DEVELOPMENT OF A FLUORESCENCE-BASED MICROPLATE ASSAY TO SCREEN STRAINS OF *LISTERIA MONOCYTOGENES* FOR SURFACE ATTACHMENT

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DEVELOPMENT OF A FLUORESCENCE-BASED MICROPLATE ASSAY TO SCREEN STRAINS OF *LISTERIA MONOCYTOGENES* FOR SURFACE ATTACHMENT

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INTRODUCTION

Listeria monocytogenes is a psychrotrophic, Gram-positive pathogen that is commonly implicated in outbreaks of foodborne disease (Chasseignaux et al. 2002). Since *L. monocytogenes* can adhere and form biofilms on numerous food processing surfaces including plastic, metal, glass, and rubber, it can easily come into contact with many foods such as milk, cheese, or meats that may contact these surfaces and pass into the food supply. In many instances it has been shown that sessile bacterial cells are more resistant to environmental changes, cleaning, and disinfection treatments (Hood et al. 1995). Therefore, there is an increasing interest in biofilms in processing plants and counteractive measures for their elimination and improvement of microbial food safety (Chavant et al. 2002).

A biofilm is generally described as a group of microbial cells that is irreversibly associated or colonized (not removed by gentle rinsing) on a surface and enclosed in a matrix made primarily of polysaccharide material (Wimpenny 2000, Hood et al. 1995). Biofilms are more than just the bacteria adhered to a surface, they also consist of all the extracellular material and any material entrapped in the biofilm matrix. Biofilms are self-regulating which means as they grow, parts of the biofilm will break off. The pieces that break off can subsequently colonize a new substrate or pass into food product. The

breaking off of bacteria into the food supply from biofilms in food processing plants is what has caused alarm in the food industry.

Biofilm formation potentially permits continual contamination of food products as each comes in contact with the established biofilm. The presence of a biofilm gives basis for a prolonged contamination of a surface and survival in the food processing environment.

The aim of this study was to develop an assay to screen various isolates of *L. monocytogenes* for their ability to form biofilms (numerically and visually) and investigate methods for their removal and/or elimination.

CHAPTER II

REVIEW OF LITERATURE

Listeria: foodborne pathogen

Listeria monocytogenes is a small, ubiquitous Gram-positive rod capable of causing morbidity and mortality in both humans and animals via listeriosis (Borucki et al. 2003). The ability of *L. monocytogenes* to grow at refrigeration temperatures has allowed it to thrive in food processing plants (Donnelly 2001). Illnesses due to *L. monocytogenes* are commonly associated with foodborne disease outbreaks. Most outbreaks are caused by serotype 4b and not the more commonly isolated serotype 1/2a. An aggregation of virulence related genes (i.e., pathogenicity island) has recently been discovered in *L. monocytogenes* (Chakraborty et al. 2000).

Although all strains of *L. monocytogenes* have now been shown to possess a pathogenicity island, they are not all equally capable of causing disease (Graves et al. 1999). It is speculated that several factors play a role in a strain's virulence, such as its ability to persist in environments where contamination may occur.

Occurrence in meat plants

Although disease from *L. monocytogenes* can be obtained by zoonotic transfer, foodborne illness is mainly transmitted to humans via contaminated foods (Chasseignaux et al. 2002). Since the main route of transmission is through foods, the prevalence of *L.*

monocytogenes in food plants has become of major concern. With prevalence ranging from 16% of samples of raw pork, 17% of raw poultry, 8% of samples from the poultry processing environments, and 26% from the pork processing environments (Chasseignaux et al. 2002) there is a demand for research to find better means of controlling *L. monocytogenes* in processing environments. With standards set at zero-tolerance for *L. monocytogenes* in ready-to-eat (RTE) meats, there is a need for more rigorous cleaning regimens in plants. *L. monocytogenes* has been found at every step of processing in the pork industry (Giovannacci et al. 1999). It has been found, with lower prevalence, starting at the farm level in the animal's feces and on hide, and even as far into the process as the cutting room in the slaughterhouse. Researchers have noticed an increased incidence of *L. monocytogenes* in plant settings compared to on-farm animal levels (Giovannacci et al. 1999). Numerous studies have suggested that *L. monocytogenes* amplification in pork may be in part to cross contamination within the plant, mainly the chilling and cutting rooms (Grahan and Collins 1991). The idea of cross contamination is further validated by the psychrotrophic nature of *L. monocytogenes* and its ability to adhere to surfaces (Giovannacci et al. 1999). In the event of biofilm formation, this allows continual contamination of food products as each comes in contact with the established biofilm. The presence of a biofilm allows for prolonged contamination of a surface and survival in the food processing environment.

Significance

L. monocytogenes is of great concern in the food industry due to its ability to form biofilms and cause listeriosis. The attachment of *Listeria* to food products or surfaces can lead to a substantial economic loss in the industry due to product loss and the potential for outbreaks. Species of *Listeria* are psychotrophic microorganisms that can grow at temperatures from 1-41^{o}C with optimum growth at 37^{o}C (Gray 1960). The significance of *Listeria* as a foodborne pathogen lies in its ability to survive and grow in food processing environments. This is aided by the ability of *Listeria* to form biofilms and which allows it to persist in processing environments that can certainly provide a constant cycle of food contamination.

Contamination in food processing plants

Listeria monocytogenes, a common contaminant in food processing plants, has been found in raw materials, the processing environment, on processing equipment, and in finished food products. Non-food contact surfaces within the processing environment that have been shown to be positive for *L. monocytogenes* are floors, walls, trucks, drains, shoes, doors and door handles, sanitizing floor mats, and foot baths (Hood et at. 1995). With such a wide array of areas, other than food processing surfaces positive for *L. monocytogenes* there is a need to look at routes and sites of contamination to better understand where the problem originates and take preventative measures.

Contamination routes and sites of *L. monocytogenes*

A thorough understanding of routes and sites of contamination in the food industry is a prerequisite for preventing unwanted bacteria in final products. Characterization of isolates by molecular subtyping has proven to be useful in tracing contamination to specific niches and points of harborage. By comparing isolates recovered at different stages throughout the processing of a food product, it is possible to pinpoint the source of contamination. Animal raw material has proven to be a contamination source of *L. monocytogenes* along with several other spoilage organisms and pathogens (Lawrence et al. 1995). Conducting molecular subtyping of strains of *L. monocytogenes* isolated from raw products versus final products resulted in little to no correlation between strains isolated from the two sources. It was noted that all or some of the molecular subtypes found in raw material were not seen in final products that contained *L. monocytogenes* (Norton et al. 2001). This discovery leads to the idea that the processing environment may be selecting for particular strains and perpetuating its own contamination. Contamination sites in meat, poultry, fish, and dairy processing plants are very similar with contamination linked to various processing machines. Conveyors have been found to be contaminated in all plant types along with machinery that is responsible for reducing a product's size. Other sites of contamination have been pinpointed to coolers and freezers (Autio et al. 1999).

Identical strains of *L. monocytogenes* have been found in both the product waste of processing machines or on the processing machines themselves and in the final

products, indicating that the processing machines have transferred the contamination to the products (Nesbakken et al. 1996). Klausner and Donnelly (1991) hypothesized that the surge in contamination from processing equipment may be due to the increasing complexity of the machinery, which in turn lowers the level of thorough cleaning that can be applied. With ample sources of contamination in a processing environment, there is a need to look at persistence of *L. monocytogenes* in processing environments.

Persistence of plant contamination

Some stains of *L. monocytogenes* have been observed to cause food plant contamination over long periods of time. Prolonged or persistent contaminations have been reported in several food industry areas, with contamination persisting for up to several years. Not all strains of *L. monocytogenes* found in processing plants lead to persistent contamination. Strains that cause persistent contamination are repeatedly recovered whereas others are only recovered sporadically. Strains of *L. monocytogenes* causing persistent contamination are not readily found in raw materials (Nesbakken et al. 1996). Although elimination of persistent contamination is difficult, it can be achieved through targeted and improved sanitation methods (Miettinen et al. 1999a).

Growth of microorganisms on surfaces

The vast majority of research done on bacteria has focused on pure cell culture lines, but research has proven that in nature, disease and industry, many bacteria survive by adhering to a substrate and residing within a biofilm (Brading et al. 1995). Bacteria within biofilms are considered sessile, and are intrinsically different than planktonic

bacteria. When bacteria are sessile they can express different genes than their planktonic counterparts. With the ability to express different genes, sessile bacteria can metamorphose their morphology, produce copious amounts of extracellular polymers, or even vary their growth rates. Another advantage that sessile bacteria have over planktonic bacteria lies within their ability to be more resistant to sanitizers and removal strategies (Hood et al. 1995).

Biofilms defined

A biofilm is a group of microbial cells that is irreversibly associated or colonized (not removed by gentle rinsing) on a surface and enclosed in a matrix made primarily of polysaccharide material (Wimpenny 2000, Hood et al. 1995). Biofilms are more than just the bacteria adhered to a surface, they also consist of all the extracellular material and any material entrapped in the biofilm matrix. Biofilms are self-regulating which means as they grow, parts of the biofilm will break off. The pieces that break off can subsequently colonize a new substrate or pass onto a food product. The breaking of off bacteria into the food supply from biofilms in the food processing plants is what has caused alarm in the food industry.

Surface colonization strategies

Bacteria that adhere to a surface attach and follow one of two colonization strategies: solitary cell colonization strategy or colonial cell colonization strategy. The solitary colonization strategy (for example, *Caulobacter* spp.) is when a single cell attaches to a surface and proliferates. A characteristic of the solitary colonization

strategy is that the progeny cells generally return to the planktonic state, this is called a shedding behavior (Brading et al. 1995). The "shedded" cells can then colonize another area and subsequently release their own progeny cells, thus spreading and covering a surface with bacteria. The other approach is a colonial colonization strategy (for example, *Pseudomonas* spp.) in which a single cell attaches, grows, and divides while forming a tightly packed group of cells on a surface. After some amount of growth, which varies from species to species, some bacteria were released back into the planktonic phase to enable the bacteria to colonize another part of the surface and spread the area of coverage. Both strategies have been described in biofilm formation. It is suggested that the strategy employed is dependant upon the species being studied (Brading et al. 1995).

Bacterial biofilm formation

Biofilms can exist in any environment including the soil, marine habitats, freshwater habitats, and any surface they may come in contact with. Bacteria in nature favor life in a biofilm because in a biofilm the bacteria are able to exploit nutrients. Biofilms take advantage of the ions and macromolecules at the surface-water interface, which is advantageous in otherwise nutritionally unfavorable conditions (Brading et al. 1995).

Initial biofilm development

Before bacteria can attach, the surface must become conditioned. Conditioning is when a wetted surface absorbs organic molecules and microbial cells. When most

surfaces are wet with water they assume a net negative charge. The negative charge of the surface attracts cations (positive) to the surface. It is not known whether bacteria, once attracted to the surface-water interface, penetrate the conditioning film and interact with the surface or if components of the bacterial cells interact with the film, but the outcome is bacterial attachment.

Production of the glycocalyx

Costerton et al. (1985) defined the glycocalyx as the polysaccharide containing structure of bacterial origin, lying outside the integral elements of the outer membrane of Gram-negative cells and the peptidoglycan of Gram-positive cells. The glycocalyx (slime layer or capsule) aids in trapping nutrients along with other microorganisms. Daughter cells often become entrapped within the polysaccharide layer and allow for thickening of the biofilm. The glycocalyx also aids in attracting charged ions and molecules which eventually results in attachment with surrounding biofilm communities yielding a continuous biofilm on a surface.

