

MICROARRAY-BASED DIFFERENTIAL GENE EXPRESSION OF LISTERIA
MONOCYTOGENES CULTURES GROWN AS BIOFILMS AND PLANKTONIC CELLS

BY

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THESIS

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Abstract:

Listeria monocytogenes is one of the most virulent foodborne pathogens prominent in the food industry. The correlating disease listeriosis infects 2500 individuals in the United States annually of which roughly 625 cases are fatal. Current research is emphasizing that these infections are due to cell cultures forming and growing as biofilms. Biofilm cells are postulated to be more pathogenic than planktonic cells due to the cells forming an exopolymer substance and differentiated strata while working together as a microcommunity. It is thought that *Listeria* grows as biofilms on food processing surfaces; sloughing of these bacterial biofilm cells causes post-contamination of food products. While previous studies explore *L. monocytogenes* grown as planktonic cells, the current study looks into the functional genomics of listerial biofilm cells. A complete genome global gene expression microarray of a *L. monocytogenes* culture grown as biofilm and planktonic cells provides critical information to better understand the pathogenicity (causes many deaths) of *L. monocytogenes*, possible drug targets, and advancements in sanitation techniques. Using a genome-wide microarray, 398 genes were observed to be up-regulated, while 306 genes were down-regulated in biofilm cells in comparison to planktonic cells. Within the up-regulated genes were those that were involved in biosynthesis, energy metabolism, adaptation and protection, transcription, transport, and motility factors, chemotaxis, and several with unknown functions. Among those genes to be down-regulated in biofilm cells were those involved in cell structure, metabolism, biosynthesis, transcriptional regulators, sugar transport, and several hypothetical proteins. Prior to completing the global differential gene expression experiment, each step in the procedure was optimized. To ensure the microarray procedure was completed correctly, a sample was grown at refrigeration temperature and the microarray results were compared to Chan et al. (18).

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Chapter 1. Introduction

It is estimated that 76 million people become sick from foodborne pathogens each year in the United States (31). Even with the advancements in scientific research, public awareness, food safety, and cures to these foodborne sicknesses, the number of individuals affected is ever increasing due to mass distribution of food, increased consumption of prepared foods, and emergence of new bacterial strains (1). One of the most fatal of these foodborne pathogens *Listeria monocytogenes* causes the disease listeriosis (76, 84). It affects 2500 people in the United States annually, killing roughly one fifth (24). In comparison to other bacterial infections, listeriosis does not affect a large number of individuals, yet a high percentage of those who do develop listeriosis suffer with complications, which can be deadly (24). Typically associated with deli meats and cheeses, transfer of listerial cells onto food products is correlated to unsanitary processing surfaces (24, 76). Immunocompromised individuals, such as pregnant women and the elderly are encouraged to avoid foods which have been associated with *L. monocytogenes* outbreaks (24, 34). The ability of this bacterium to sicken many individuals is due to the bacteria's ability to invade and spread quickly intracellularly and extracellularly as a result of genes located on its Pathogenicity Island (41, 69). A Pathogenicity Island is a grouping of genes (genomic island) acquired via horizontal transfer which encode genes that contribute to virulence factors (43).

Biofilms are biologically active microcolonies composed of attached microorganisms and foreign material. It is currently accepted that the majority of microorganisms are surface-associated (9, 82). Biofilms are known to decrease efficiency of food processing equipment and cause post-processing contamination via sloughing (9, 50). Being comprised of one or several species of microorganisms and other foreign matter, cells grown as biofilms are known to be

more pathogenic than cells in the planktonic state (82). This increased pathogenicity has been shown to be a result of their strong attachment, exopolymer matrix, genetic differentiation between strata, and antibiotic resistance (2, 33). Although research has not yet been able to separate and analyze each strata, it is known that some cells are directly associated with the surface which possess no flagella and live in an anaerobic environment, while others are located closer to the exterior and subsequently function as the nutrient gatherers and metabolizers (33). The genomics of biofilm cells has been widely researched, concluding that functional differentiation and gene expression give rise to a variety of attributes. Therefore the ability of a microorganism to form a biofilm is considered a virulence factor (64).

Chapter 2. Literature Review

A. *Listeria* species

A1. Phylogeny and Biochemistry

Listeria is a member of the phylum *Firmicutes* and is closely related to *Bacillus subtilis* due to high similarity in 23s rRNA and 1,428 orthologous genes (76). The genus *Listeria* is composed of six species: *L. grayi*, *L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri* and *L. welshimeri* (69). Out of these six species, *L. monocytogenes* is pathogenic in humans while *L. ivanovii* is pathogenic in other animals, mainly sheep (34, 38, 39, 76). Additionally, *L. monocytogenes* is classified by three lineages and 13 corresponding serotypes and genotypes (66, 76). Lineage I is comprised of serotypes 1/2a, 1/2c, 3a, and 3c. Lineage II is comprised of serotypes 1/2b, 3b, 4b, 4d, 4e, and 7. Lastly, lineage III is comprised of serotypes 4a and 4c (38, 66, 76). Ninety to ninety five percent of food outbreaks are recorded to be associated with serotypes 1/2a, 1/2b, and 4b (34, 57, 66, 69, 76, 84). Furthermore, serotype 4b has been reported as being the most virulent, therefore causing the larger outbreaks (39, 46, 76, 84). Historically, the *Listeria* species was first reported in rabbits and guinea pigs by Murray, Webb, and Swann from Cambridge University in 1926 (70, 76, 84). It was later named to honor British surgeon Dr. Joseph Lister (70).

Listeria is an opportunistic gram positive, non-spore forming rod-shaped facultative anaerobic bacterium (38, 39, 41, 69, 84). Biochemically, most *Listeria* strains are catalase positive and oxidase negative (76). Well adapted for the food industry, strains of *Listeria* are known to grow in high salt concentrations (10% (w/v) NaCl), on foods whose water activity is above 0.93, and in a wide range of pH levels ranging from 4.5 to 9.2 (1, 38, 61, 69, 76, 84). Atypical to most foodborne pathogens, *Listeria* can grow at refrigeration temperatures (4°C), (1,

34, 76, 84, 91). For locomotion, *Listeria* has peritrichous flagella which exhibit a tumbling effect at 20-25°C (69, 76). Known to cause human infection, listerial cells grow and attach best at 37°C (human body temperature) in a nutrient-rich medium (60, 61, 62).

A2. Listeriosis

The causative agent of listerial illness is listeriosis of which nearly all cases are foodborne (69, 76, 84). Annually in the United States, the Center for Disease Control (CDC) estimates that *L. monocytogenes* is responsible for about 2500 cases and accounts for 27.6% of all foodborne disease deaths (1, 24, 34, 41, 69, 70, 76, 84). In addition to exhibiting a low prevalence worldwide (ranging between 0.1 and 11.3 million depending on the country), the incidence in the United States is gradually decreasing (by 36% between 1996 and 2006) (24). On the other hand, there has been an increased rate of listeriosis in several European countries over the past decade (1). Of all the cases, roughly 91% are hospitalized and 20-30% of total cases are fatal (34, 41, 50, 66, 76, 84, 85, 88).

