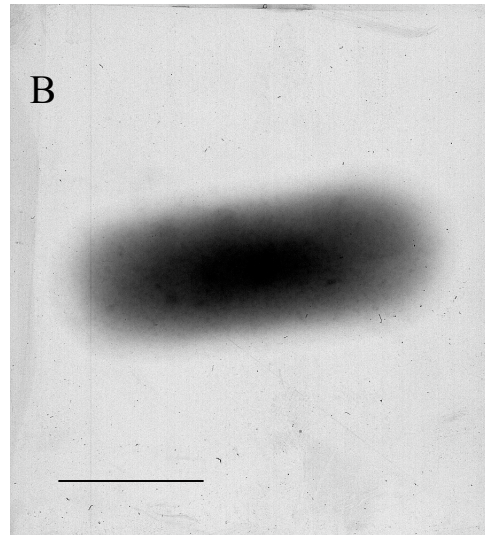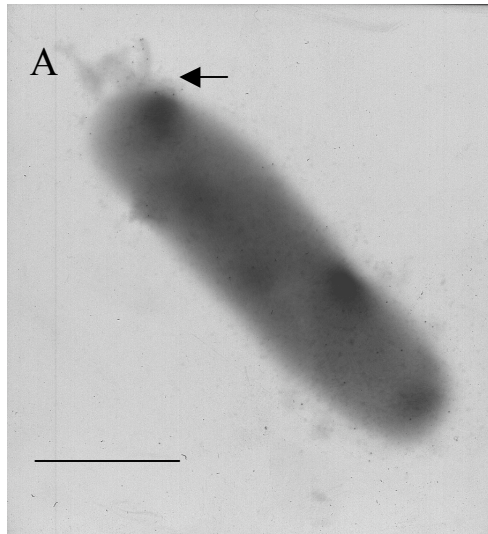
FIGURES 16A and B. *Escherichia coli* DH5α grown for 1 week at 37°C and probed with STDT104 anti-CP antibodies followed by secondary antibodies conjugated to 12 nm colloidal gold. Note that some binding does occurs in A but not in B. Magnification = 25,000 x. Scale bar = 1 μm.
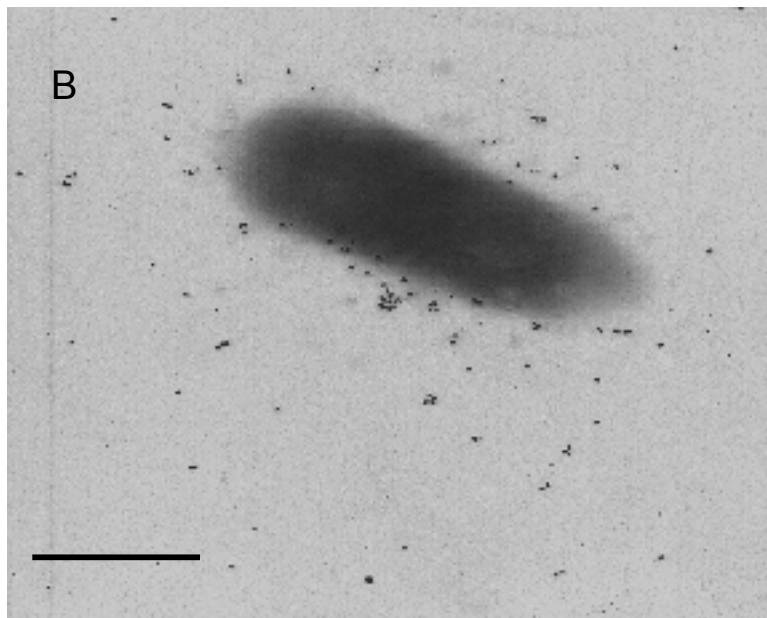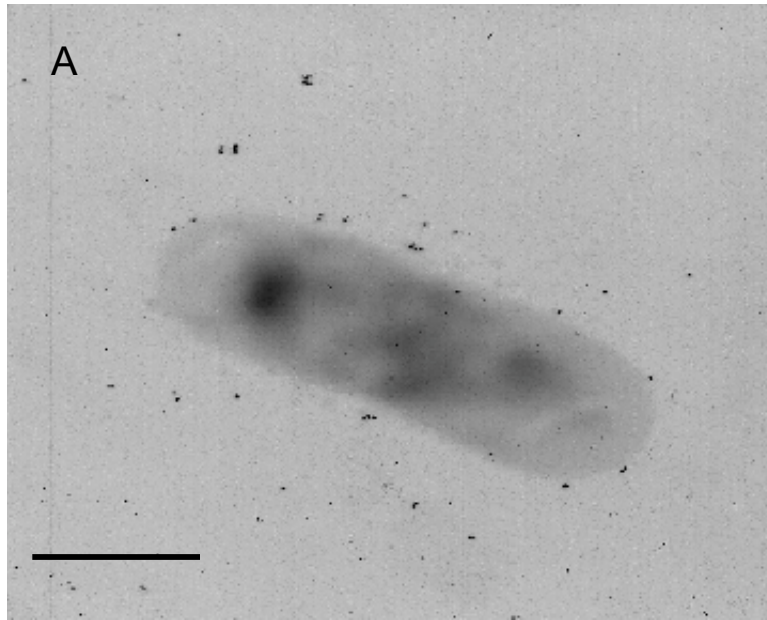
FIGURES 17A and B. *Salmonella enterica* Serovar Typhimurium DT104 rugose variant (Rv) grown for 1 week at room temperature probed with Concavalin A lectin conjugated to 20nm colloidal gold. This lectin, which is specific to mannose and glucose, bound to cells with moderate affinity (A) and high affinity (B), a variation in binding affinity noted in preliminary trials using FITC-conjugated lectins. Magnification: 25,000 x. Scale bar = 1μm.