The maturing biofilm

As the biofilm matures it becomes three-dimensional as more microorganisms, daughter cells, and molecules become entrapped in the glycocalyx. Exopolysaccharides and three-dimensional structure are thought to play an important role in the resistance of biofilms to sanitizers (Kumar et al. 1998). The cells toward top of the biofilm have water, oxygen, and nutrients readily accessible to them much like their planktonic counterparts; whereas, cells embedded deep within the glycocalyx, such as the cells

attached to the surface, are less likely to have nutrients, water, and oxygen readily available to them. Channels throughout the maturing biofilm are often formed to allow for nutrient and water movement to the lower layers of the biofilm that are attached to the surface (Davey et al. 2000).

Steps in biofilm formation

Attachment

There are two proposed models for attachment of bacteria to surfaces, the two-step and the three-step mode. In the two-step model, the first step involves when bacteria are in close proximity with a surface and absorption takes place. This step is reversible.

The second step is after the bacteria are absorbed onto the surface it is allowed to sit and produce extracellular material that allows for anchorage of the bacteria to the surface, irreversible (Hood et al. 1995).

The three-step model relates attachment with distance of the bacteria from the surface. The first range of distance is >50 nm, where long-range forces such as van der Waals forces are employed for reversible attachment. The second range of distance is around 20 nm from bacteria to surface where long-range and electrostatic forces are in use; this step is reversible, but with time it is irreversible. The third range of distance is <15 nm where short-range forces act such as chemical bonding and hydrophobic interactions which result in an irreversible attachment (Hood et al. 1995). Regardless of which model is used to describe bacterial attachment it is agreed that in both instances the

ultimate attachment of bacteria to a surface is reliant upon time for the bacteria to produce adhesive extracellular material to anchor itself and not be easily removed.

Passive versus active adhesion

Passive and active adhesion is directly associated with the presence or absence of attachment structures. In passive adhesion, the bacteria have attachment structures prior to attachment to a surface. Bacteria that undergo passive adhesion have holdfasts or pili and possibly a flagellum that may aid in attachment and proximity to a surface. In active adhesion, the bacterium lacks adhesion structures and needs extended exposure times on a surface to produce adhesive extracellular material, such as exopolysaccharides, that anchor it to the surface (Brading et al. 1995).

Properties of the cell

Many attributes of the cell play a role it its ability to form a biofilm. A cell's hydrophobicity, presence of flagella or fimbriae (surface structures), cell wall, cell morphology, and production of exopolysaccharides play a role in the ability of a cell to attach to a surface (Brading et al. 1995, Hood et al. 1995, Chae et al. 2000). A cell's hydrophobicity may play a role in attachment because with increasing non-polar natures of one or both substrates involved with attachment, such as a bacteria and a surface, the hydrophobic interactions increase as well. Although most bacteria have negative surface charges, they still have hydrophobic components on their cell surface such as fimbriae. Fimbriae have been closely examined and have shown to possess a high percentage of hydrophobic amino acid residues. The fimbriae play a role in bacterial cell surface

hydrophobicity and are also speculated to aid in overcoming a repulsion barrier between bacteria and a surface.

The bacteria's cell wall also effects attachment to a surface. A cell can be Gram-positive or Gram-negative, and with each comes distinct characteristics. A Gram-negative cell wall has proteins, lipids, and peptidoglycan with lipopolysaccharides (LPS) on its surface. The LPS can vary widely from species to species allowing for variance in cell surface hydrophobicity thus effecting attachment abilities (Brading et al. 1995). The Gram-positive cell wall is mainly peptidoglycan with lesser amounts of teichoic acids, polysaccharides, and proteins. With a multitude of surface components, the combinations for attachment are numerous.

The morphology of a cell is also a factor in attachment. Bacteria that are rods and coccoids have low surface roughness when compared to bacteria that are filamentous (Brading et al. 1995). Mucoid strains adhered more readily than did non-mucoid strains. Non-mucoid strains were able to attach with increased time but eventually reduced after a peak period; whereas, the mucoid strain peaked with attachment and continued at a steady state (Brading et al. 1995).

There have been other studies where attached bacteria were treated with proteolytic enzymes that caused a release of bacteria from a surface (Danielsson et al. 1977). This indicates proteins play a part in the attachment of some bacteria to a surface. Korber et al. (1989) used flagella-positive and -negative strains of *Pseudomonas fluorescens* and observed that motile strains attached more readily and evenly across a

surface than did the non-motile strains. Korber suggests that flagella play a role in attachment by overcoming repulsive forces given off by the surface/substratum.

Substratum effects

The surface to which a biofilm attaches may have characteristics that make it more favorable for bacterial attachment. Levels of attached bacteria to a surface are proportional to surface roughness (Hood et al. 1995). The reason more bacteria are found on rougher surfaces (i.e. scratches in surfaces) is due to a decrease in shear forces the bacteria come in contact with along with higher surface area. Rougher substrates can be due to the use of harsh chemicals or cleaning with abrasive materials such as steel wool.

Conditioning films

On processing surfaces in the food industry, organic and inorganic molecules from products such as meat or milk get absorbed on surfaces creating what is termed a conditioning film. As more and more molecules accumulate at the solid-liquid interface, favorable conditions for attachment of bacteria and subsequent formation of a biofilm are created due to higher amounts of nutrients available as compared to in the fluid phase (Kumar et al. 1998). Transfer of nutrients within a biofilm is more efficient than nutrient attainment in the planktonic state.

Characteristics of the aqueous medium

The aqueous medium in the environment may also effect attachment of bacteria to a surface. Factors such as pH, temperature, ionic strength, and nutrient levels have an influence on a surface's favorability for bacterial attachment. The aqueous medium not

only controls the amount of dissolved nutrients, but also may play a part in substratum interactions (Brading et al. 1995). Flethcher (1988) conducted an experiment in which he examined the effect of cations (Na^{+}, Ca^{2+}, La^{3+}, and Fe^{3+}) on attachment of *P. fluorescens*. His findings suggested that the addition of each cation resulted in a decrease in separation distance between the bacteria and the glass surface. Flethcher proposed his findings were due to the reduction of repulsive forces between the negative charges on bacterial surface polymers and the glass surface. In a study done by Cowan et al. (1991) it was shown that an increase in nutrient concentration of the aqueous medium resulted in a proportional increase in the amount of attached bacteria.

Detachment

Shear removal and sloughing

Shear removal or erosion of biofilms is defined as the continuous removal of small particles of biofilm as a result of shear forces (Kumar et al. 1998). As a biofilm becomes thicker, it requires an increase in shear force at the biofilm-fluid interface to remove portions of it. Biofilm erosion can be compared to erosion in nature, a constant loss of small biofilm portions and water flowing downhill steadily wearing away rock (i.e., The Grand Canyon) respectively. Shear removal or erosion is a very slow process; although, biofilm sloughing is very rapid. In sloughing, large portions of biofilm are lost, and is usually associated with thick biofilm in environments rich with nutrients (Brading et al. 1995). Sloughing and erosion can occur simultaneously. Erosion or shear removal

is an ongoing process; whereas, sloughing is thought to be a sporadic event in which conditions within a biofilm become unfavorable and detachment is optimal.

Abrasion

In conjunction with shear removal and sloughing, abrasion is another process by which bacteria are detached from a biofilm. Abrasion is collision of solid particles with a biofilm. The resulting collisions knock free parts of a biofilm. Thin biofilms are less affected by abrasion; whereas, thick highly-developed biofilms are more affected. This is due impart to the available area in which a solid particle can collide with a biofilm. A highly-developed three-dimensional biofilm would protrude off of a surface and in turn be more likely to come in contact with a particle. The thin biofilm which is in close proximity with the surface can still be effected by abrasion (flow perpendicular to surface), but if the flow is horizontal to the surface the particles are more likely to flow over the surface of the biofilm.

Established biofilm communities: advantages of sessile growth

Genetic regulation of biofilm formation

Research done on biofilms is producing evidence that up- and down-regulation of a number of genes occurs as quickly as a cell has interaction with a solid substrate. The ability of an organism to genetically adapt to a new environment is fundamental to survival and fitness. Genetic adaptation can come about by genetic recombination, genetic uptake, mutations, or regulation of existing genes by up or down regulation (Jefferson 2004). On the forefront of today's research is the ability of bacteria to go from

a planktonic phase and grow in a sessile community via adaptation. There have been numerous studies on what genes appear to be needed for biofilm formation, and genes that are regulated in a biofilm (Jefferson 2004).

In biofilm communities, cells are in close proximity to one another. This living situation provides an ideal setting for the exchange of chromosomal DNA and/or plasmids. Conjugation occurs at a higher rate in biofilms than in planktonic cells (Jefferson 2004). Horizontal gene transfer has become important for biofilm evolution and genetic diversity (Davey et al. 2000). By the exchange of genetic material, biofilms can also spread genes encoding antibiotic resistance.

Quorum sensing

An individual bacterial cell is able to sense other bacteria of the same species and respond accordingly by differentially expressesing specific genes. When one cell communicates with another it is called quorum sensing (QS). QS involves the direct or indirect stimulation of a response regulator by a signal molecule. The major QS signal molecules are N-acyl homoserine lactones in Gram-negative bacteria and post-translationally modified peptides in Gram-positive bacteria (Ulrich 2004). The ability of bacteria to communicate and respond helps in the formation of a denser, thicker biofilm.

Predation

Bacteria living within a biofilm not only reap more benefits by having higher amounts of nutrients available to them, a higher potential for genetic transfer, and heightened communication, but they are also less likely to be preyed upon or be out

competed. By being in a biofilm, a bacteria's survival improves by avoidance of being preyed upon by scavenging protozoa. A biofilm has a complex exopolysaccharide matrix that aids in attachment to surfaces, but also provides protection from predation by binding the bacteria to a biofilm making removal less likely. The exopolysaccharide may also be responsible for slowing the penetration of digestive enzymes secreted by surface predators (Korber et al. 1995).

Biofilm structure

Extracellular polymeric substances

The main components of a biofilm are bacterial cells and extracellular polymeric substances (EPS) matrix. Extracellular polysaccharide can vary in chemical and physical properties but is mainly comprised of exopolysaccharides. The EPS can account for 50-90 % of the total organic carbon of a biofilm, and is therefore regarded as the primary matrix material for the biofilm (Kumar et al. 1998). The anionic property of biofilms (cationic in some Gram-positive) allows for association with calcium and magnesium which help in cross-linking polymer strains allowing for higher binding force in mature biofilms. The anionic property is attained from the presence of uronic acids such as D-glucuronic, D-galacturonic, and mannuronic acids or ketal-linked pyruvates (Sutherland 2001). Due to hydrogen bonding, the EPS is highly hydrated. The EPS can also be hydrophobic, but most EPS are both, hydrophilic and hydrophobic (Sutherland 2001). Sutherland's (2001) research suggests that there are two properties of the EPS that may effect biofilm formation. One important property is the structure of the polysaccharide.

Most bacterial EPS possess a backbone structure that contains either 1, 3- or 1, 4-β-linked hexose residues. This type of linkage tends to be more rigid, and in some instances poorly soluble or insoluble. Secondly, Sutherland (2001) notes that the EPS varies from one area of a biofilm to another area and the amount of EPS is dependant upon age of the biofilm. It is also speculated that the EPS may also contribute to the antimicrobial resistance properties of biofilms by hindering entrance of antibiotics into the biofilm (Donlan 2000).