While four percent of those individuals with listeriosis are asymptomatic, the majority of the population exhibits symptoms (67). Initially, normally healthy individuals who have consumed *L. monocytogenes* cells exhibit flu-like symptoms a few hours after consumption (1, 3, 34). As the infection spreads to the gastrointestinal tract 12 to 24 hours after consumption, fever, chills, nausea, diarrhea, and gastroenteritis appear (3, 24, 84). If not treated or resolved, the illness enters the central nervous system (within a few days to three weeks) and results in invasive listeriosis (1, 3, 24, 69). The resulting listerial meningitis has a mortality rate of 70% (3, 34, 41, 39). Respective symptoms with the bacterial infection entering the meninges are headache, loss of balance, stiff neck, and convulsions (24). This can also progress into septicemia where roughly 50% of those affected die (1, 34, 41, 76). Other complications due to

listeriosis include hepatic, splenic and brain abscess, osteomyelitis, pericarditis, myocarditis, necrotizing fasciitis, and joint infection (1).

The symptoms listed earlier in the aforementioned list are typically experienced by normally healthy individuals, while complications and the latter symptoms are more likely to develop in immunocompromised individuals (70, 50). This target population includes the elderly, those undergoing immunosuppressive therapy, or those with diabetes, autoimmune diseases, or alcoholism (24, 32, 34, 66, 84). Invasive listeriosis targets the immunosuppressed population of pregnant women and their fetuses. With a 20-fold increase in likelihood of sickness, these women may only develop flu-like symptoms but pass along the infection to their underdeveloped children (24, 76, 84). Transmission of listeriosis from mother to child can occur either prenatally or neonatally (3). Prenatal listeriosis due to *in utero* transplacental transmission results in 3rd trimester fetal abortion, stillbirth, or premature delivery to roughly 33% of fetuses (1, 3, 36, 70, 76, 79, 84). Infected mothers can alternatively pass listeriosis along to their children via the birth canal (1, 76, 84). The resulting neonatal infection has similar symptoms of sepsis and meningitis in the newborn child (24, 50, 70, 76, 79, 84).

The severity of sickness depends on the strain, the dose consumed, and the individual who consumed the contaminated food (3, 36, 69). Due to multiple contributing factors, an exact infectious dose has not been determined (69, 84).

Since listeriosis is not as prevalent as a streptococcal infection, there is no routine clinical test, although once detected via a blood test, the infected can receive antibiotic treatments including penicillin or ampicillin and aminoglycosides (1, 3, 24, 34, 63, 69, 84). Using blood, stool, cerebrospinal fluid, or placenta, clinical tests are performed for detection (1). The United States Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) and

International Organization for Standardization (ISO) methods use Polymerase Chain Reaction (PCR) for rapid detection (34). Further methods used in the United States and Europe include pulsed-field gel electrophoresis (PFGE) subtyping, enzyme-linked immunosorbent assays (ELISA), and DNA hybridization (1, 32, 84). Ultimately educating the public about food safety through the International Commission on Microbiological Specification Food Safety Objectives can lead to better risk management and hopeful decrease in foodborne disease outbreaks (88). This includes using Good Manufacturing Practices (GMPs), Hazard Analysis Critical Control Point system (HACCP), and good hygiene practices (32, 69, 88). Many agencies including the FDA, CDC, Food and Agriculture Organization (FAO) in conjunction with the World Health Organization (WHO) have implemented assessments regarding *L. monocytogenes* in the food industry.

A3. Prevalence in food industry

Listeria is designated by United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) as a zero tolerance microorganism (32). This allows only <0.04 CFU/g of *L. monocytogenes* in a 25g sample (88). This zero tolerance microorganism ensures that no adulterated food enters the food trade. *L. monocytogenes* cells, normally found in soil, water, and sewage pass into the food chain at various points (1, 32, 39, 41, 76). A less common source of listeriosis is contaminated raw vegetables in a farm environment (3, 24, 70, 84). Entering the food chain through infected feed, the bacterium infects animals (not including humans), but proves to be asymptomatic (1, 24, 41, 69, 76). Consequent animal products such as raw poultry, fish, and milk have a risk of infecting consumers (3, 69, 70, 84). Processing, including pasteurization of milk, kills listerial cells and therefore removes the threat of further infection (24). The most common cause of this bacterial contamination is post-processing

contamination from food processing equipment and a lack of sanitation or appropriate cleaning measures (1, 41, 76). The greatest outbreaks are attributed to deli meats with more than 100 cases per year and more than five cases per billion servings (3, 24, 70, 84). Other less prominent food contaminations have been found in soft cheeses, ice cream, Ready-To-Eat (RTE) meals, and other dairy and meat products (3, 66, 70, 84). Most of the aforementioned products are stored at refrigeration temperatures at which *Listeria* can grow. Among the most recent outbreaks, 22 Canadians died from cold cut contamination in June 2008 (55). A few months later in October, 13 perinatal and two adult deaths were reported from cheese in Quebec (54). Also in the same year, soft cheese was the cause of 119 cases and five deaths in Santiago, Chile (57). In late 2009, Austria and Germany reported a cheese outbreak with 45 cases and eight confirmed deaths (56). Additionally, Denmark reported a significant increase in listeriosis cases between 2003 and 2009 (83). The British Columbia Centre for Disease Control reported a recall for *Listeria*-contaminated cheese in March 2010 (11). Furthermore, Ottawa and Quebec cold cuts, cheeses, and RTE meals resulted in 14 cases and five deaths in Canada and seven cases and two deaths in Texas according to the Public Health Agency of Canada after a massive recall (12, 13, 14, 30, 68). In May 2010, legal action was taken against a German supermarket store for selling *Listeria*-contaminated cheese which resulted in seven deaths (51).

A4. Pathogenicity

Following ingestion, listerial cells enter the intestine beginning their infectious spread throughout the body. Initial attachment to intestinal epithelial cells is mediated by internalin A (InlA) of the bacteria with E-cadherin of the host cells (46, 58, 67, 70). Jacquet et al. has identified InlA as a virulence marker due to its presence in pathogenic strains (46). Additionally, Olier et al. determined that strains with truncated InlA proteins are less invasive in chick embryos (67).

Another internalin InlB activates signaling cascade pathways such as MAPK, tyrosine kinase MET, and induces clathrin endocytosis (41, 58). Working together, InlA and InlB and several other bacterial surface proteins mediate internalization of *L. monocytogenes* into a single membrane vacuole (58, 67). To gain entry in the nutrient-rich cytoplasm, *L. monocytogenes* expresses a phospholipase PlcA and a cholesterol-dependant pore-forming hemolysin Listeriolysin O (LLO) which create holes in the single membrane (41, 58, 70). Being a toxin, LLO must be tightly regulated to ensure only the vacuole membrane is destroyed. Overexpression of LLO results in lysing of the host cell, consequentially exposing the bacterium to the host immune system (41). Much research has been conducted regarding LLO due to its unique properties for *L. monocytogenes*. LLO activity is activated by an acidic environment (pH 6) which is atypical of other cytolysins (41). Additionally, LLO has been identified to help with initial adhesion as well as to participate in activation of various signaling pathways including NF- κ B67 and MAPK68 (41, 58). With this evasion from the innate immune system, the bacterium is free to multiply within the nutrient-rich host cell.