FIGURE 18. *Salmonella enterica* Serovar Typhimurium DT104 rugose variant (Rv) grown for 1 week at room temperature probed with Helix Pomatia lectin conjugated to 20nm colloidal gold. This lectin, which is specific to terminal N acetyl galactosamine, did not bind to the cells. Magnification: 25,000 x. Scale bar = 1 μm.

Confocal observation of CP

In order to understand more precisely the importance of the capsular material and the localization of CP-producing cells in biofilms of Rv/25, Rv/37 and Stv, Z-series scans of all xy focal planes of biofilms were observed using CSLM. Perspectives of the biofilm structures were analyzed by comparing sagittal and overhead sections of biofilms after 45h of incubation (Figure 19). Rv/25 formed pillars of cells that were approximately 12μm high (Figure 19A). The thickness of the biofilm reached 20μm in some areas of the glass coverslip (data not shown). Cells on the top of biofilms appeared to be loosely suspended over the glass surface and attached to cells at lower depths by a structure that could not be visualized. Observation of overhead sections of the biofilm showed that Rv/25 formed clusters of densely packed cells that were often associated with production of CP based on fluorescent-linked antibody detection. Furthermore, the number of cells involved in the primary colonization of the glass surface was greater for Rv/25 than for Rv/37 and Stv. Rv/25 also formed a more confluent biofilm on glass surfaces while Rv/37 and Stv formed random islands of monolayer biofilms surrounded by uncolonized areas. Furthermore, monolayers of Rv/37 and Stv cells were not as dense as those observed for Rv/25 (Figures 19B, C, E and F). Occasionally, biofilms of Rv/37 and Stv did develop into multilayered structures; however, these structures contained few cell-cell interactions and did not achieve the depth observed for Rv/25.
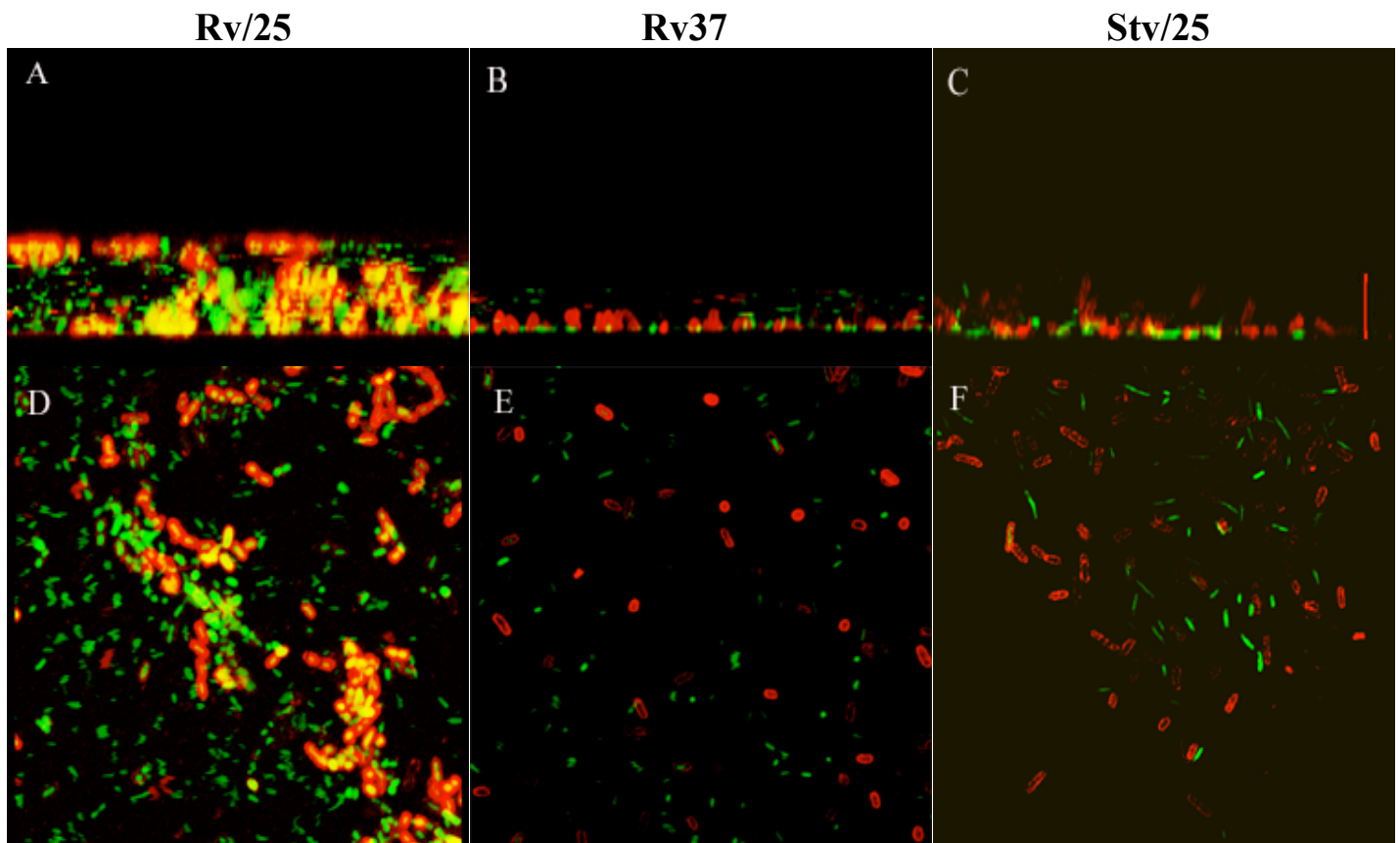
FIGURES 19A, B, C, D, E and F. Sagittal (A, B and C) and overhead (D, E and F) images of *Salmonella enterica* serovar Typhimurium DT104 (STDT104) rugose variant (Rv) and smooth mutant (Stv) biofilms formed on glass coverslips. Cultures were grown in Luria-Bertani broth (LB) without salt with shaking (100 rpm) for 45h at 25°C and 37°C. Coverslips were rinsed with sterile distilled water to remove unattached cells and biofilms were dually stained with the nucleic acid stain, SYTO 9 and STDT104 anti-capsular polysaccharide (CP) polyclonal antibodies followed with Alexa Fluor labeled secondary antibodies. Stained biofilms were examined under a confocal scanning laser microscope using a 100x oil immersion objective (numerical aperture, 1.4). Sagittal and overhead images were acquired from a collection of 36 consecutive Z-series scans of each biofilm, each taken at 0.4μm and 0.9μm intervals, respectively. Side bar = 10μm.