Biofilm architecture

As a biofilm develops on a surface, a characteristic biofilm architecture emerges. Throughout the time span of research on biofilms, it has been debated whether or not the structure observed under the microscope is a random arrangement, regulated formation, or the product of stochastic processes (Davey et al. 2000). To get an insight on biofilm architecture, a mutant strain of *Pseudomonas aeruginosa*, unable to produce the QS molecule acylhomoserine lactone, was observed for biofilm architecture versus it wild-type counterpart. What was observed was that the mutant strain of *P. aeruginosa* had radically altered biofilm architecture from that of the wild-type (Davey et al. 2000). This suggested that QS molecules are needed in biofilm architecture construction, thus that biofilms architecture is a regulated process. In a study done by Danese et al. (2000), they observed that a biofilm formed by *Escherichia coli* had a biofilm architecture similar to that of *Pseudomonas*, but when a mutant strain of *E. coli* unable to synthesize colonic acid, the major EPS synthesized by this organism, was studied they observed that the

biofilm architecture was not like that of the wild-type. This suggests that EPS plays a role in the normal formation of biofilm architecture. Danese et al. (2000) did observe that the mutant was not affected when attaching to the surface, suggesting colonic acid was not an early adhesion factor. It is suggested that the EPS may stabilize interactions between the bacteria and the surface (Davey et al. 2000).

Microscopy methods for studying biofilms

In order to understand biofilms it is important to have an understanding of their dimensions and spatial arrangement. There are several microscopic methods applicable for the study of biofilms, each having its advantages and disadvantages.

Light microscopy

Light microscopy has many various techniques which have been used to study biofilms such as bright-field, dark-field, phase-contrast, and fluorescence. The light microscope works by using glass lenses to bend and focus light. This bending and focusing of light allows small objects to be enlarged. The resolution of a light microscope is dependant upon the aperture of its lenses (Beech et al. 2000).

Bright and dark field

Most biological materials do not have contrast themselves, so the bright field microscope is limited in its use unless the specimen is stained. In bright field microscopy, specimens are visible because of the change of the speed and the path of the light passing through the specimen (refraction). In order to visualize a specimen clearly with bright field, the light rays passing through it have to be refracted enough to cause

interference and produce ample contrast (differences in light intensities) to make the specimen visible. Most biological specimens are mainly water and consequently have very low contrast unless they have been stained. Fine detail is also a problem with bright field even if the specimen is stained, and stained specimens are usually dead. Dark field microscopy uses images that are produced by a hollow cone of diffracted light which highlights the specimen (Beech et al. 2000). As the name dark field implies the background is dark and the specimen is bright. Dark field microscopy is a specialized technique which amplifies the contrast of specimens and their background, forming a bright image of the specimen superimposed onto a dark background. Small details like flagella can be visualized with this microscopic method.

Fluorescent microscopy

Fluorescent microscopy works with the idea that the specimen is emitting light. When a fluorescent specimen is exposed to ultraviolet light, violet, or blue light the result is the emission of the absorbed light as longer, specific wavelength that is used to produce an image (Beech et al. 2000).

Phase contrast

Phase contrast microscopy allows one to view a live or unstained specimen. The phase contrast microscope works by utilizing the different refractive indexes of materials. The process involves slowing the light waves as they traverse a material. Light waves that are not refracted are shifted by ¼ of a wavelength by a secondary phase ring located between the objective and the eyepiece. Light waves that are refracted are not passed

through the secondary phase ring. When the light waves that were shifted are coupled with the light waves that were not, an improved contrast of the material on the slide can be observed (Beech et al. 2000). This method is advantageous for work with biofilms because no staining or preparation is needed.

Confocal scanning laser microscope (CSLM)

CSLM is rising in popularity due to its ability to produce blur-free, crisp pictures of thick specimens such as biofilms. The microscope rejects any out-of-focus images by having a pinhole in front of the microscope's detector (Beech et al. 2000). This allows only focused images to be detected. The microscope scans the specimen by deflection of the laser beam or by stage movement. The analogue signal is detected by the photo-multiplier, which is subsequently converted to a digital signal that ends up as a pixel-based image on a computer screen attached to the microscope (Beech et al. 2000).

Electron microscopy (EM)

There are many different forms of EM that have been used to study biofilms. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) both require fixation with chemicals and further dehydrating of samples prior to viewing. Both TEM and SEM require extensive sample treatment and both are more costly than other microscopy methods. TEM and SEM (Fig. 9) offer high resolution imaging of cell/surface interactions which is a key area of interest in biofilms (Beech et al. 2000).

***Listeria* and biofilms**

In recent years *L. monocytogenes* has raised increasing concerns in the food industry. It is thought that contamination of foods by *L. monocytogenes* is likely due to food processing environments where cells have attached to surfaces and subsequent contact with food products (Borucki et al. 2003). The persistence of *L. monocytogenes* on contact surfaces is due to its ability to adhere and produce biofilms. *Listeria monocytogenes* can readily attach to stainless steel and plastic material, which are consequently the prevalent materials used for construction in the food industry (Stepanovic et al. 2004, Chae et al. 2000). Once *L. monocytogenes* has established itself on a surface, the biofilm has inherent capabilities to be resistant to disinfection regimens employed it the food industry (Borucki et al. 2003). Typical sanitizers used in sanitation are iodine, chlorine, and acidic anionic compounds (Chae et al. 2000). Life in biofilms has provided a way for *L. monocytogenes* to persist in processing environments.

There are 13 serotypes of *L. monocytogenes*, yet only three (1/2 a, 1/2 b, and 4b) are commonly associated with human disease (Chae et al. 2000). In a study done by Borucki et al. (2003) strains of *L. monocytogenes* were divided into two divisions molecular typing. Division I included serotypes 1/2 b and 4b, and division II included serotypes 1/2 a and 1/2 c. Borucki et al. (2003) found that biofilm formation correlated with phylogenetic division but not with serotype. Their study also showed that division II strains, not normally associated with foodborne outbreaks, had increased biofilm formation. Although contradictory to this study, it still proposes the idea that there are

biofilms constituted of specific serotypes of *L. monocytogenes* that are more prone to cause human illness. The study only showed that division II had increased biofilm formation over division I, whereas division I still formed biofilms.

Strains of *L. monocytogenes* have diverse abilities to form and grow into mature biofilms (Chae et al. 2000). This diverse ability to form biofilms may be a crucial determinant in persistent strains found in processing environments or on food products manufactured by the food industry.

OBJECTIVE OF PRESENT STUDY

The need to control *L. monocytogenes* biofilms in the RTE meats industry is gaining greater recognition and importance. Studies and books to date mainly deal with the structure of, surface conditions surrounding, genetic differences, and detection of biofilms with little being done to find methods of removal and eradication.

The objectives of this study were to create an assay aimed at evaluating adherence by various strains of *L. monocytogenes* and subsequent detachment strategies. The assay served as a way to screen a strains ability to form a biofilm along with providing a model to test removal strategies against. A successful removal or destruction method for biofilms would be useful in helping to control *L. monocytogenes* biofilms in the RTE meat industry that may otherwise contaminate food and possibly result in foodborne illness.

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CHAPTER III

DEVELOPMENT OF A FLUORESCENCE-BASED PLATE ASSAY TO SCREEN STRAINS OF *LISTERIA MONOCYTOGENES* FOR ATTACHMENT TO SURFACES AND THEIR SUBSEQUENT REMOVAL

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INTRODUCTION

Listeria monocytogenes is a Gram-positive, facultative, psychrotrophic bacterium which is pathogenic to both humans and animals. It often is present on raw meats making it difficult to eliminate from processing environments producing ready-to-eat (RTE) meat products. *Listeria monocytogenes* is also capable of producing biofilms on processing equipment (stainless steel, plastic, and rubber surfaces) making its eradication even more difficult and allowing for potential contamination of RTE food products. Wong (1998) found that not only could *L. monocytogenes* adhere to stainless steel and rubber, but under favorable conditions, it could multiply on stainless steel. Biofilms are self-regulating which means as they grow, either individual cells or large parts of the biofilm will break off. The pieces that break off can subsequently colonize a new substrate or pass into a food product. *Listeria monocytogenes* presents a formidable problem to the RTE meat industry, as both USDA and FDA have established a 'zero-tolerance' for its presence in RTE foods.

In the U.S., there are about 2500 cases of listeriosis per year with 20-40% mortality (Mead 1999). Bacteria within biofilms are considered sessile, and are intrinsically different than planktonic bacteria. When bacteria are sessile they can express different genes than their planktonic counterparts. With the ability to express different genes, sessile bacteria can metamorphose their morphology, produce vast amounts of extracellular polymers, or even vary their growth rates. Another advantage

that sessile bacteria have over planktonic bacteria lies within their ability to be more resistant to sanitizers and removal strategies (Hood et al. 1995 and Chae et al. 2000).

Several methods have been developed to try and quantify the number of cells in a biofilm. O'Toole et. al. (1998) used crystal violet to stain biofilm cells and take an absorbance reading for cell number estimation. The problem with using crystal violet to stain cells is that there is no ability to differentiate between live and dead cells, and no way to decipher different species of bacteria. Some strains of bacteria may inherently stain darker than others thus giving a higher biofilm level when in actuality this is not the case. Other concerns with the use of crystal violet are that different strains may produce varying levels of extracellular polysaccharides which may take up crystal violet and also the variability in destaining one experiment to the next. Narisawa et. al. (2005) made modifications to the crystal violet method by staining biofilm cells in a microtiter plate and then extracting the crystal violet from the cells by rinsing with 70% ethanol. The rinse was transferred to a fresh microtiter plate and an absorbance reading taken at 590 nm. This method still allows for dead cells to be stained and possible loss of accuracy due to transfer of rinse and amount of decolorizer used. Along the same lines as crystal violet staining, is the use of acridine orange for biofilm enumeration and visulation. Fessia et. al. (1991) used acridine orange to stain biofilm cells of cogulase-negative Staphylococci (CNS). Acridine orange is a nucleic acid stain and has the same problems that crystal violet has, staining dead cells with no way to standardize how much stain

cells absorb thus leading to varying absorbance readings and negatively affecting the viability of the cells.

The purpose of this study was to develop a convenient assay that could make use of a high throughput format to identify strongly adhering strains among those in our collection that have been isolated from live animals, raw retail meats, and RTE meat processing environments for subsequent studies related to biofilm formation by *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial cultures and growth conditions

Initial attachment and detachment assays were developed using four strains of *L. monocytogenes* (Scott A-2, serotype 4b; V7-2, serotype 1/2a;, 39-2 retail hotdog isolate; 383-2 ground beef isolate). Bacterial strains were cultured by transferring 100 ul of thawed frozen culture suspension into 9 ml of brain heart infusion (BHI) broth, incubated overnight (24 hours) at 30ºC, and subcultured twice times before use. Frozen culture stocks were prepared by centrifuging 9 ml of culture and resuspending the pellet in 2 ml of BHI broth (containing 10% glycerol) and storing at -76^{o}C. Upon use of the frozen culture, the vials were placed on the lab bench until partially thawed, vortexed, and 100 uL removed aseptically for inoculation of 9-ml media tubes. Colony enumeration was performed on general-purpose agar for 24 hours at 37^{o}C (tryptic soy agar, TSA; Difco, Becton-Dickenson, Franklin Lakes, NJ). Additional strains of *L. monocytogenes* were obtained from our culture collection and contained strains isolated from retail hotdogs (Wang and Muriana, 1991), raw meats, and RTE meat processing facilities.