To spread the infection to other cells, *L. monocytogenes* overtakes host cell machinery to induce actin polymerization with the help of bacterial surface protein ActA (41, 69). This propulsion allows for undetected movement into neighboring cells intracellularly (41, 69). Pamer showed that infection with ActA-deficient bacteria is less pathogenic (70). Entrance into a second cell creates a double-membrane around the bacteria. These layers are lysed by LLO and phospholipase PlcB. Thus, the bacteria enter the cytoplasm of the second cell and multiply again. This begins the cycle of replication, spread from cell-to-cell, escape, and replication as shown in Figure 1. With this mechanism of infection, *L. monocytogenes* is able to cross the intestinal

epithelium in addition to the blood brain barrier (BBB) and the feto-placental barrier (41, 58, 67, 69).

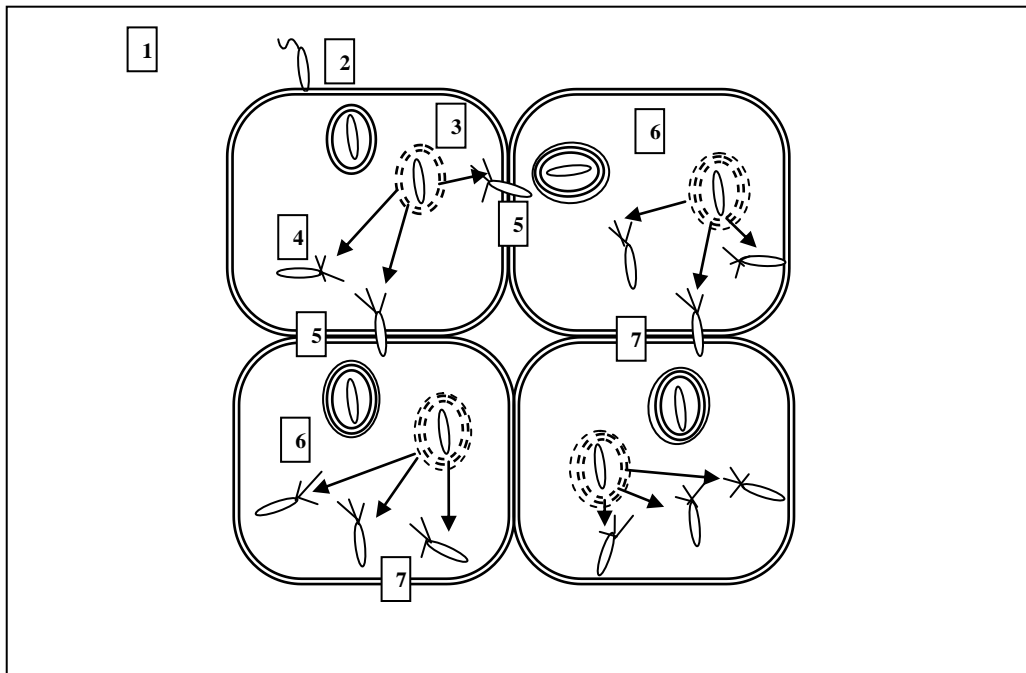


Figure 1: *Listeria* pathogenicity cycle. 1: Ingestion of *L. monocytogenes*. 2: Adhesion and internalization with surface proteins InlA and InlB. 3: Escape from vacuole with LLO and PlcA. 4: Multiplication in cytoplasm. 5: Actin polymerization to spread from cell-to-cell with ActA. 6: Escape from double membrane vacuole with PlcB and LLO. 7: Further cycle of invasion.

A5. Genomics and Proteomics

Currently there are only two strains of *Listeria* whose genomes have been completely sequenced (*L. monocytogenes* strain EGD-e and *L. innocua* CLIP 11262) (39, 66). Upon partial genomic comparisons, only a few genetic differences are noted between strains and serotypes, thus indicating high genomic stability (17, 38, 39, 66). Microarrays have been used to compare genomics of listerial lineages (26). Nelson et al. determined that 83 and 51 genes are specific to serotype 1/2a and 4b, respectively, while between 50 and 100 genes are strain specific (66). Nelson et al. also postulates that pathogenicity and adaptation to survive in various environments are due to these minor differences in genes (66). Differing between strains of *L. monocytogenes*,

the genome contains roughly three million base pairs and a G + C content of approximately 36-42% (39, 65, 76).

Many of the prominent virulence and virulence-associated genes are found in *Listeria* Pathogenicity Island 1 (LIPI-1). Under the 27-kDa polypeptide transcriptional regulator PrfA, virulence genes are expressed in response to environmental changes (41, 48). According to the National Microbial Pathogen Data Resource (NMPDR), the following genes are included in the 9-kb LIPI-1: *prfA*, *plcA*, *hly* (encoding LLO), *mpl* (Zinc metalloproteinase precursor), *actA*, *plcB*, *orfX* (distant similarity with viral glycoprotein gp160 of HIV type 1), *orfA* (virulence cluster protein A), and *orfB* (virulence cluster protein B) (38, 65). Several factors influence PrfA translation. It is widely accepted that PrfA is thermoregulated, being translated when grown at 37°C and inactivated below 30°C (23, 48). Additionally, PrfA is known to be activated under stress conditions such as in nutrient-poor environments (48, 75). Iron has been shown to play a role in catalase, superoxide dismutase, and LLO activity (27). Additionally, activated charcoal has been shown to repress virulence gene expression while a high osmolarity induces expression of *hly* (48).

Under various growth or stress conditions, *Listeria* cells have been known to change their gene and protein expression (69). Folio et al. determined 161 proteins to be either up- or down-expressed when comparing mid-log and stationary phase cells (28). Chan et al. reported 105 genes up-regulated and 74 genes down-regulated in log and stationary phase cells when grown at 4°C (18). Additionally, 170 genes were up-regulated and 102 genes were down-regulated in log and stationary phase cells when grown at 37°C (18). Within the genes found to be down-regulated when grown at 4°C were genes involved in encoding virulence factors (18). Bowman et al. determined differential gene expression of listerial cells under hydrostatic pressure

processing (8). Up-regulated genes included those with functions of DNA repair mechanisms, motility, peptidoglycan biosynthesis, and protein translocase systems (8). During heat-shock treatment, *L. monocytogenes* cells differentially express two percent of their genome (87). Ultrahigh-temperature processing of milk containing *L. monocytogenes* cells up-regulated genes including those involved in transport and binding proteins, transcriptional regulators, energy metabolism, and protein synthesis (53). Although there are several studies experimenting with listerial proteomics and genomics, there is still much information to be learned with further research.