Discussion

> Rv synthesizes thin aggregative fimbriae and polysaccharides at 25°C
>
> but primarily polysaccharides at 37°C

Anriany *et al*. (2001) described a strain of STDT104 (Rv/25) that formed rugose colonies and pellicles in a temperature-sensitive fashion. The aim of this study was to investigate if STDT104 produces a polysaccharide associated with its extracellular matrix and if so, its role in rugosity, pellicle and biofilm formation.

Rugosity and biofilm formation have been associated with the ability of some organisms such as *H. adhaerens*, *V.cholerae* El Tor and *V. cholerae* O139 to synthesize extracellular polysacharides (Quintero and Weiner, 1995; Yildiz and Schoolnik, 1999; Preston *et al*., 1995). *S. enteritidis* was shown to form rugose colonies (lacy phenotype) in a temperature-dependent fashion that were associated with the production of an extracellular matrix composed of high molecular weight O-antigens and other proteins (Guard-Petter *et al*., 1996). Recently, cellulose was identified as a component of the extracellular matrix of *E. coli*, *S. typhimurium* and *S. enteritidis* (Zogaj *et al*., 2001; Solano *et al*., 2002). The function of cellulose was presumed to stabilize the interaction between cells in a biofilm by forming non covalent bonds with thin aggregative fimbriae, the primary component of the extracellular matrix (Zogaj *et al*., 2001).

Synthesis of thin aggregative fimbriae by *E. coli* and *Salmonella* has been associated with the formation of rugose colonies on agar and pellicles in broth. These organelles, which permit organisms to form tight intercellular bonds and adhere to

abiotic surfaces, bind CR and serum proteins such as fibronectin (Bian and Normark, 1997; Romling *et al.*, 1998). For example, *Salmonella typhimurium* strain SR-11 has been reported to express thin aggregative fibers at ambient temperature that are responsible for the formation of rough and dry colonies that bound CR dye (Romling *et al.*, 1998).

The possible presence of thin aggregative fimbriae and a polysaccharide in the extracellular matrix of Rv was screened using LB agar plates supplemented with CR as described elsewhere (Zogaj *et al.*, 2001). Bacterial synthesis of either thin aggregative fimbriae or cellulose results in colonies with either a brown or pink coloration, respectively.

In this study, Rv formed red and rugose colonies at 25°C but pink and smooth colonies at 37°C. Additionally, Rv bound Calcofluor at both temperatures (Figure 5). These preliminary results indicated that Rv possibly synthesized fimbriae and polysaccharides at ambient temperature but mostly polysaccharides at 37°C. Furthermore, previous studies have associated the synthesis of both extracellular components with the ability of the organism to form rigid pellicles in the air-medium interphase whereas the synthesis of either component resulted in formation of fragile pellicles (Solano *et al.*, 2002). In this study Rv was more adherent to abiotic surfaces and formed rigid and hydrophobic pellicles of interconnected cells in the air-medium interphase at 25°C. At 37 °C, Rv did not adhere as well to surfaces and exhibited some autoaggregation in liquid culture that was easily disrupted by gently shaking. These observations support the possibility that Rv synthesizes polysaccharides predominantly at 37°C. However, Rv colonies grown on agar at 37°C did exhibit

isolated regions where CR was readily absorbed (Figure 4). These regions may represent sub-populations that are capable of secreting thin aggregative fimbriae albeit not in sufficient quantities to allow pellicles or biofilms to form. Analysis of Rv biofilms using confocal microscopy showed that at 37°C, the organism occasionally formed pillars of cells that were much shorter than those obtained at 25°C (data not shown).