Fluorescent plate assay

A method for microplate incubation of various strains was devised and compiled partially from similar procedures and conditions found in the literature. Strains to be tested were subcultured overnight (24 hours) in BHI broth held at 30^{o}C. The overnight culture was diluted 100,000-fold (i.e., from 10^{9} cfu/ml to 10^{4} cfu/ml) in fresh BHI broth

and 200 ul was transferred to designated wells of a 96-well black microwell plate with a clear lid (Nunc^{Tm}, Denmark). The edge of the plate was wrapped in petri film to prevent evaporation and incubated at 30^{o}C for 24 hrs (the temperature was chosen the same as the culture incubation temperature). After incubation, the microplate was washed 3x with Tris buffer (pH 7.4, 0.05M) in a Biotec Elx405 Magna plate washer (Fig. 1A) (Ipswich, Suffolk, UK). The washing was followed by the addition of 200 ul of fresh (sterile) BHI broth to each experimental well and again wrapped in petri film, incubated at 30^{o}C, and again washed 3x with Tris buffer (pH 7.4, 0.05M) after another 24 hrs. After the final washing, 200 ul of carboxyfluorescein diacetate (5,6-CFDA; Sigma-Aldrich, St. Louis, MO) fluorescent substrate solution was added. The CFDA fluorescent substrate working stock was prepared by adding 10 ul of a 2% CFDA solution in dimethyl sulfoxide [DMSO] to 1 ml of cold Tris buffer [pH 7.4, 0.05M]. Various incubation times and temperatures with CFDA substrate were examined for effective fluorescence response. Following incubation with CFDA, the plates were washed 3x with Tris buffer (pH 7.4, 0.05M) with the plate washer and replaced with 200 ul of the same. The plate was then read from above in a Tecan GENios fluorescent plate reader (Fig. 1B) (Phenix Research Products, Hayward, CA) with excitation at 485 nm and detection at 535 nm.

Attachment (detachment) quantification assays

We examined the use of various enzymes to cause release of attached cells for the purpose of subsequent enumeration. Initially, we evaluated various proteases including pronase E, trypsin, papain, pepsin, and thermolysin (Sigma-Aldrich) [all constituted in

Tris buffer (pH 7.4, 0.05M) at 1,000 IU/ml] as well as BAX^{Tm} protease (Qualicon) which was used according to the manufacturer's directions (12.5 ul per 1 ml Tris buffer, pH 7.4, 0.05M). We also examined the effect of lipoprotein lipase B, lipase, alpha amylase, and cellulase (VWR). Each enzyme (except for BAX^{Tm} protease) was used at 100 IU per 200 ul microwell plate assay.

Planktonic cells in broth BHI culture were also tested for viability in the presence of the same level of enzyme used in the plate assays in order to determine the effect of enzyme treatment on cell viability. Overnight 9 ml cultures of the 4 test strains of *L. monocytogenes* described earlier were centrifuged at 4500 x g for 30 min in a Sorvall RC5 Plus centrifuge at 5^{o}C; the supernatant broth was discarded and the cell pellets were resuspended in 9 ml of Tris buffer, pH 7.4. Eight hundred microliter samples of the resuspended cells were placed into an eppendorf tube along with 200 ul of enzyme/Tris buffer (pH 7.4) such that the final concentration of enzyme was 100 IU per 200 ul. A control was used for each strain consisting of buffer without enzyme. After 1 hr at 37^{o}C, appropriate dilutions were made of both controls and enzyme-treated planktonic cells using 0.1% buffered peptone water (BPW) and plated on BHI agar followed by 48 hr incubation at 30^{o}C before enumeration.

Non-proteolytic enzymes were tested with RediPlate 96 EnzChek^{Tm}, a microplate format assay to test for metallo-, serine, acid-, and sulfhydryl protease activity in order to insure the absence of protease activity in non-proteolytic enzyme preparations used above. The assay was performed according to manufacturers' directions, generating a

green fluorescent signal upon hydrolysis, and read in the Tecan GENios plate reader with excitation at 485 nm and detection at 535 nm.

A 'detachment assay' was run on attached cells using the 48 hr microplate assay described above. After 48 hrs of incubation, the microplates were washed 2x with Tris buffer (pH 7.4) using the automated plate washer followed by manually pipetting 200-ul of enzyme solution followed by incubation at 37^{o}C for 1 hr and the detached cells were then harvested for plating. Attached cells in microplate wells were subjected to a final rinse with either Tris buffer, pH 7.4, 0.05M (i.e., controls) or Tris buffer containing 100 IU of enzyme per 200 ul (i.e., experimental samples). After incubation at 37^{o}C, the liquid in the wells was harvested and plated for microbial enumeration of detached cells. All plating was done on BHI agar plates incubated at 37^{o}C for 48 hours. After the detachment assays, microplates were washed with the automated plate washer and subjected to the CFDA-based fluorescence assay for comparison of attached-cell (control wells without added protease) and detached-cell (from residual attached cells) fluorescence with microbial cell counts recovered by the enzyme treatments.

Fluorescence microscopy

Fluorescence microscopy was conducted with cultures in a modified attachment assay using 8-compartment CultureSlides^{Tm} (Falcon, Becton-Dickenson, Bedford, MA) that are polystyrene chambers fixed onto glass slides with the intention that after culturing, the liquid is removed, the chambers washed and disassembled, and the bottom surface of the chamber is a microscope slide useful for microscopic observation.

Overnight cultures of select strains of *L. monocytogenes* were diluted 10^5-fold (i.e., ~10^4 cfu/ml) in fresh/sterile BHI broth and 200 ul of the resulting dilution was placed into respective chambers on the culture slides. Cultures were incubated under the same conditions as the microplate assay (48 hrs, 30°C), rinsed by manual pipette aspiration using Tris buffer (pH 7.4, 0.05M), and incubated with CFDA-based substrate as previously described. Chambers were removed using the manufacturer's tool and the bottom slides were examined by fluorescence microscopy using a Nikon Eclipse E400 fluorescent microscope (excitation @ 450-490 nm, detection @ 500nm) using a BA 515 B-2A filter. Pictures were taken with a digital camera attachment.

Scanning electron microscopy

Scanning electron microscopy (SEM) images were obtained by comparison of 8 strains of *L. monocytogenes* selected from the results with our microplate assays. We selected 4 strains that demonstrated high level fluorescence in our attachment assay in comparison with 4 strains that gave low level fluorescence. The cultures were grown in the presence of glass microscope coverslips placed in a sterile 24-well microplate (Falcon) with 500 ul of culture at ~10^4 cfu/ml in fresh BHI broth and incubated overnight at 30°C. As with our microplate attachment assay, the cells were removed and wells/coverslips were washed 2x with Tris buffer (pH 7.4, 0.05M) and replaced with 500 ul of fresh BHI broth for further incubation. After a total of 48 hrs, the wells/coverslips were washed 2x again with Tris buffer (pH 7.4, 0.05M) for transfer to the Electron

Microscopy Core Facility at Oklahoma State University for SEM analysis performed by Terry Colberg.

Effects of incubation temperature on attachment

We examined attachment at 10^{o}, 20^{o}, 30^{o}, and 40^{o}C to determine if there was a preference for attachment at particular temperatures, as all of these temperatures would likely be employed at various points within processing facilities where *L. monocytogenes* may be found as an environment contaminant.

Experimental design and statistical analysis

All trials were carried out in triplicate replications. Standard deviations were obtained for the multiple test samples within the various replications. Statistical analysis was performed for multiple comparisons of the means and standard deviations obtained for different treatments. Analysis of variance (ANOVA) was performed using the Holm-Sidak test for pair wise multiple comparisons to determine significant differences ($P < 0.05$) using the software program SigmaStat 3.1 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

CFDA-based fluorescence assays provided excellent correlation and linearity (r^2=0.9979) when cell populations were serially-diluted and tested for fluorescence with the 5,6-CFDA substrate (data not shown). *Listeria monocytogenes* ScottA-2 was incubated in microplate wells at 30°C for attachment and then examined at various temperatures for uptake and response with the CFDA substrate. The results showed that the shortest substrate incubation time (15 minutes) at each of the three temperatures yielded the highest fluorescence levels (Fig. 2A). The reason for the observed decrease in fluorescence with increased substrate incubation time is not clear. Although one may suspect leakage of the cellular cytoplasmic fluorophore, the diacetate modification (i.e., 5,6-CFDA) is supposed to prevent cytoplasmic leakage of the hydrolyzed product due to the presence of negative charges at cytoplasmic pH levels. Since the rate of decrease of fluorescence was least when cells were incubated at 25°C, we chose this substrate incubation temperature for the remainder of the study. We examined shorter substrate incubation periods at 25°C and found that a 15 min incubation period provided higher fluorescence levels from attached cells than did either 5 or 10 minutes (Fig. 2B). The low early levels of fluorescence may be due to a minimum time necessary for the substrate to enter the cell and become hydrolyzed to the fluorescent by-product while the subsequent decreasing fluorescent levels may be due to metabolic quenching as any leakage from the

attached cells would still be retained in the microplate wells and still capable of contributing to the flourescence observed (Fig. 2B).

We screened more than 60 strains of *L. monocytogenes* isolated from RTE meat processing facilities, raw retail meats, and RTE meats for their ability to adhere in our attachment assays. Of the strains tested, more than a 50-fold difference in fluorescence signal was obtained between various strains suggesting that some may be demonstrating greater levels of attachment than others (Fig. 3).

Strains were differentiated into strong vs. weakly adherent based on our attachment assays (Fig. 4A). Although we considered higher levels of fluorescence to correspond to higher levels of attachment, one possible explanation for the variations in signals from our microplate fluorescence assays may also have been that different strains were able to take up and hydrolyze the substrate better than others. In that case, the fluorescence signals may merely represent strain differences in biochemical handling of the substrate rather than differences in attachment. We therefore compared the fluorescence of attached vs. planktonic cells in suspension to determine if there were strain differences to explain what we observed after attachment assays. When these same strains at the same cell levels in liquid suspension were treated with the fluorescent substrate, we obtained equivalent or higher levels of fluorescence (Fig. 4B) with those that were previously deemed to be weakly fluorescing in our attachment assays (Fig. 4A). Considering that the planktonic fluorescence assay was performed with an equivalent

number of cells of all strains tested (Fig. 4C), we conclude that the attachment assay is representative of relative adherence levels of the various strains.

In order to confirm adherence by more quantitative means, we compared 8 strains of *L. monocytogenes* (4 strongly fluorescing and 4 weakly fluorescing strains from attachment assays) for their ability to attach in head-to-head comparison when tested under the same conditions using the microscope slide chambers. The degree of attachment was measured microscopically and by static count. After incubation for attachment and substrate uptake, the chambers were removed and slides examined by both light- and fluorescence microscopy. The results confirmed that the strains which yielded strong fluorescence signals were present in much higher numbers on the slides than the strains giving weak fluorescence signals in attachment assays (data not shown). These same strains were again incubated under identical conditions in microplates with glass chips that were washed 5x with buffer before submitting for SEM analysis. Those strains that were chosen from our attachment assays for high fluorescence signals and shown to have high levels of attachment by light- and fluorescence microscopy, were also found to be strongly adhering by SEM analysis (Figs 5A-5D). The same strains that showed consistently low levels of fluorescence in the attachment assays, also showed low levels of attachment by SEM analysis, relative to the more highly adhering strains (Figs. 5E-5H).

Another approach that we have used to quantify the relative levels of attachment has been to examine proteolytic release (or 'detachment') from microplate well surfaces.