B. Biofilms

B1. Definition

Broadly defined, a biofilm is a surface-associated three-dimensional community composed of differentiated strata and surrounded by an exopolymer substance (19, 61, 71, 82). Between these layers are water channels allowing for constant nutrient nourishment and waste removal (9, 19, 20, 82). Biofilms range in thickness from micrometers to millimeters (9). This has been visualized as a mesh-like material via scanning electron and scanning confocal laser microscopy (61, 82). Minimal research has been conducted observing the layers formed within biofilms. Derlon et al. experimented with various shear stresses to observe the tightness of binding of these stratified microcolonies (21).

Biofilms were first discovered in the 1920's on ship hulls and later in the 1980's in various locations including natural, industrial, and medical sites (79, 82). Within recent years, biofilms have become an interest to the scientific community. The United States National Institute of Health (NIH) has announced that roughly 80% of microorganisms are surface-associated (52, 64). Since then, it is accepted that most bacteria grow preferentially as biofilms

(9, 82). Of concern to the food industry are the spoilage and pathogenic bacteria sloughing off and causing post-processing contamination (79). Current research has yet to find a physiological marker associated with bacterial biofilms (71). Being considered a virulence factor, biofilm formation is of great interest to bacteria researchers (64).

B2. Development cycle

The first step of biofilm formation is the initial adhesion of planktonic cells to a surface (37, 47, 81). This adsorption includes weak electrostatic interactions and van der Waals' forces, providing reversible adhesion (9, 19, 47, 85). *L. monocytogenes* adheres to abiotic surfaces such as stainless steel, rubber, plastic, polymers, polystyrene, and glass, most of which are present in the food processing environment (20, 37, 85, 91). They are also capable of adhering to biotic surfaces including plant and animal tissues (including food products) (20, 37, 85). Many physiochemical factors determine biofilm formation including surface electrical properties, environmental temperature, pH, oxygen and water activity, osmolarity, interaction with other microorganisms, nutrient availability, and bacterial hydrophobicity and motility (19, 23, 35, 37). Habimana et al. has determined that surface hydrophobicity, microbial-host interactions, and flagella are the most important factors in biofilm determination (37). Shi and Zhu have suggested that bacterial adhesion is heightened by high free energy and a damp surface (79). Research by Chavant et al. indicates that incubation temperature has no impact on the initial attachment of cells to surfaces (19). More recently, Zameer et al. determined that maximum biofilm growth occurs at 30°C (91). Older research by Kim and Frank proves that glucose and amino acid levels are not significant in biofilm formation, while the level of phosphate in minimal medium is significant (49). Other research determining nutrient requirements was conducted by Folsom et al. and Gorski et al. using a variety of growth media (29, 61). Extensive research concludes that

adhesion is strain- and serotype-specific (29, 47, 61). Serotype 1/2a cells adhere the fastest and produce a larger biofilm in comparison to other serotypes as determined by Kalmokoff et al. and Folsom et al. (29, 47). Although bacterial attachment is mainly via adhesions, it has been postulated that surface proteins and other cell surface appendages play a role in adhesion (47, 79). Adhesions such as poly-beta-1,6-N-acetyl-d-glucosamine (PGA) and surface proteins LapA are known to aide in attachment (35). Shi and Zhu mention that *Staphylococcus aureus* expresses Bap (biofilm-associated protein), *Vibrio parahemolyticus* expresses VP1443, and *Salmonella* expresses BapA (79). No such protein has been found to be specific to *Listeria*.

Although most microorganisms prefer attachment to hydrophobic surfaces, *L. monocytogenes* is an exception (35, 79). Chavant et al. has confirmed via microscopy and enumeration that the basic and negatively charged surface proteins of listerial cells allow for greater and faster biofilm formation on hydrophilic surfaces such as stainless steel in comparison to hydrophobic surfaces such as polytetrafluoroethylene (19). Some gram negative bacteria such as *Salmonella* and *Escherichia coli* express fimbriae which contribute to the cell surface hydrophobicity (35, 79). *L. monocytogenes* possess fibrils for a similar function. Research by Kalmokoff et al. determined that strains with higher numbers of fibrils adhere better to surfaces (47). Another listerial cellular appendage functioning in cell-to-surface adhesion is flagella. Indirectly acting in attachment, flagella bring the cells to the surface (35, 52, 79). The importance of flagella gene expression in biofilm formation has been researched by Kumar et al (50). Todhanakasem et al. determined that flagella knockouts inhibit biofilm formation (85). In addition to flagella, *E. coli* biofilms rely on motility, chemotaxis, and pili for initial attachment (52). For locomotion, other bacteria, such as *Pseudomonas aeruginosa* have pili (82). Chen et al. determined that *inlA* and *inlB* also play a role in attachment to glass surfaces (16).

Once the initial monolayer of cells have adhered to a surface, biofilm cells change from reversible adhesion to irreversible attachment (19, 82). The number of cells in biofilms increase either by binary division (replication) or by recruitment of more cells from the environmental fluid (47, 81, 82). This step also involves the production of exopolymers called the extracellular polymeric substance (EPS) as seen in Figure 2 (42, 47, 82). Zameer et al. determined that EPS formation occurs within 48 hours of initial incubation (91). This 'matrix' material is composed of carbohydrates, proteins, phospholipids, nucleic acids, and other substances from the immediate environment (64, 79, 82). Hefford et al. viewed this EPS and conjugately-labeled layers via microscopy and completed further proteomic studies regarding this EPS and strata (42). Meng-Ying et al. indicates the importance of EPS in gram-positive and gram-negative bacteria biofilm formation (52). This EPS serves as a scaffold for structural support as well as a chemical-retardant barrier (6, 64, 82). Musk and Hergenrother have determined that EPS provides protection from antimicrobial agents (64). A specific anionic polysaccharide alginate has been involved in *P. aeruginosa* biofilms as the main agent in antimicrobial trapping and initiation of biofilm formation (2, 82). Stoodley et al. determined that disruption of EPS results in greater biofilm destruction due to antimicrobials (82). Goller and Romeo stipulate that cells sample environments before choosing to irreversibly attach (35). After forming gradient layers with an EPS covering, each cell differentiates to best help the microcolony survive (19, 33). Fux et al. states that cells at the surface specialize in oxygen and glucose consumption (33). Being at the surface, they are exposed to the nutrient-rich environment and they are able to provide byproducts to interior cells (82). The metabolic role of cells toward the center of the biofilm differs from that of exterior cells. Not being exposed to oxygen at the center, these cells create an anoxic and anaerobic environment with restricted metabolic activity (2, 33, 40, 64). In addition,

accrue of cellular waste products creates an acidic environment in the interior cells (64). Musk and Hergenrother discussed that these anaerobic and acidic niches antagonize antimicrobials, thus leading to better survival of biofilm cells in comparison to planktonic cells (64). Stoodley et al. determined that addition of carbon nutrients to *Pseudomonas putida* and *E. coli* interior cells restores their metabolic activity, thus converting them to planktonic cells (35, 82). This resembles a survival technique used by spores. Interior cells are hibernating and growing more slowly until they are exposed to further nutrients (2, 64). These adaptations allow for greater protection from environmental stresses where only a few of the outer cells may be damaged (2, 40). Fux et al. describes that interior cells are physiologically similar to planktonic cells grown to stationary phase (33, 81).