In contrast to Rv/25, Stv did not bind CR at ambient temperature after 4 days of incubation. However, upon extended incubation at 25°C (> week), Stv bound CR on agar plates and formed pellicles in broth. This delayed ability to exhibit multicellular behavior correlated with the ability of Stv to adhere to the walls of test tubes at levels similar to Rv/25 after growth for 9 days (Figure 7). This suggests that Stv, a spontaneous smooth mutant, may possess a defect in the biogenesis of thin aggregative fimbriae. At 37°C, colonies of Stv did acquire a pink coloration as a concentric circle in the middle of the colony that also bound Calcofluor. However, the ability of Stv to adhere to the walls of test tubes was inferior to Rv/25 (Figure 7).

CP extraction and the specificity of antibodies

Based on these preliminary results, RV was grown at 37°C to favor the conditions where the polysaccharide is the predominant component of the extracellular matrix. Furthermore, the culture was grown to late stationary phase (48 h) since the production of the extracellular matrix in *Salmonella* spp has been reported to occur during this phase (Zogaj *et al*., 2001).

Although small amounts of protein were detected in the final extracellular extract, proteins were neither detected in gels probed with Coommasie blue nor on nitrocellulose membranes probed with the more sensitive Sypro staining kit (Figure 9A, lane 7). Anti-CP polyclonal antibodies showed high affinity to the CP of Rv but not to the CP of *H. adhaerens*. This observation was expected since the CP of these organisms have distinctly different compositions. The CP of Rv possesses glucose as the predominant monosaccharide (Figure 8). The CP will be fully characterized after studying the types of linkages between the monosaccharide monomers of the CP extract. The CP of *H. adhaerens* has been reported to be primarily composed of N-acetyl-galactosamine (Quintero and Weiner, 1995). The affinity of the anti-STDT104-CP antibodies for the CP was supported further by the observation that polyclonal antibodies raised against the LOS of *Microbulbifer degradans*, a marine bacterium, did not react with the CP of Rv (data not shown).

Anti-CP antibodies also reacted with two proteins present in *E. coli*, Rv and Stv that migrated slightly above and below the 78kd molecular weight marker. The bands could represent proteins that were protected from the proteinase K treatment during the procedure to purify the CP extract due to their close association with the extracellular matrix of *E. coli* and *Salmonella*. For example, CsgB is a nucleator that is exposed to the surface of *E. coli* and polymerizes CsgA into curli fibres (Romling *et al.*, 1998). AdrA represents a putative transmembrane protein of *E. coli* involved in the biosynthesis of cellulose (Zogaj *et al.*, 2001).

In order to reduce the strength of the signals observed for *E. coli* cell lysate sample preparation, anti-STDT104-CP cross-reacting antibodies were adsorbed out

with *E. coli* acetone powder. Performing the adsorption procedure five times reduced the intensity observed for *E. coli* while slightly increasing the binding intensity for STDT104-CP. This probably occurred as the anti-CP serum sample became more concentrated with antibodies specific for STDT104-CP epitopes. The use of serum that had been adsorbed nine times with the powder further reduced the intensity of the bands observed for *E. coli*; however, the binding intensity was much reduced for the Rv-CP sample (Figure 10). Therefore, serum adsorbed five times was used for label Rv and Stv cells for immunoelectron microscopy (Figure 9C).

Electron microscopy revealed that antibodies bound to the capsular region of cells of Rv/25, Rv/37 and Stv. However, not all cells were labeled with gold particles suggesting that only a portion of all cells synthesize CP. This was further supported by confocal images that showed that only some cells were labeled with the antibodies. Furthermore, Rv/25 did not label as well as Rv/37 and Stv. This could be due to the presence of a dense matrix of thin aggregative fimbriae surrounding cells, which blocks access of antibodies to their specific carbohydrate epitopes. Also, Rv/25 cells probed with ConA, a lectin specific for glucose and mannose residues, labeled Rv/25 cells much more extensively than HP, which has specificity for terminal galactosamine. This supports the finding that the CP of Rv is mainly composed of glucose and mannose. ConA bound to cells around the capsule and to a peripheral material surrounding the cell (Figures 17A and B). HP lectin did not bind to either region with the same intensity (Figure 18).

Confocal images clearly demonstrate that Rv/25 better colonizes surfaces than Rv/37 when grown at 37°C or than its mutant counterpart (Fig 20). Furthermore,

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Rv/25 forms pillars of cells in 45h that can reach a thickness of approximately 20μm. This was not observed for Rv/37 or Stv. This suggests that the presence of another organelle, possibly thin aggregative fimbriae, may be responsible for superior attachment of Rv/25 cells to abiotic surfaces. As observed by immunoelectron microscopy, Rv/25, Rv/37 and Stv are capable of producing CP. However, CP-producing cells are not necessarily associated with primary colonization of surfaces since non-CP-producing cells are also present on surfaces. It appeared that CP-producing cells tend to be more concentrated towards the top layers of biofilms of Rv/25.

Chapter 5: TESTING FIELD SAMPLES COLLECTED FROM BROILER FARMS AND PROCESSING PLANTS USING FLUORESCENT PROBES

Rationale

The contamination of food products and food-production surfaces with biofilms poses a serious risk to the health of consumers and compromise the profitability of the food industry. Current approaches to reduce contamination in production and processing facilities include proper choice of surface materials and washes using detergents and disinfectants (Kumar and Anand 1998; Tide *et al*., 1999). However, these cleaning procedures do not seem to adequately disinfect surfaces contaminated with bacterial biofilms. For example, although hypochlorite and chill tanks are used to reduce bacterial populations in poultry processing plants (Carr *et al*., 1999), a recent study has shown that live-haul poultry containers and truck beds were inefficiently decontaminated and did not remove coliforms and *Salmonella* from surfaces (Carr *et al*., 1999).