In order to rely on counts from 'detachment' assays, we had to insure that the enzymatic treatments had no adverse affects on the viability of the treated cells; otherwise the counts would not be representative of what was previously attached. We found little or no affect on cell viability using a general protease, trypsin, papain, pepsin, thermolysin, BAX™ protease, lipoprotein lipase B, or cellulase (data not shown). Also, some non-proteolytic enzyme preparations such as alpha-amylase and lipase were found to contain considerable proteolytic activity when tested with the EnzChek assay (data not shown). Because of their lack of protease activity, we compared the effect of lipoprotein B lipase and cellulase with that of BAX™ protease prior to our microplate fluorescence assay and for quantitation of *L. monocytogenes* after detachment from microplates (Fig. 6). When control wells for strongly adhering strains were treated with buffer instead of enzyme, we obtained typical fluorescence signals when performing our microplate assay, although little or no signal was obtained with controls for several weakly adhering strains also included in the assay (Fig. 6A). When wells containing strongly attaching strains were treated with BAX™ protease or cellulase, we obtained complete loss of fluorescence, and nearly complete loss with lipoprotein B lipase (Fig. 6A). The data suggests that substrates for the 3 types of enzymes may be involved in attachment by *L. monocytogenes* or more likely, that the cellular constituent may be embedded in the peptidoglycan layer which contains protein, carbohydrate, and lipid moieties that can all be acted upon by the enzymes tested. When we examined the ability of the same enzymes to detach attached *Listeria*, the data complemented that obtained with

fluorescence in that BAX^{Tm} protease and cellulase gave the highest recovered plate counts while those obtained with lipoprotein B lipase treatment slightly lower for all 4 strongly adhering strains (Fig. 6B). In this series of assays, all attached wells were washed 5x prior to final treatment of control wells (i.e., with buffer) or test wells (with enzyme) to obtain samples for plating (Fig. 6B). For the strongly adhering strains (*L. monocytogenes* 50, 62, 77, 99-38), the data shows that greater than 3-log lower levels were recovered when treated with buffer than when treated with enzymes, meaning that only about 0.1% of what is attached comes off in the buffer wash (Fig. 6B). However, the weakly adhering strains (*L. monocytogenes* 34, 35) showed approximately 5-log lower levels of attached cells than the highly adhering strains and the controls show comparable levels of release with buffer treatment as with enzymatic detachment (Fig. 6B). The differences in recovery of cells after buffer vs. enzyme treatments for the strong vs. weakly adhering strains is further demonstrative of their relative levels of attachment capability.

These same strains were tested for attachment to each of 4 types of surfaces (glass, plastic, stainless steel, rubber) as determined by detachment recovery after 2 days of incubation on same-size pieces of material. Similar to what we observed with microplate wells, attachment of the strongly adherent strains was approximately 5-log levels higher than what was observed for the weakly adherent strains (Fig. 7A). It has been suggested that one possible cellular constituent that might be responsible for attachment could be flagella, a known entity involved with cellular attachment, and that

our attachment incubation temperature could be straddling the temperature limits for expression. Flagella for *L. monocytogenes* are known to be absent above 25°C (Way et al., 2004; Dons et al., 1992) and we therefore incubated cells at two temperatures above (30°C, 40°C) and two temperatures below this level (10°C, 20°C) to see if any differences were observed for attachment levels that would indicate temperature involvement with this trait. The levels recovered from detachment assays did not show any major temperature-related differences suggestive of temperature-based genetic expression (Fig. 7B). As expected, the levels recovered from microplates incubated at 10°C were less that than observed for the other three temperatures, especially with the strongly adherent strains, likely due to the reduced growth rate at 10°C compared to the other, higher temperatures (Fig. 7B).

The data contained herein presents an important and practical distinction between strains isolated in meat processing plants depending on whether they are strong- or weakly-adhering strains. Meat and poultry processors can not determine which strains may enter their plants on raw meat ingredients. A strongly-adhering strain as shown in Fig. 5 may prove more difficult to remove from plants producing RTE meat products and perhaps more readily promote the initiation of biofilms on processing equipment and environmental surfaces. Such strains are able to adhere strongly irrespective of the type of surface or temperature (Fig. 7). The prospects of viable *L. monocytogenes* on environmental or food-contact surfaces has significant consequences that can result in the manufacture *Listeria*-contaminated RTE meats that may lead to consumer illness and

death, product recalls, reduced confidence and/or loss of retail customers, and increased USDA-FSIS regulatory actions. The data presented here further emphasizes the importance for microbial interventions that eradicate *L. monocytogenes* from processing environments. Although we used enzymatic detachment as a means of quantifying strain attachment, this approach may also be useful as part of a sanitizing regimen similar to its use in laundry detergents to eradicate protein-based stains.

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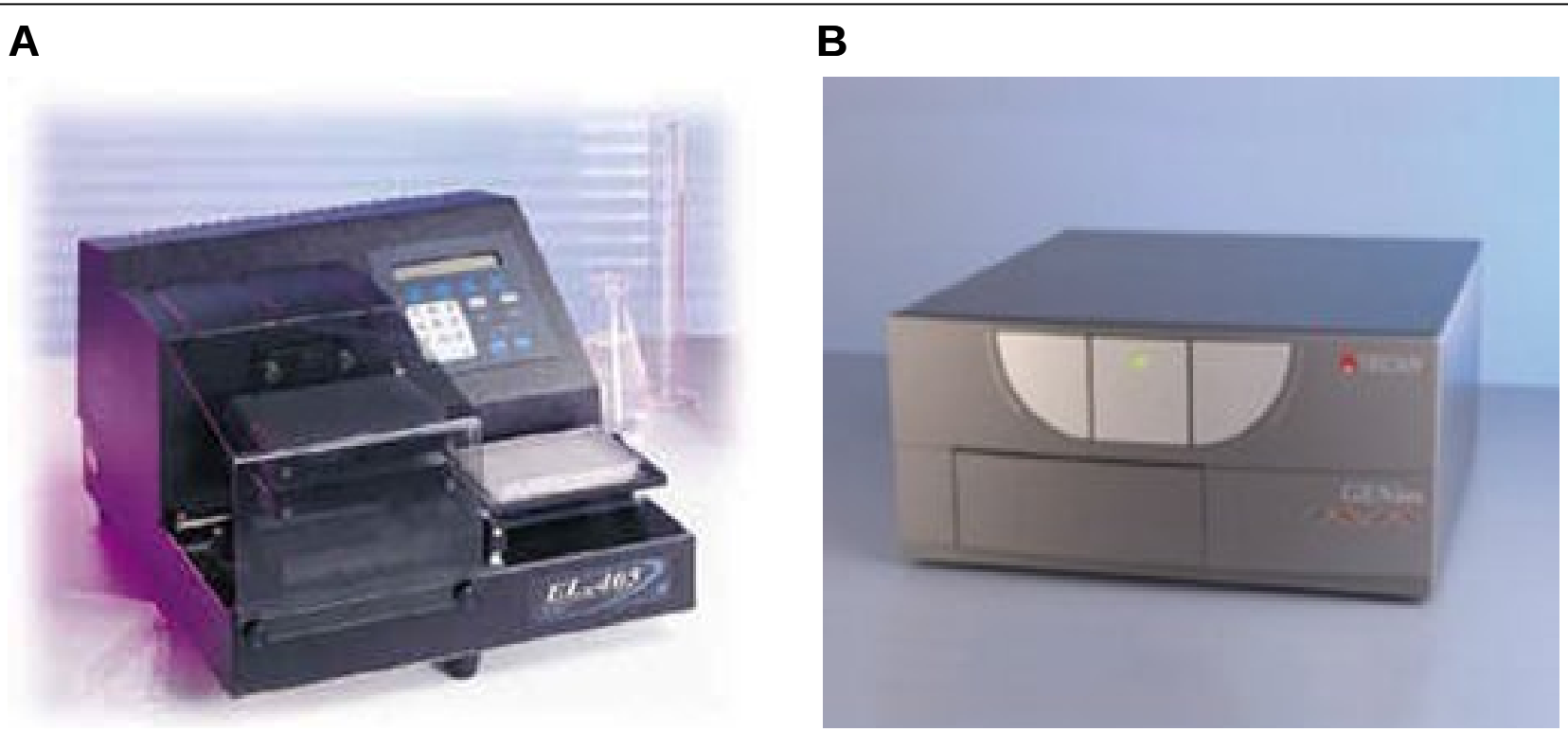

Figure 1. Main equipment used for our microplate attachment assay. Panel A, Bio-Tec ELx405 Magna plate washer with 96-pairs of needles to aspirate and replenish buffer solutions. Panel B, Tecan GENios fluorescent plate reader that can incubate and read microplates.

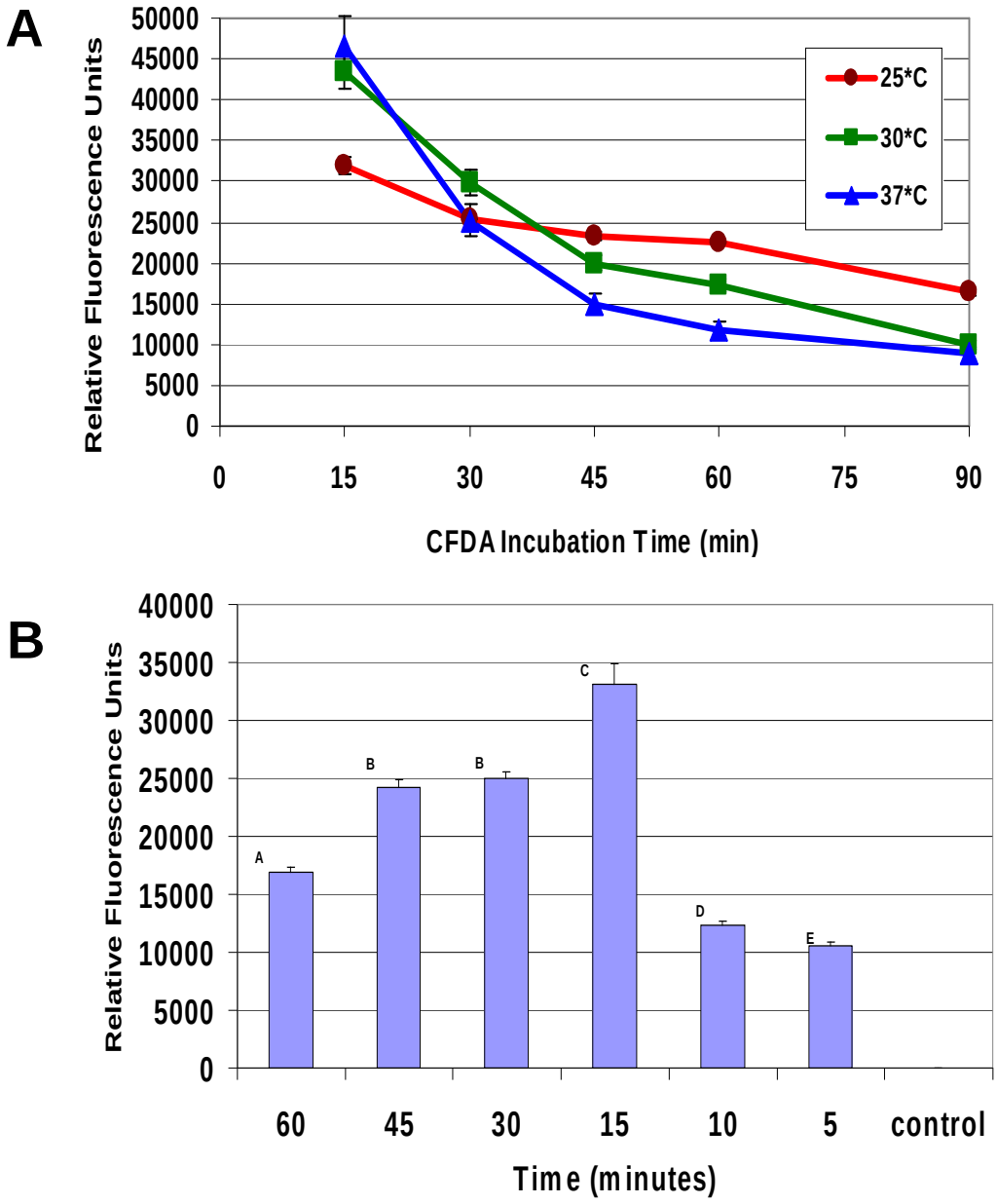

Figure 2. Optimization of the 5,6-CFDA assay for *Listeria* attachment. Panel A, examination of fluorescence obtained after different substrate incubation times at 25°, 30°, and 37°C. Panel B, fluorescence obtained at various incubation times at 25°C.