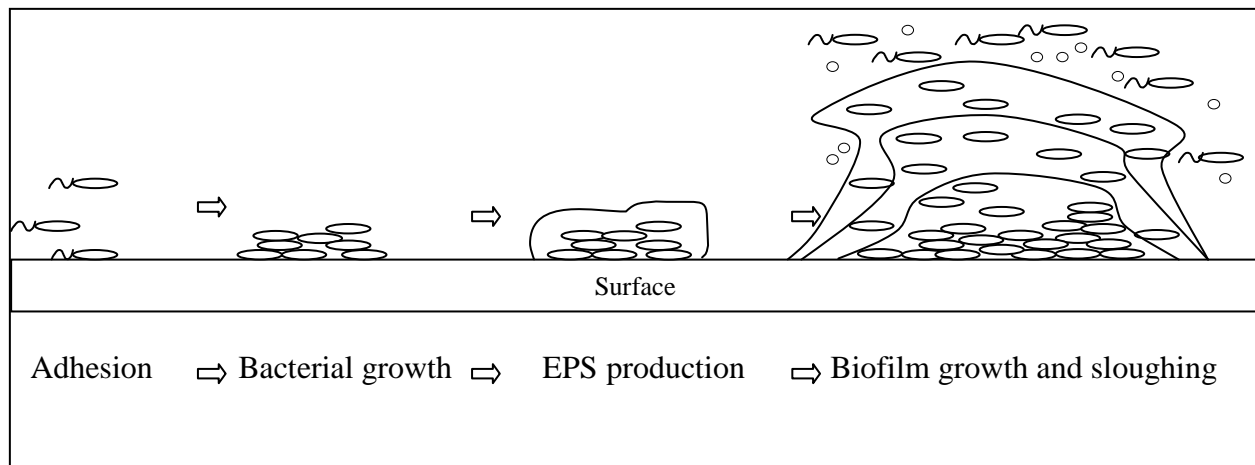


Figure 2: Biofilm formation. Initially, cells are brought to a surface via flagella. Reversible adhesion becomes irreversible attachment. Next, an EPS is formed and cells begin to differentiate and form strata. Breaking off of biofilm cells creates water channels which aide in waste removal.

Since biofilms are dynamic structures, there is constant addition and removal of cells. Extensive research by Parsek and Singh determined that this detachment or sloughing can be single cells or clusters (71). These cells either actively or passively enter their new environment and revert back to planktonic cells, fully regaining their metabolic capabilities as shown by 2D protein gel electrophoresis (19, 71, 82). It is still not known how the EPS is removed to allow

this process to occur. It is postulated that enzymes play a role in dissolving these exopolymers (71). The empty spaces created by the departing cells become water channels, bringing nutrients to cells and removing waste products (64, 82). Additionally, death of cells can result in sloughing (81). This results in post-processing contamination of the food product (9, 19, 50).

B3. Resistances

Showing an antimicrobial resistance of up to one thousand times than that of planktonic cells, biofilm cells are a major concern to the scientific community (2, 6, 52, 64, 71, 80). These antimicrobial methods include: drying; cleaning; or applying UV light and disinfectants (23, 50, 79). Chemical treatments include antimicrobials, chelating agents, sodium chloride, calcium chloride, and surfactants (81). After killing the cells, proper sanitation must be administered to remove the debris prior to resuming use of equipment. Additionally, dead cells dilute the killing agents, making them less effective (40).

It has been postulated that this antimicrobial resistance is due to several factors including the physical protection of the EPS, the changed genetic expression from susceptible planktonic cells in the differentiated cells inside the biofilm, acquired through horizontal gene transfer, and quorum sensing (2, 33). The EPS creates a diffusion barrier through which antimicrobials cannot pass (2, 33). The stress that the antimicrobial exhibits on the biofilm induces changes in gene expression that aide in resistance (40, 71). Furthermore, it is thought that biofilm formation is a stress response (2).

Genetic differentiation plays a critical role in biofilm resistance to killing agents (64). Anderson and O'Toole have determined specific tightly regulated resistance genes that are expressed in biofilm cells (2). These include expression of efflux pumps, bacterial targets, and

enzymes which alter the antibiotic (64). Induction of these gene expressions may be a result of the already differentiated layers or can be brought on by quorum sensing of the antimicrobial (2).

Quorum sensing has been identified to play a key role in biofilm formation and perhaps in antimicrobial resistance (33, 52, 79). Quorum sensing is a method of communication of bacterial colonies by which signal molecules (autoinducers) are sent to sense the surrounding environment (6, 82). By sensing population density, signals are received by the colony which induces genetic changes to best survive (6, 64). After initial attachment to a surface, biofilm cells interact with their immediate surroundings, thus aiding in biofilm growth and survival as seen in *Bacillus* and *Streptococcus* species (6, 64). In many species, the autoinducer LuxS is used to sense population density (35). An increase in LuxS-like activity results in formation of a denser biofilm (79). *Aeromonas hydrophila* possess AHL quorum sensing to aide in their biofilm formation (52). Planktonic cells do not possess this function (6).

Antibiotic resistance genes for both planktonic and biofilm cells are horizontally transferred through conjugation (33, 71). This is very common in hospital settings, producing a hazardous environment in which bacteria can change within one life cycle (71). Being located in close proximity to one another and in high density, biofilm cells horizontally transfer antibiotic resistance genes more easily than in other conditions (71). Along with horizontal transfer, genes can be transferred from one bacterium to another via phage transduction (71). Mah et al. demonstrated this antimicrobial resistance is due to genetic factors in *P. aeruginosa* biofilm cells (59).

Biofilm cells are known to also resist host cell defenses (20, 80). The host immune system secretes antibodies which should penetrate the cells. Biofilm cells resist these host cell

defense mechanisms in various ways including by hiding antigens and releasing enzymes such as catalase (6, 33).