Similarly, in the dairy industry, cleaning-in-place procedures (CIP) inadequately sanitized equipment, resulting in post-pasteurization contamination of milk and milk-products. Biofilms have been shown to accrue on gaskets, endcaps, vacuum breaker plugs and pipelines in milk production facilities (Austin and Bergeron, 1995). Biofilms also cause damage in waste water treatment facilities and in drinking water distribution systems usually by clogging membranes and pipes, affecting the efficiency of the operation (Kumar and Anand, 1998).

Other production areas that may harbor biofilms include floors, waste water pipes, bends in pipes, conveyor belts, stainless steel surfaces and cracks formed from corroded equipment (Kumar and Anand, 1998). For example, *Listeria monocytogenes* has been shown to attach and accumulate on stainless steel and Buna-N rubber surfaces, which are two types of material that are widely used in the food industry (Somers *et al.* 1994). Biofilms can also form on food animal tissue contributing to the spread of bacteria from contaminated to uncontaminated carcasses during the slaughtering and processing stages of food production (Kumar and Anand, 1998).

In conclusion, biofilms pose a problem to the food industry by compromising the quality and safety of food products and may cause equipment to malfunction.

Materials and methods

Locales

A commercial broiler house and a processing plant in the Eastern Shore of Maryland were sampled for biofilm contamination.

Broiler house

In broiler housing, sampling was restricted to surfaces consistently exposed to moisture such as water lines, hoses, foggers and feeders.

Using a template, each surface area of approximate 50 cm^2 was swabbed for 15 sec. Each swab was placed in 10 ml of buffered peptone water (BPW) contained in a 15ml centrifuge tube and transported to the laboratory for immediate culturing of *Salmonella* spp. The protocol used has been described elsewhere (Mallinson *et al.*,

2000). Briefly, each swab in BPW was incubated for 24 h at 41°C and 0.5 ml was transferred to 10 ml Tetrathionate broth (TTH). The tube containing TTH was incubated for 24h at 37°C and 30 μl aliquots were streaked for isolation on Miller-Mallinson agar (MM). Triple Sugar Iron (TSI) and Lysine Iron Agar (LIA) slants were inoculated with suspect colonies. Antisera to *Salmonella* O antigen was used to confirm each suspect isolate. Delayed secondary enrichment (DSE) was performed on all samples that tested negative after the first BPW-TTH enrichment procedure to detect *Salmonella* in viable but non-culturable states.

In addition to swabs, each surface was scraped using sterile razor blades and the biofilm-suspected materials were placed in whirl-pack bags. In the laboratory, a small amount of each sample was incubated in BPW and tested for *Salmonella* using BPW and TTH as described above. Small amounts of sample were stained with Calcofluor and observed under epifluorescence microscopy. Briefly, small amounts of presumed biofilm material were mixed with a drop of dH$_2$O and air fixed to glass slides. After fixation, each slide was incubated with 200μl of Calcofluor solution for 2h at 37°C in the dark. After incubation, the staining solution was gently rinsed using dH$_2$O and the sample was analyzed.

Water samples were stained in a similar way. Aliquots of 200μl of each sample were transferred to an Eppendorf tube and an equal volume of Calcofluor was added. The solution was thoroughly mixed and incubated for 2h at 37°C. The sample was centrifuged (10,000 rpm for 5 min), the supernatant was discarded and the pellet was suspended again in an equal volume of dH$_2$O. The procedure for staining with lectins was the same.

Furthermore, a naturally contaminated plastic tray was collected. The tray was triple washed (30 sec each time) with pressurized hot water. The surface was sprayed 5 times (approximate volume of 1 ml per spray application) with Calcofluor (80μl/ml) and was left at room temperature (RT) for 15 min. The staining solution was rinsed and the surface was exposed to UV light (figure 20).

Processing plant

The processing plant was sampled (total of 17 samples) in identical locations before and after the beginning of operations. Sampling of surfaces was restricted to the rehang belt, shackles and a metal shoot at the end of belt area. Areas from the center and edge of the belt (approximately 100 in^2) were sampled using sterile stainless steel surgical blades. Each area was scraped and the blade was placed inside a sterile 15 ml centrifuge tube for transport to the laboratory. Gloves were changed between the collection of each sample. After each area was sampled, the location was also swabbed (each swab was composed of two cotton swabs moistened in skim milk) and processed for *Salmonella* using BPW and TTH as previously described. Water samples also were collected from the following areas: neck cutter, reprocessing area, carcass wash and chiller overflow and tested for *Salmonella*.

In this trial, some swabs were also tested for the presumptive presence of enterococci spp. Three ml of swab suspension in BPW were transferred to 7 ml of Enterococcosel media (Becton-Dickinson, Cockeysville, MD) and incubated for 36 h
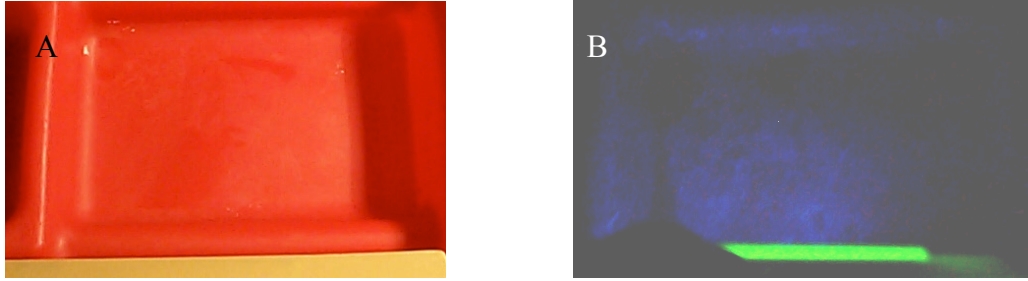
FIGURE 20. A naturally contaminated poultry-feeding tray probed with Calcofluor. Broiler feeding tray that appears clean after being triple washed with pressurized hot water (30 sec each time) (A); Same tray exposed to UV light after being stained with Calcofluor and rinsed (B). Regions that fluoresce represent areas that are not clean, potentially contaminated with biofilms.