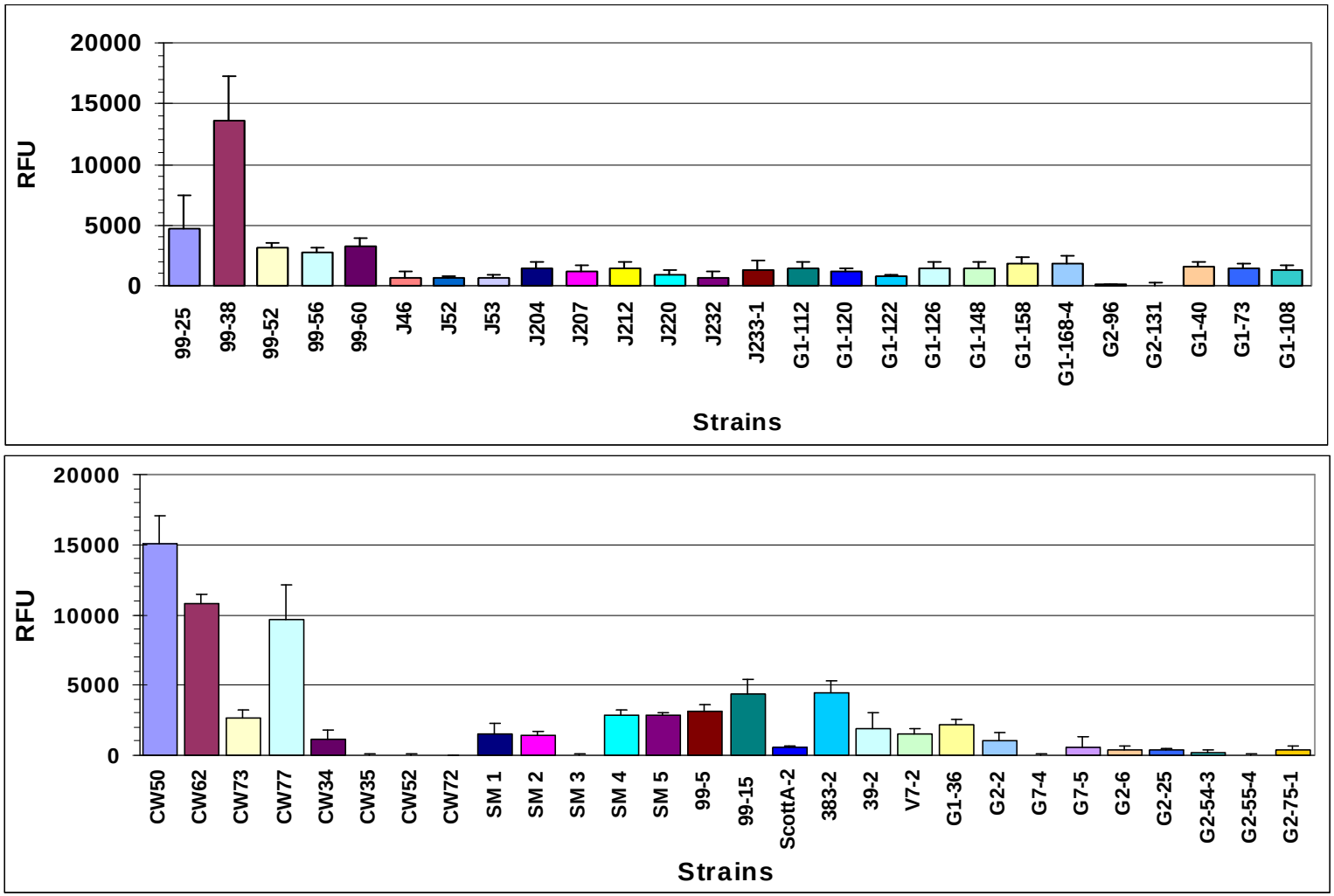

Figure 3. Microplate fluorescence attachment assay of various strains of *L. monocytogenes* from retail ground beef (99-series strains), from various retail ground pork and poultry samples (G- and SM-series), from environmental surfaces from commercial processing plants making RTE meats (J-series), and strains isolated from retail franks (CW-series).

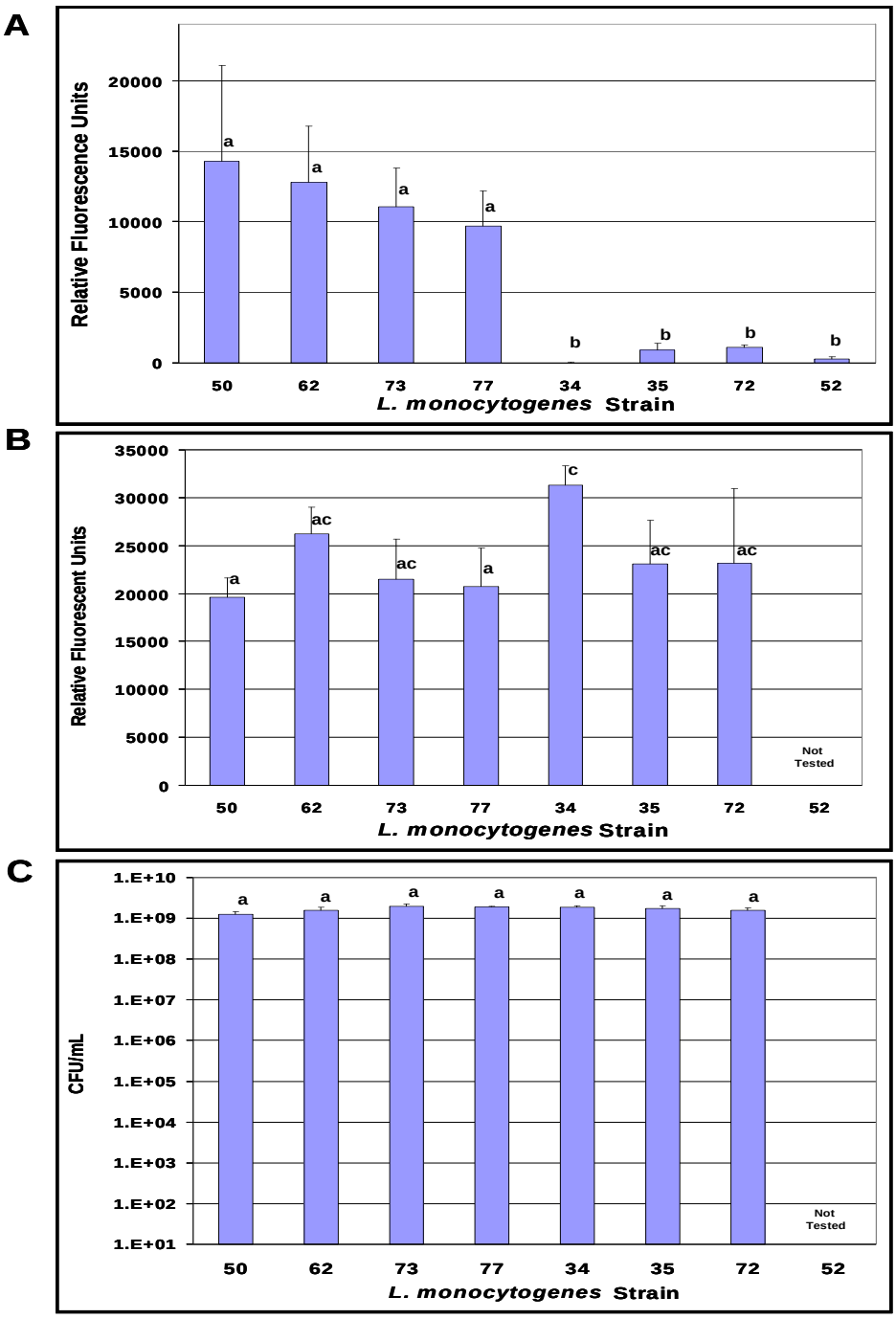

Figure 4. Comparison of fluorescence for strains of *L. monocytogenes* as attached or planktonic cells. Panel A, fluorescence from cells after attachment to microplates and incubation with 5,6-CFDA. Panel B, fluorescence from the same strains tested in liquid suspension after incubation with 5,6-CFDA. Panel C, plate counts of the planktonic cells in suspension from Panel B. Means sharing the same letter are not significantly different from each other; means with different letters are significantly different ($P < 0.05$). All data are presented as the means of triplicate replications.

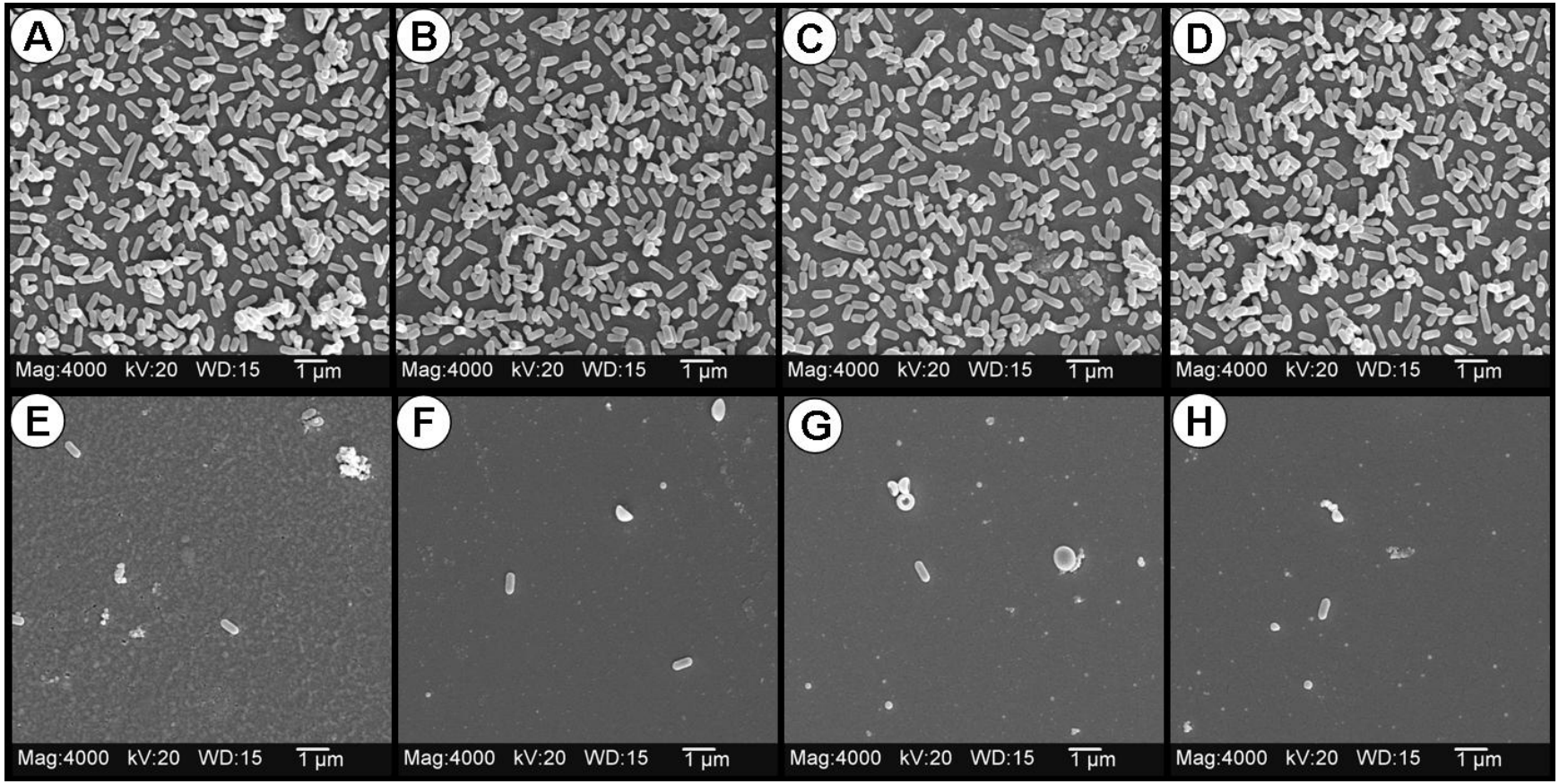

Figure 5. Scanning electron microscopy (SEM) images of various strong and weakly attaching strains of *L. monocytogenes* screened using the fluorescence microplate attachment assay. The strains of *L. monocytogenes* are: Top row: CW50 (A), CW62 (B), CW77 (C), 99-38 (D); bottom row: CW34 (E), CW35 (F), CW52 (G), SM-3 (H).