B4. Proteomics and Genomics

Although planktonic and biofilm cells are genomically identical, expression of certain genes are different (either up-regulated or down-regulated) which results in phenotypic changes (6, 20, 71, 79, 82, 91). Using 2D gel electrophoresis, Matrix-assisted Laser Desorption/Ionization- Time-Of-Flight (MALDI-TOF) and Multiple Steps of Mass Spectrometry (MS/MS), proteomic studies have determined differences in protein expression in biofilm and planktonic cells (42). Hefford et al. noted 19 proteins more highly expressed in biofilm cells in *L. monocytogenes* 568 (42). Their functions include stress response, biosynthesis, energy generation, regulatory functions, and protein synthesis (42). Tremoulet et al. determined 22 *L. monocytogenes* proteins to be up-regulated and nine proteins to be down-regulated in biofilm cells (86). Due to a lack of proteomic information at the time of the study, few functions were assigned with the proteins (86). The only down-regulated protein identified was that of flagellin (FlaA), while the up-regulated enzymes pyruvate dehydrogenase (PdhD) and 6-phosphofructokinase are known to be involved in carbon metabolism (86). *P. aeruginosa* biofilms showed a six-fold or greater increase in protein expression for over 800 proteins (82). Three hundred of these were expressed at undetectable levels in planktonic cells (82). The main functional groups of these up-regulated proteins are adaptation and protection, membrane transport, secretion, metabolism, and phospholipid biosynthesis (82). Similarly, *E. coli* biofilms have between 13 and 20 % of the proteome either up-or down-regulated (64).

With advancements in technology, genomic studies have been conducted exploring gene expression of biofilms. Kumar et al. mentioned the two component regulatory system degU was

important in *L. monocytogenes* biofilm formation (50). While mutants of *prfA* and *sigB* (sigma factor B) formed wild type biofilms, mutants of *agr* (accessory gene regulator) and *ami* (autolysin-adhesion gene) impaired biofilm initial attachment (50, 79). Genes regulated by the *agr* peptide sensing system are involved in several virulence factors for *L. monocytogenes* and *S. aureus* (79). The mutant gene system *rpoS* (RNA polymerase sigma S involved in stress tolerance) inhibited *E. coli* biofilm formation and induced a larger and more antibiotic resistant biofilm in *P. aeruginosa* (2). Schembri et al. used a microarray to determine up-regulated genes in *E. coli* biofilm cells functioning in low oxygen adaptation, encoding transport proteins, oxidoreductases and heavy metal resistance (77). Many genes found by Schembri et al. had no function assigned, therefore postulating that they might play a role in resistance or biofilm formation (77). Other research regarding *E. coli* biofilms have identified 22 genes up-regulated with functions including stress response, fimbriae, and many unknown functions (72). Further research concluded that some of these unknown functions included energy production and envelope biogenesis (5). Also determined by DNA microarrays, six percent of *B. subtilis* genes and one percent of *P. aeruginosa* genes were differentially expressed in biofilms (33). Whiteley et al. showed that genes within the one percent of differentially expressed genes of *P. aeruginosa* included those related to antibiotic sensitivity (89). Microarray analysis of *S. aureus* biofilms showed 48 genes induced and 84 genes repressed (4). Furthermore, *S. aureus* biofilms revealed up-regulated genes participating in cell envelope synthesis, intracellular adhesion, formate fermentation, urease activity, oxidative stress response, and ammonium production (73). *Streptococcus mutans* biofilms have 139 genes up-regulated and 104 genes down-regulated (78).

B5. Biofilms in nature

Biofilms are widespread in natural, industrial, and clinical environments, being both beneficial and detrimental (20, 71). It has been shown that most biofilms are multispecies in which mutualistic symbiotic relationships are formed to better adapt to growth conditions (35, 71, 79, 82). In addition, these species work together to confer more severe infections on the host (9, 71). To date, the most studied biofilms include strains *S. aureus*, *Vibrio cholera*, *E. coli*, and *Pseudomonas fluorescens* (85).

Biofilms composed of microorganisms are naturally present throughout the world in various environments. These microcolonies can include both bacteria and other material, including archae as found in hot springs and acid mine drainage runoff (82). Goller and Romeo mention communities growing near plant rhizospheres, while Parsek and Singh have noted *Legionella* biofilms present in aquatic locations (35, 71). Not only are they present in the natural environment, but also within animals, such as in bovine rumen (82).

Biofilms in the industrial setting can be either beneficial or detrimental. In wastewater treatment facilities and bioremediation sites, the resistance of biofilm cells proves helpful (2). On the other hand, biofilms can grow in water distribution systems, encasing pathogenic bacteria and sloughing them into drinking water (71). Upon successive chlorine and sanitizing agent treatments, these pathogenic cells within the biofilms can obtain resistance, making more severe problems (71, 79). Buildup of biofilm cells in pipes and on ships increases energy consumption and reduces heat transfer (79, 81).

Recently, biofilms have been named as the causative agent for many medical ailments. Fux et al. estimates that roughly 60% of bacterial infections in a clinical setting are due to biofilms (33). One of the first medical biofilms found includes gram-positive cocci developing subgingival plaque which in turn causes periodontitis (33, 35, 40, 64, 82). More recently,

researchers have determined that biofilms cause chronic lung infections in cystic fibrosis patients (2, 64, 71). Due to surgical contamination, biofilms grow in or on catheters, sutures, medical implants, and prostheses impeding the healing process and causing complications (2, 33, 35, 64, 81). Other common biofilm-associated illnesses include chronic otitis media (ear infections), endocarditis, osteomyelitis, infectious kidney stones, and urinary tract infections (33, 40, 64, 71).

B6. Biofilms in food industry:

Microbial contamination associated with biofilms costs the food industry millions yearly (9). This can be accredited to cross contamination or post-processing contamination from faulty food processing equipment (9, 79). Due to the complexity of this equipment, many surface materials are available on which bacterial biofilms can grow (9). In addition to growing in the processing environments, biofilms can also grow on food products (9). As a result of this issue, food companies are encouraged to maintain GMPs, HACCP plan, and implement Cleaning-in-Place (CIP) procedures (79). Brooks and Flint have extensively written about biofilm prevalence in the food industry, discussing ineffective cleaning methods and financial issues due to loss of product (9). Some of the most well-researched foodborne pathogens known to form biofilms on food products and food processing equipment include *E. coli* O157:H7, *L. monocytogenes*, *Yersinia enterocolitica*, and *Campylobacter jejuni* (79).

B7. Listerial biofilm

Listeria monocytogenes form detrimental biofilms on a variety of surfaces including food, floor drains, conveyor belts, pipes, and storage tanks such as silos (79). In addition, listerial biofilms are typically found in conjunction with *Pseudomonas* species in pipes and silos (9, 19). Although strains from lineage I have been measured to form larger biofilm in comparison to other lineages, lineage I is known to cause few foodborne illnesses (7, 25). Using time-lapse

laser-scanning microscopy, listerial biofilms were found to have a bell-shaped structure (74). Much research has been conducted exploring antimicrobial treatments for biofilm destruction. Longhi et al. concluded that biofilm cells cease to attach to a surface and do not express antibiotic resistance genes when protease serratiopeptidase is applied (58). Listerial biofilms attach more tightly to glass and stainless steel and at a range of temperatures including 4°C, 12°C, and 22°C (23). Caly et al. showed that listerial biofilm cells attach equally well to polystyrene and stainless steel at NaCl levels up to 11% (10). Constantly sloughing dead cells and attaching new cells, listerial biofilms are able to survive on food production equipment for several years with one such case describing a listerial biofilm surviving 10 years (84).