at 37°C. Samples were plated on Enterococcosel agar (Becton-Dickinson). Both broth and plates were observed for a change to a dark coloration of colonies. Some colonies were picked and Gram stained. Scraped samples were stained with Calcofluor and lectins as previously described.

Results

Broiler House

Results from this trial using the probes directly in the house were inconclusive. Calcofluor was shown to be highly sensitive against biofilms grown *in vitro* (Eriksson de Rezende *et al*., 2003); however, when tested *in situ*, the dye bound to wood chips, which contains cellulose, and other debris resulting in false positives. Furthermore, the stain was applied to metal surfaces such as water pipes and gently removed using sterile water. Stained surfaces faintly fluoresced when exposed to UV light partially as a result of the indoor sunlight. Furthermore, it was not possible to determine if the fluorescence was due to binding of the dye to biofilms, to dirt or Calcofluor's inherent background fluorescence.

All swabbed surfaces tested negative for *Salmonella* and scraped material did not indicate the presence of biofilms.

Processing Plant

Only two samples tested positive for *Salmonella* after growth in BPW and secondary enrichment in TTH broth. One consisted of a sample of water collected from the chiller tank. The second consisted of water collected from a dripping

carcass. These two samples tested positive for biofilms using Calcofluor as a probe, where strongly fluorescing and well-demarcated biofilms could be easily viewed (data not shown). Results using lectins were less conclusive for specific organisms. Biofilm-like structures bound strongly to GNA and Lotus A (binding score of 3) and weakly to PSA, AAA and MPA, which was expected based on empirical data accessing the specificity of lectins to pure culture biofilms (Table 3). However, a binding score of 2 was noted for BPA, which in previous trials had shown to have no affinity for biofilms of *Salmonella* DT104 and *S. berta*. Another sample collected from a dripping carcass was highly contaminated with *E. coli* and also tested positive for biofilm with Calcofluor.

The remaining samples tested negative for *Salmonella* and staining with Calcofluor failed to identify biofilm-like particles; however, these samples were contaminated with gram positive organisms based on gram stain and culture observations.

Interestingly, one shackle was sampled in the morning when the premises were presumed disinfected. This shackle had a visible accumulation of a lipid-like material, and tested positive for *E. coli* in the laboratory.

Swabs of surfaces taken during the morning before the processing of birds were lightly contaminated with gram negative organisms. All samples tested negative for *Salmonella* and four of nine samples tested positive for lactose fermentors. However, eight samples of nine were presumptively positive for enterococcus spp. The three samples collected in the afternoon were negative for *Salmonella* and lactose fermentors.

Biofilms formed in most tubes containing swabs and BPW after prolonged incubation at RT. Calcofluor bound to these biofilms, which fluoresced with a strong intensity.

Discussion

The development of probes that detect biofilms on surfaces in food preparation facilities will help food inspectors and producers develop strategies targeted at identifying and treating areas that are prone to contamination. In preliminary field trials, Calcofluor was assessed as an *in situ* probe to detect biofilms; however, several practical problems were encountered during these studies.

The general lack of cleanliness of broiler houses made the use of Calcofluor impractical. Calcofluor bound to dust-covered surfaces and wood shavings, the most common source of bedding in houses. In those rare cases when dirt was not an impediment to staining (e.g., some water pipes), natural light in the house quenched the fluorescence. This problem, compounded by the ability of the dye to emit fluorescence in an unbound state, made the interpretation of fluorescing surfaces difficult; therefore, it may be necessary to transfer samples collected in the field to a laboratory for proper inspection of contamination using Calcofluor.

Similar problems were encountered in a trial at a processing plant. Calcofluor was applied to the surface of the rehang belt area and penetrated the porous plastic-based material. Such areas continued to fluoresce after surfaces were vigorously washed with a pressure water hose giving the appearance that they were permanently stained.

Swab and scrape samples collected from these areas tested negative for *Salmonella* and lactose fermenting bacteria (e.g., - *E. coli*).

Calcofluor also was used to stain metal surfaces that were continuously exposed to carcasses coming directly from the chill tank. These surfaces were covered by a visible film of a lipid-like substance and fluoresced faintly after the staining procedure. Scrape and swab cultures tested negative for *Salmonella* and other gram negative bacteria. It was proposed that an emulsifier be included in the Calcofluor staining solution to help dissolve fat residues that may be shielding biofilms from detection by the probe; however, this formulation was not tested.