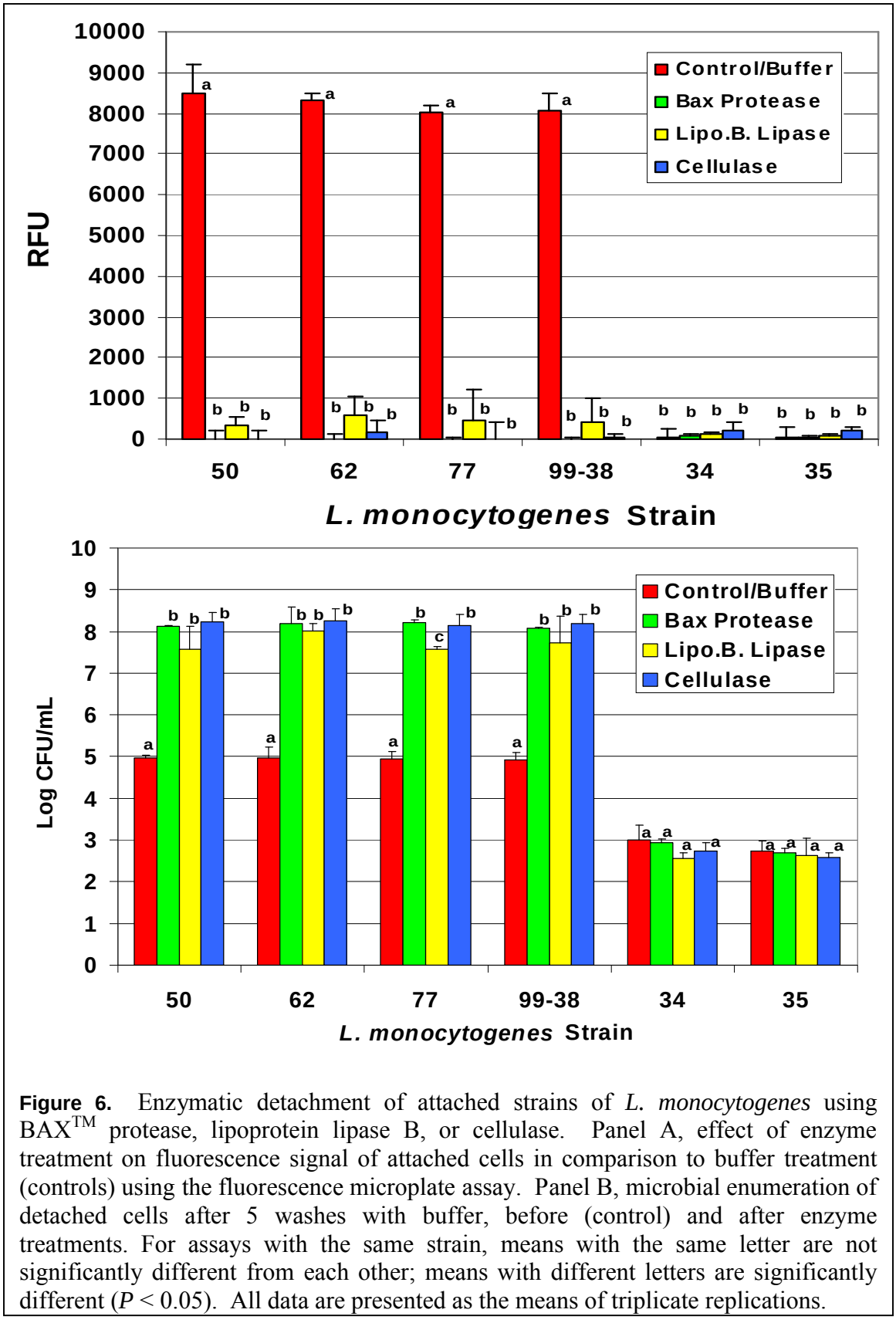

Figure 6. Enzymatic detachment of attached strains of *L. monocytogenes* using BAX™ protease, lipoprotein lipase B, or cellulase. Panel A, effect of enzyme treatment on fluorescence signal of attached cells in comparison to buffer treatment (controls) using the fluorescence microplate assay. Panel B, microbial enumeration of detached cells after 5 washes with buffer, before (control) and after enzyme treatments. For assays with the same strain, means with the same letter are not significantly different from each other; means with different letters are significantly different ($P < 0.05$). All data are presented as the means of triplicate replications.

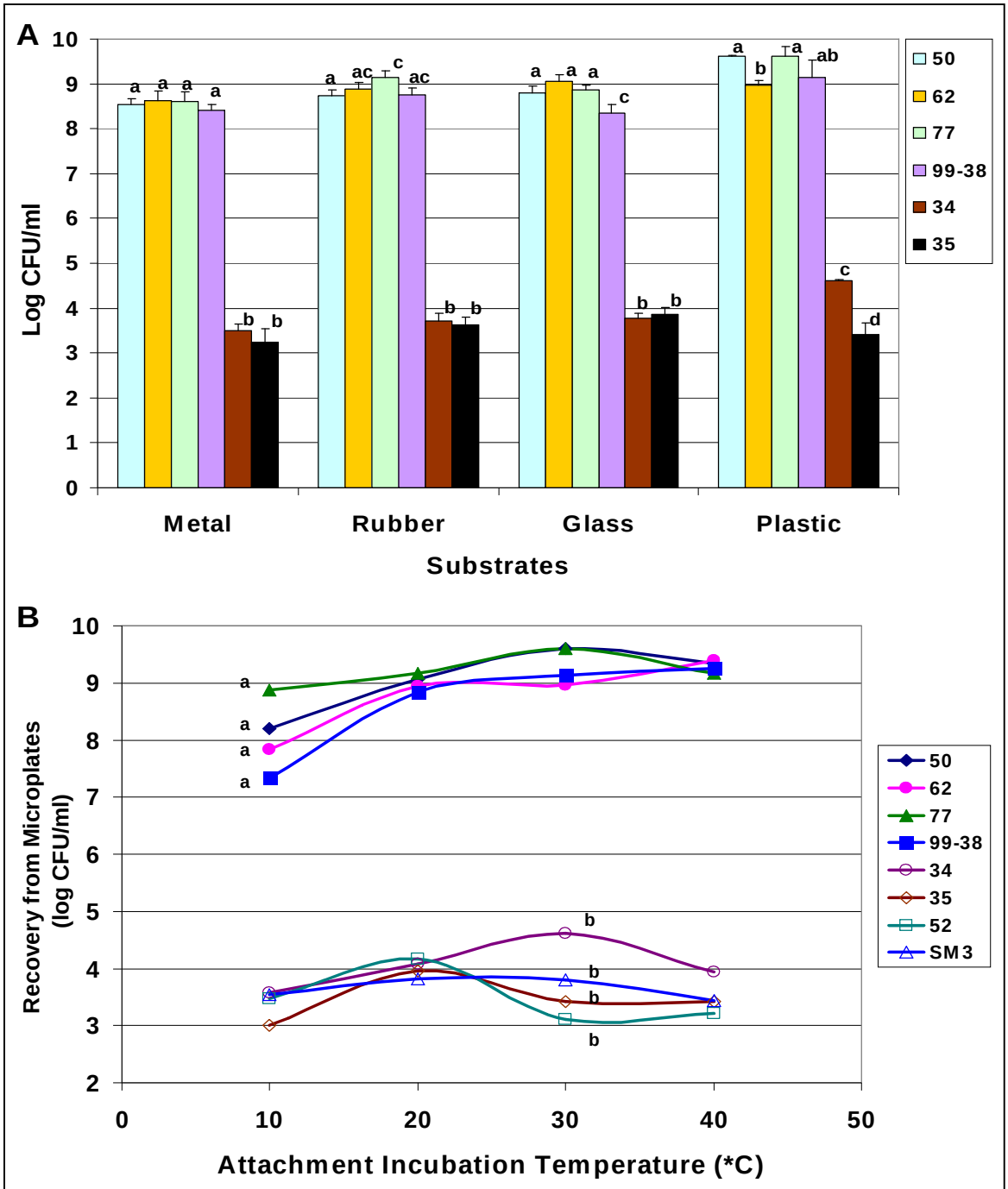

Figure 7. Attachment characteristics of strong and weakly-adhering strains of *L. monocytogenes*. Panel A, microbial plating of BAX™ protease detached cells after attachment to various substrates. For a given substrate, means sharing the same letter are not significantly ($P > 0.05$). Panel B, microbial plating of BAX™ protease detached cells after attachment for 48 hrs (20°, 30°, 40° C) or 96 hrs (10°C) at various incubation temperatures for attachment. Data sets with the same letter are not significantly different ($P > 0.05$) whereas those with different letters are significantly different ($P < 0.05$). All data are presented as the means of triplicate replications.