Chapter 3. Materials and Methods

A. Bacterial strain

Listeria monocytogenes strain 10403S was used throughout this research project. Stock cultures were stored at -80°C in tryptic soy broth (TSB) (BD Diagnostic Systems, Franklin Lakes NJ) in the Department of Food Science and Human Nutrition at the University of Illinois culture collection. Planktonic and biofilm cultures for this experiment were grown from a working culture which was stored at -20°C as ten milliliter aliquots of early log phase cells with an optical density of 0.4 measured at 600 nm ($OD_{600} = 0.4$) in TSB. Prior to completing the microarray experiment as diagramed in Figure 4, each step was optimized.

B. Growth of planktonic cell culture

Working stocks were thawed and incubated at 37°C overnight (12 hours) while shaking at 300 RPM on an Innova 2100 platform shaker (New Brunswick, Edison, NJ). A one milliliter aliquot of the overnight culture was added to nine milliliters sterile TSB and incubated at 37°C in a shaker-incubator at 300 RPM until early log phase ($OD_{600} = 0.4$ as measured by Nanodrop under Cell Culture setting (ND1000; Thermo Fisher Scientific, Waltham, MA)). A one milliliter aliquot of the previous sample was used to inoculate 99 mL sterile TSB in a 250 mL Erlenmeyer flask and was grown at 37°C in a shaker-incubator at 300 RPM until early log phase ($OD_{600} = 0.4$). A third and final inoculation was made into 75 mL sterile TSB with starting $OD_{600} = 0.1$ - 0.2 in a 250 mL Erlenmeyer flask and was grown at 37°C (with no shaker) until early stationary phase ($OD_{600} = 1.0 \pm 0.25$) while being topped with a foam stopper-foil cap. Cell culture density was checked hourly and diluted samples were plated onto tryptic soy agar (TSA) (BD Diagnostic Systems). Plates were incubated at 37°C for 36 hours to detect contamination by visually checking for atypical cells. Cell count was determined via plating.

B1. Variation 1 of planktonic cell growth

A ten milliliter thawed working stock was used to inoculate 90 mL sterile TSB in a 250 mL Erlenmeyer flask and topped with a foam stopper-foil cap. The beaker was incubated at 37°C overnight (12 hours) while being shaken at 300 RPM on a platform shaker.

B2. Variation 2 of planktonic cell growth

A working stock was thawed and incubated at 37°C overnight (12 hours) while shaking at 300 RPM on a platform shaker. A one milliliter aliquot of the overnight culture was added to nine milliliters sterile TSB and incubated at 37°C on a shaker at 300 RPM until early log phase ($OD_{600} = 0.4$ as measured by Spec 20 ;Thermo Spectronic). A one milliliter aliquot of the previous sample was used to inoculate nine milliliters sterile TSB in a test tube and was grown at 37°C on shaker at 300 RPM until early log phase ($OD_{600} = 0.4$). A final inoculation was made into a test tube containing sterile TSB with starting $OD_{600} = 0.1-0.2$ and final volume of ten milliliters. This was grown at 37°C (with no shaker) until early stationary phase ($OD_{600} = 1.0 \pm 0.25$). Cell culture density was checked hourly using Spec 20.

B3. Variation 3 of planktonic cell growth

The same procedure as mentioned in Materials and Methods section B was performed with the exception that the final inoculation was placed in 4°C. A ten microliter sample was taken daily and the OD_{600} was measured. Cells were ready to harvest once they reached early stationary phase ($OD_{600} = 1.0 \pm 0.25$).

C. Harvesting planktonic cells

The liquid sample was placed in 50 mL plastic centrifuge tubes (Fisher Scientific). They were balanced and centrifuged at 6,000 x g for ten minutes at 4°C (in Eppendorf AG centrifuge 15 amp version, Hauppauge, NY). The supernatant was pipetted out (and discarded) and the

pellet was resuspended in five milliliters sterile potassium phosphate buffer (PPB) (Fisher Chemical) (50 mM; pH 7; adjusted with 0.1N KOH (Fisher Chemical)). All aliquots from a sample were combined into one 50 mL centrifuge tube. It was centrifuged at 10,000 x g for five minutes at 4°C. After removing the supernatant, the pellet was resuspended in one milliliter sterile PPB and transferred to a two milliliter microcentrifuge tube and stored at -20°C until further use. Total cell samples were frozen for a maximum of 36 hours prior to RNA extraction.

C1. Variation to harvesting planktonic cells

The liquid sample was placed in 50 mL centrifuge tubes. They were balanced and centrifuged at 6,000 x g for ten minutes at 4°C. The supernatant was pipetted out (and discarded) and the pellet was resuspended in five milliliters sterile PPB. All aliquots from a sample were combined into one 50 mL centrifuge tube. It was centrifuged again at 10,000 x g for five minutes at 4°C. After removing the supernatant, the pellet was resuspended in five milliliters sterile PPB and again centrifuged at 10,000 x g for five minutes at 4°C. The supernatant was removed and replaced with one milliliter sterile PPB, transferred to a two milliliter microcentrifuge tube and stored at -20°C until further use.

D. Growth of biofilm cell culture:

A working stock was thawed and incubated at 37°C overnight. A one milliliter aliquot of the overnight culture was added to nine milliliters sterile TSB and incubated at 37°C on platform shaker at 300 RPM until early log phase ($OD_{600} = 0.4$ as measured by Nanodrop). A one milliliter sample was used to inoculate 99 mL sterile TSB and was grown at 37°C on platform shaker at 300 RPM until early log phase ($OD_{600} = 0.4$). This sample was used to inoculate three 250 mL Erlenmeyer flasks containing 100 g sterile glass beads (6 mm diameter) (Walter Stern, Port Washington, NY) and 75 mL sterile TSB with starting $OD_{600} = 0.1-0.2$. The three flasks

were topped with foam stoppers and foil caps and incubated at 37°C (with no shaking) for five days as shown in Figure 3. Liquid medium was removed by pipetting and replaced with 50 mL preheated fresh TSB every 24 hours. A sample was taken daily and dilutions were plated onto TSA. Plates were grown for 36 hours. On the fifth day, the sample was ready to harvest.



Figure 3. Picture of biofilm cells grown on 100 g glass beads after 24 hour incubation while being topped with a foam stopper-foil cap.

D1. Variation 1 of biofilm cell growth

A working stock was thawed and incubated at 37°C overnight (12 hours) while shaking at 300 RPM on a platform shaker. Three stainless steel chips each (48 total; type 304; No. 4 grade; 2.54 x 2.54 cm) (62) were placed in a Pyrex glass petri dish. A 0.1 mL aliquot of the overnight culture was pipetted onto each stainless steel chip and gently spread with a sterile spreader (Fisher Scientific). The petri dishes were placed in a dessicator (with water at the bottom; 100%

RH) and kept at room temperature for three hours. Each chip was washed with 20 mL PPB. One hundred microliters of sterile TSB was added to each chip and spread with the pipette tip. The petri dishes were placed back in the dessicator. The dessicator was incubated at 37°C for 24 hours. For four days, each chip was washed with PPB and an aliquot of TSB was added.