A concern was raised that Calcofluor, despite its affinity for carbohydrates, could bind non-specifically to fat residues and yield faint false positives. A sample of water collected from the overflow of a chill tank showed that Calcofluor bound to biofilms but not to particles of fat (Figure 21). Arrow points to unstained fat globule). Interestingly, this sample tested positive for *Salmonella* raising questions about the effectiveness of the chill tank to decontaminate broiler carcasses.
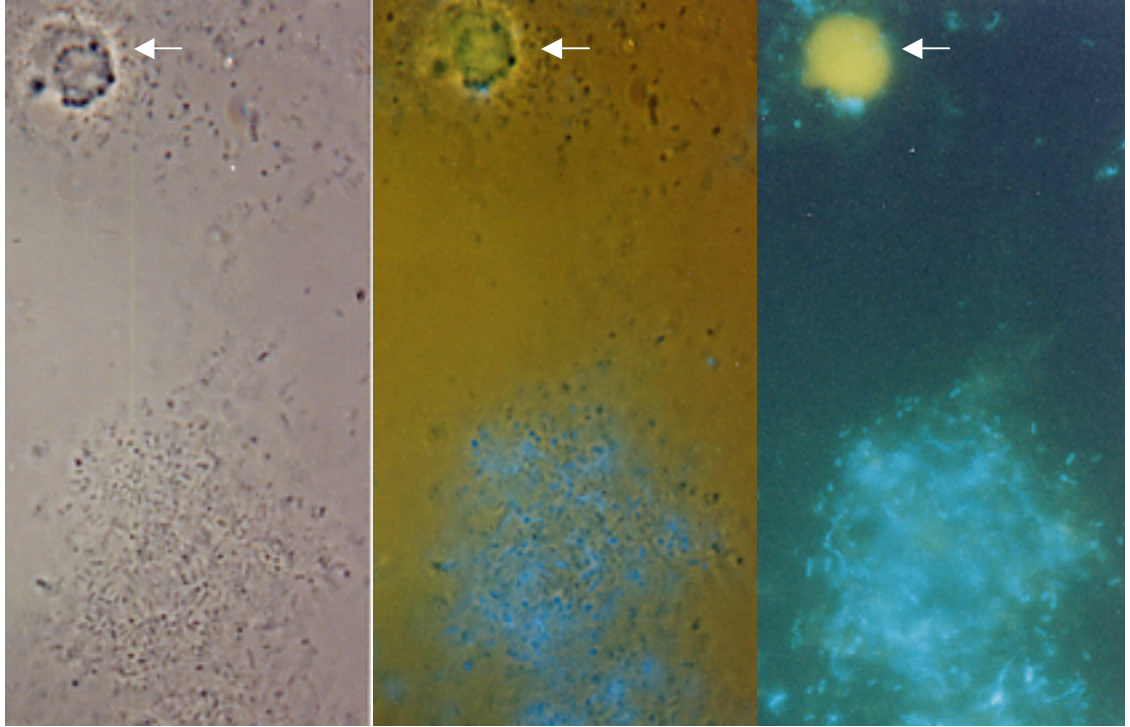
FIGURE 21. A water sample from a poultry processing plant chiller tank probed with Calcofluor and viewed under phase (A), phase and fluorescence (B) and fluorescence only (C). The presumed multispecies biofilm fluoresced under UV light. Fat globule does not bind Calcofluor (arrow). 630 x magnification.

In conclusion, using Calcofluor directly on food production areas for detection of biofilms was not practical. In poultry houses, Calcofluor bound to dust-covered surfaces and wood shavings yielding many false positives. Similarly, the probe bound to porous surfaces (e.g., rehang belt) in food processing facilities in ways that appeared to be nonspecific (dye was absorbed to all areas that it contacted). Furthermore, these areas also yielded negative swab culture results.

Another problem experienced during these trials was the difficulty in differentiating Calcofluor's natural background fluorescence from its more intense fluorescence when to β-D glucans polymers. Lastly, critics of Calcolfuor were concerned that the direct use of this in food preparation areas could compromise the safety of their products. Despite these problems, the author believes that Calcofluor could be used to probe samples from areas of high risk for biofilm contamination (e.g., corners, drains) as long as testing occurs in a nearby laboratory, which many food production facilities possess.

Overall conclusions

Calcofluor demonstrated to be a good non-specific presumptive stain for biofilms grown in vitro. However, results obtained from field studies revealed some of the limitations of this dye as a biofilm probe.

Lectins have more specificity for capsular material than Calcofluor so they were tested as presumptive, species-identifying, rapid probes of food-borne pathogens. Initially, cross-reactivity between lectins against same pathogen presented as a major concern; however, this problem partially solved by using lectins at lower

concentrations (10µg/ml). After this modification, lectins proved to be useful as genera-specific probes.

Studies on biofilms formed by STDT104 revealed that this organism produces an additional EPS, a capsular polysaccharide (CP), that appears to be constitutively synthesized. Unlike the curli, it is not believed to be a primary adhesin, however it is shown to be a component of the biofilm matrix. Fluorophore Assisted Carbohydrate Electrophoresis (FACE) analysis indicated that the isolated CP is principally comprised of glucose and mannose, with galactose as a minor constituent. This differs from the known colanic acid-containing CP of *E. coli* and other enteric bacteria. Specific antibody and lectin analyses reveal the CP to surround the cell during all growth phases at 25 and 37° C. The results from this study suggest that CP is yet another potential virulence factor in this multi- resistant, non-Typhi *Salmonella*.

Field studies showed that the use of Calcofluor as an in situ probe is impractical; however, it could be used in the laboratory as a non-specific probe to test scraped surfaces from food processing areas for biofilm contamination.