APPENDIX

FLUORESCENCE ASSAY TO SCREEN STRAINS

OF *LISTERIA MONOCYTOGENES* FOR

SURFACE ATTACHEMENT

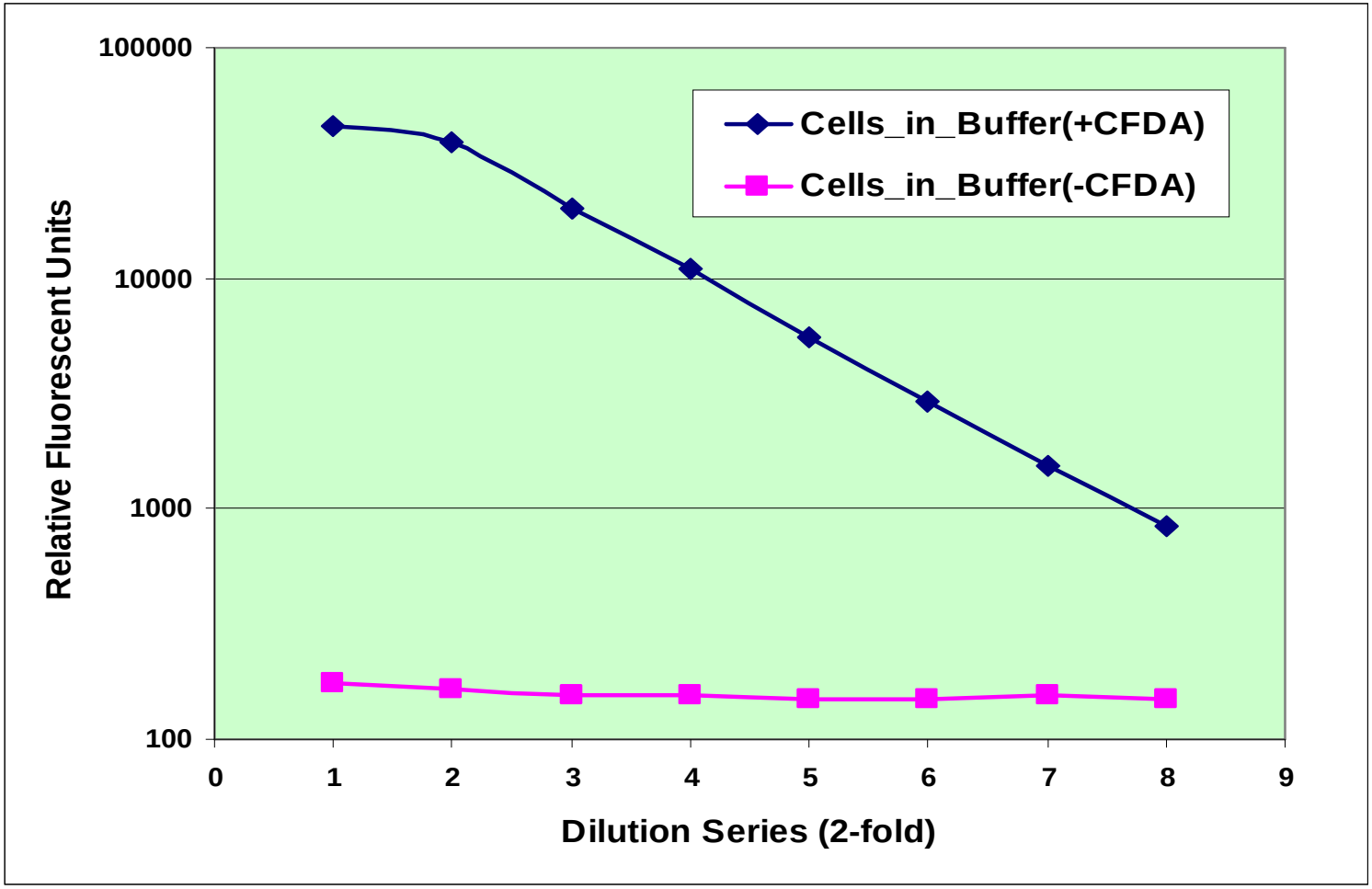

Figure 1. CFDA-based fluorescence obtained with serial dilutions of overnight cells in 50 mM Tris buffer (pH 7.4) using *L. monocytogenes* strain Scott A-2 in comparison to cells in Tris buffer without addition of 5,6-CFDA.

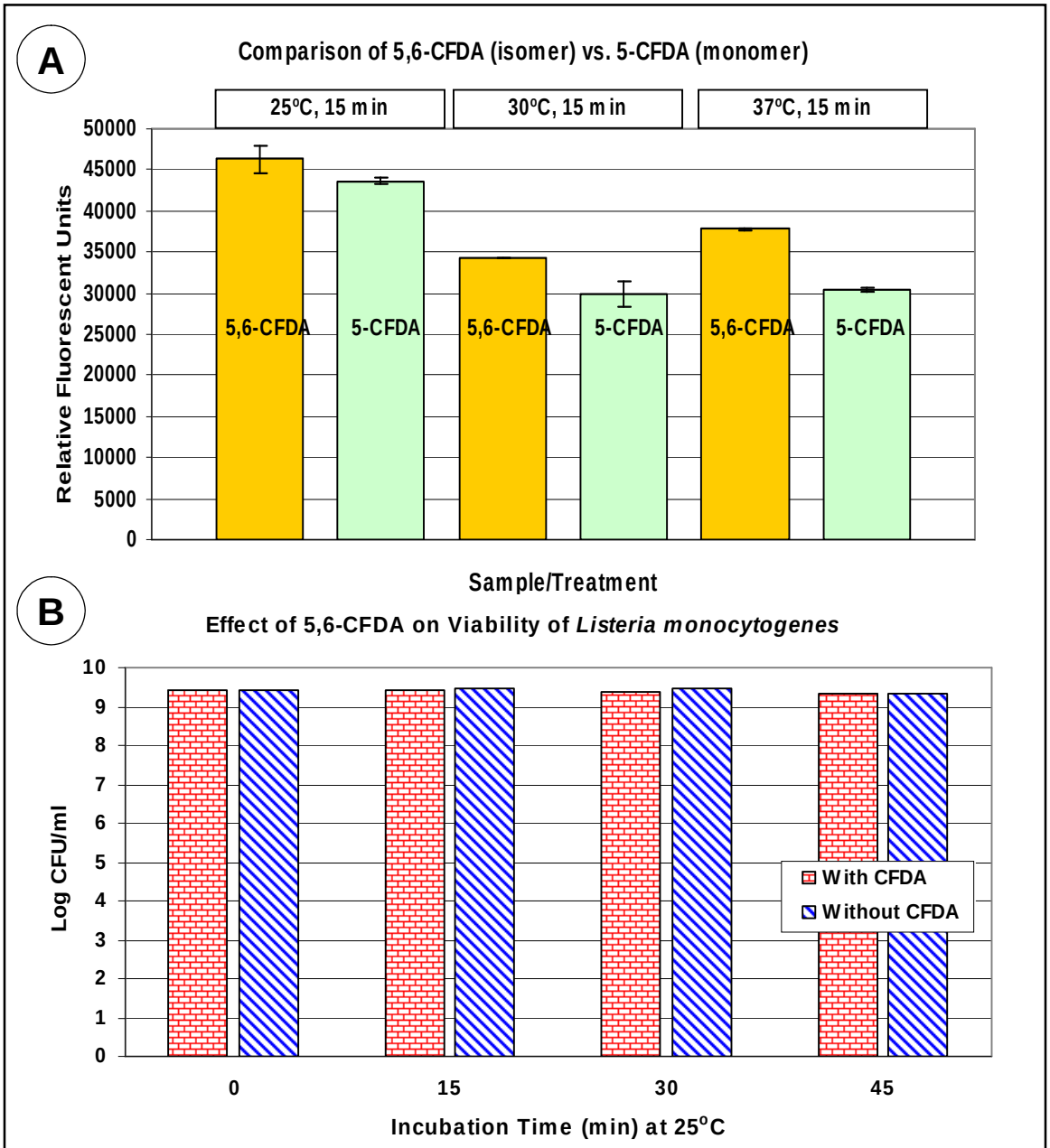

Figure 2. Characteristics of CFDA incubation temperature and on cell viability with *Listeria monocytogenes*. Panel A, comparison of signal obtained from attachment assays using the 5,6-CFDA mixed isomer with the 5-CFDA monomer incubated at 25°C, 30°C, and 37°C for 15 min for substrate utilization. Panel B, evaluation of the effect of 5,6-CFDA substrate incubation on viability of *L. monocytogenes* at 25°C.

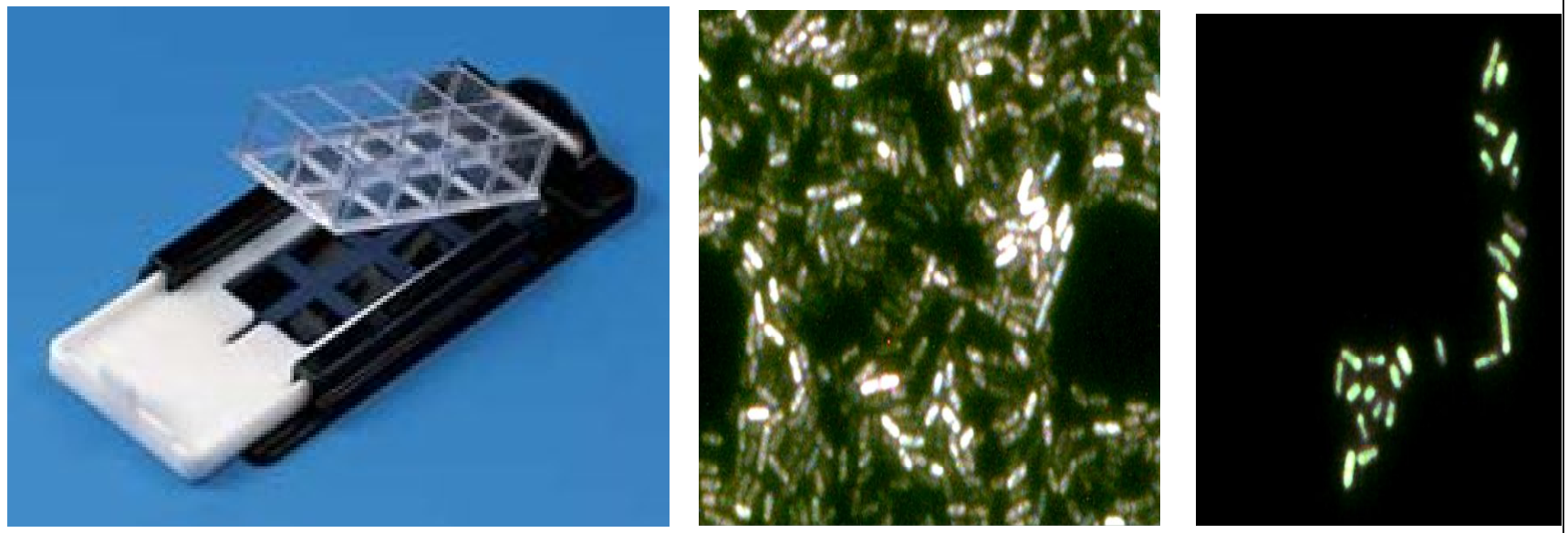

Figure 3. Microscope slide chamber and fluorescence microscopy of strongly and weakly-adhering strains. Panel A, microscope slide chamber with removable wells for direct microscopic comparison of attachment abilities of various strains. The bottom surface of the well is the microscope slide. Panel B, fluorescence microscopy of *L. monocytogenes* CW 50, a strong biofilm forming strain. Panel C, fluorescence micrsocopy of *L. monocytogenes* CW 35, a weak biofilm forming strain, attached to the bottom surface of the slide chamber.

VITA

RACHEL K. WRIGHT

Candidate for the Degree of

Master of Science

Thesis: DEVELOPMENT OF A FLUORESCENCE-BASED MICROPLATE ASSAY TO SCREEN STRAINS OF *LISTERIA MONOCYTOGENES* FOR SURFACE ATTACHMENT

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Organizations: Institute of Food Technologist

Name: Rachel K. Wright Date of Degree: May, 2006

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: DEVELOPMENT OF A FLUORESCENCE-BASED MICROPLATE ASSAY TO SCREEN STRAINS OF *LISTERIA MONOCYTOGENES* FOR SURFACE ATTACHMENT

Pages in Study: 66 Candidate for the Degree of Master of Science

Major Field: Food Science

Scope and Method of Study: Our objectives were to develop a fluorescence-based method to screen for strains of *L. monocytogenes* attached to microplate surfaces and to establish optimum conditions for its evaluation.

Findings and Conclusions: Four strains of *L. monocytogenes* were tested at various incubation temperatures (25°, 30°, and 37°C) to determine a good screening regimen. A Biotec Elx405 Magna plate washer was used to remove planktonic cells and wash attached cells. Retained cells were incubated at various time/temperatures with 5,6-carboxyfluorescein diacetate (5,6-CFDA) to determine optimal detection of attached cells using four laboratory strains of *L. monocytogenes*. Fluorescence from the internalized fluorescing substrate byproduct was determined with a Tecan GENios fluorescent plate reader. Attachment of additional strains was examined using this procedure and identified strong and weakly adhering strains showing > 50-fold difference in fluorescence measurements that was further confirmed by microscopic analysis and enzymatic detachment that allowed enumeration of the retained cells on microbiological media.

ADVISER'S APPROVAL: Dr. Peter M. Muriana