Future research

The efficacy of Calcofluor as a practical and fast probe for biofilm contamination needs to be tested on a large number of fresh samples collected from food-processing facilities. Preferably, this trial would take place in the microbiology laboratory of a food plant. Surfaces in direct contact with carcasses (e.g., rehang belts) should be sampled but special attention must also be given to floor drains, which has been reported to harbor biofilms that can aerosolize during washing procedures (Zotolla and Sasahara, 1994).

The conditions and the functions that promote STDT104 to synthesize a CP must be further studied. To accomplish this it is vital to use targeted mutagenesis (transposons) to delete specific genes responsible for CP synthesis and/or assembly. Creating a STDT104 incapable of synthesizing CP would allow us to determine if this polymer has any protective, cell aggregative, or surface binding functions. We could also determine if the absence of a capsule affects the three dimensional structure of STDT104 biofilms grown at ambient temperature. Additionally, NMR (nuclear magnetic resonance) studies could reveal the existing linkages between the individual monosaccharides comprising the CP of STDT104.

Appendix 1

Lectins used in this project and their specificities:

AAA = *Anguilla anguilla* [α-Fuc].
ABA = *Agaricus bisporus* [β-Gal and Gal(β1,3)GalNAc].
BPA = *Bauhinia purpurea* [α-GalNac and β-GalNAc].
ConA = *Canavilis ensiformis* [α-Man, α-Glucose (Glc), α-GlcNAc (N-acetyl-glucosamine) and branched Man with α-Fucose (Fuc) as determinant].
GNA = *Galanathus nivalis* [α- Mannose (Man) and Man (α1,3)].
GSI = *Griffonia simplicifolia* [α- Galactose (Gal), α- GalNac (N-acetyl-galactosamine)].
Jacalin = *Artocarpus integrifolia* [α- Gal, α- GalNac, and Gal(β1,3)GalNAc].
Lch = *Lens culinaris* [α-Man, α-Glc, α-GlcNAc and branched Man with α-Fuc as determinant].
Lotus A = *Lotus tetragonolobus* [α-Fuc and Fuc(α1,2)Gal(β1,4)[Fuc(1,3)GlcNAc].
MPA = *Maclura pomifera* [α-Gal, α-GalNac, and Gal(β1,3)GalNAc].
PSA = *Pisum sativum* [α-Man, α-Glc, α-GlcNAc and branched Man with α-Fuc as determinant].
UEA-I = *Ulex europaeus* [α-Fuc and Fuc(α1,2)Gal(β1,4)GlcNAc].
VFA = *Vicia faba* [α-Man, α-Glc, α-GlcNAc and branched Man with α-Fuc as determinant].

Abbreviations

cAMP = Cyclic adenosine monophosphate
CP = Capsular polysaccharide
°C = Degrees Celsius
DLVO = Derjaguin, Landau, Verwey and Overbeek theory
EPS = Exopolymeric substance
ES = Exopolysaccharide
FITC = Fluorescein isothiocyanate
g = Gam
GalNAC = N-acetyl galactosamine
Gal = Galactose
Glc = Glucose
kDa = Kilodalton
L = Liter
LAL = Limulus Amebocyte Lysate
LPS = Lipopolysaccharide
Man = Mannose
μg = Microgram
μl = Microliter
μm = Micrometer
PAGE = Polyacrylamide gel electrophoresis
PBS = Phosphate buffered saline
Rham = Rhamnose
Rv = *Salmonella enterica* serovar Typhimurium DT104 rugose variant
Rv/25 = *Salmonella enterica* serovar Typhimurium DT104 rugose variant grown at 25°C
Rv/37 = *Salmonella enterica* serovar Typhimurium DT104 rugose variant grown at 37°C
SCLM = Scanning confocal laser microscopy
SDS = Sodium dodecyl sulphate
STDT104 = *Salmonella enterica* serovar Typhimurium DT104
Stv = *Salmonella enterica* serovar Typhimurium DT104 smooth mutant
TSA = Trypticase soy agar
TSB = Trypticase soy broth
LB agar = Luria-Bertani agar
LB broth = Luria-Bertani broth.

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Resume

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- 2002-2006: The Ohio State College of Veterinary Medicine. DVM candidate.

- 2001-2004: University of Maryland, College Park, MD, Masters in Cell Biology and Molecular Genetics.

- 1994-1998: University of Maryland, College Park, MD. Bachelors in Life Sciences with high honors in Microbiology.
- 1992-1994: Montgomery College, Rockville, MD. General Studies.

Work experience

- 1997-1999: Virginia Maryland Regional College of Veterinary Medicine: Researcher. Gained extensive experience in laboratory techniques used to isolate and diagnose gram positive and negative bacteria.

- 1999-2001: Department of Cell Biology and Molecular Genetics, University of Maryland, College Park. Researcher. Extensive experience in molecular techniques (e.g., western blots, protein and carbohydrate isolation and analysis) and microscopy (e.g., light, fluorescence, transmission electron and confocal).

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- "Identification of Hot-Spots and Quantification of *Salmonella* and Fecal Coliforms as a Function of Environmental Parameters in Poultry Houses". N. L. Tablante, **C. E. de Rezende**, E.T. Mallinson, S. W. Joseph, R. A. Morales,

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Selected Publications:

- Mallinson, E.T., R. G. Miller, **C. E. de Rezende**, K. E. Ferris, J. deGraft-Hanson, and S. W. Joseph, 2000. Improved plating media for the detection of *Salmonella* species with typical and atypical hydrogen sulfide production. J Vet Diag Invest 12: 83-87.

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