D2. Variation 2 of biofilm cell growth

The same procedure was used as in Materials and Methods section D, but with the biofilm being grown on 33g (monolayer) of glass beads rather than 100g (multilayer).

D3. Variation 3 of biofilm cell growth

The same procedure was used as in Materials and Methods section D, but with the biofilm being grown for three days rather than five days.

D4. Variation 4 of biofilm cell growth

The same procedure was used as in Materials and Methods section D, but with the third inoculation being grown while continuously shaking at 300 RPM every day.

E. Harvesting biofilm cells

To detach biofilm cells from glass beads, liquid medium was removed by pipetting and discarded. Fifty milliliters PPB were added to the flasks and they were shaken at 200 RPM for three minutes. The liquid media was removed by pipetting, placing liquid buffer media in 50 mL plastic centrifuge tubes. In addition to measuring absorbance to ensure late log/early stationary phase cell culture ($OD_{600} = 0.9-1.9$), samples were plated on TSA for 36 hours to verify no contamination was present in the sample. The centrifuge tubes were centrifuged at 6,000 x g for ten minutes at 4°C. Supernatant was removed by pipetting and discarded. Ten milliliters sterile PPB was added to each tube and the pellet was resuspended. The liquid from these three tubes were combined into one tube to complete one sample. It was centrifuged at 10,000 x g for five

minutes at 4°C. Supernatant was removed by pipetting and discarded. The remaining pellet was resuspended in one milliliter sterile PPB and was transferred to a two milliliter microcentrifuge tube before being frozen at -20°C. Total cell samples were stored for a maximum of 36 hours prior to RNA extraction.

E1. Variation 1 of harvesting biofilm cells

To detach biofilm cells from stainless steel chips, on day five, each chip was washed with 20 mL PPB. The chip was placed into a 50 mL centrifuge tube containing five milliliters PPB. The tubes were shaken by hand for 15 seconds, followed by vortexing for 1.5 minutes. The liquid from each tube was collected into 50 mL centrifuge tubes. These were centrifuged at 10,000 x g for ten minutes at 4°C. The supernatant was discarded and replaced with five milliliters sterile PPB. The pellet was resuspended. The tube was centrifuged at 10,000 x g for 15 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in two milliliters sterile PPB. This was transferred to a ten milliliter centrifuge tube and stored at -20°C.

F. RNA extraction

The frozen sample was thawed before cell lysis. A calculated 200 µL pellet was placed into a two milliliter microcentrifuge tube and was centrifuged at 10,000 x g for 2.5 minutes in a Micro 14 microcentrifuge (Fisher Scientific). The supernatant was removed and discarded. The pellet was resuspended in one milliliter TRI Reagent (Sigma-Aldrich, St. Louis, MO) by pipetting. Glass disruption beads (0.5:1 vol/vol; 0.1mm diameter) (Research Products International Corp, Mt. Prospect, IL) were added to the two milliliter tubes and chilled for three to five minutes. The cells were disrupted in FastPrep FP120 Cell Homogenizer (Thermo Scientific) at 4°C for eight times 30 seconds. The tube sat at room temperature for five minutes and later was centrifuged at 13,000 x g at 4°C for 12 minutes in a Sorvall Fresco microcentrifuge

(Thermo Scientific). The supernatant was removed for further use. The sample sat at room temperature for five minutes. Twenty microliters chloroform (Sigma-Aldrich) was added, vigorously shaken for 15 seconds, and incubated at room temperature for five minutes. The tube was centrifuged at 12,000 x g at 4°C for 15 minutes. The upper layer was removed and placed into a new two milliliter microcentrifuge Eppendorf tube. After addition of isopropanol (Sigma-Aldrich) (0.5 mL) to the tube, it was vortexed for five seconds and incubated at room temperature for ten minutes. The tube was centrifuged at 12,000 x g at 4°C for eight minutes. Supernatant was discarded and 75% ethanol (one milliliter) was added and vortexed to dislodge the pellet. The tube was centrifuged at 7,500 x g at 4°C for five minutes. The ethanol was discarded and the pellet was air dried on ice for five to ten minutes. Prior to storage at -20°C, the pellet was resuspended in 100 µL diethylpyrocarbonate (DEPC)-treated water (Sigma-Aldrich).

F1. Variation 1 to RNA extraction

The same procedure was used to extract RNA as in Materials and Methods section F, with the exception that no glass disruption beads were used.

F2. Variation 2 to RNA extraction

The same procedure was used to extract RNA as in Materials and Methods section F, with the exception that the supernatant after initial thawing and centrifuging was not removed.

F3. Variation 3 to RNA extraction

The same procedure was used to extract RNA as in Materials and Methods section F, with the exception that the extraction of the biofilm sample was completed immediately following harvesting (with no freezing).

G. RNA purification and quantification

The 100 μ L sample was purified using an RNeasy Mini Kit (Qiagen, Valencia, CA) at room temperature. Briefly, as per the protocol, 350 μ L of RLT buffer (containing β -mercaptoethanol (Sigma-Aldrich)) was added to the sample and was vortexed. Two hundred fifty microliters absolute ethanol was mixed in by pipetting and was applied to an RNeasy mini column. The column attached to a two milliliter collection tube was centrifuged at 8,000 x g for 30 seconds. The collection tube was completely emptied. Five hundred microliters RPE buffer was added to the column and was centrifuged at 8,000 x g for 30 seconds. Flow-through was discarded again. Another 500 μ L RPE buffer was added. The column was centrifuged at 8,000 x g for two minutes. The column was transferred to a fresh two milliliter collection tube and was centrifuged at 8,000 x g for one minute. The column was transferred to a 1.5 mL final collection tube. Fifty microliters RNase-free water (Applied Biosystems Ambion, Austin, TX) was added to the column and was left to sit for four minutes. The column was centrifuged at 8,000 x g for one minute. The process was repeated with another 50 μ L RNase-free water. The sample was stored at -20°C.

A two microliter sample was used to measure the concentration (in ng/ μ L) and purity ($A_{260/280} > 1.8$ and $A_{260/230} > 1.8$) using a NanoDrop on the Nucleic Acid setting. A sample was analyzed with the Agilent 2100 Bioanalyzer (Santa Clara, CA) with a starting concentration between 40 and 500 ng/ μ L.

H. cDNA synthesis

Purified total RNA was reverse transcribed into single-stranded cDNA using iScript Select cDNA synthesis (Bio-Rad, Hercules, CA) using random primers included in the kit. The protocol was followed with no exceptions. Briefly, one microgram total RNA sample was added to four microliters 5x iScript selection reaction mix, two microliters random primer, and one

microliter iScript reverse transcriptase. Nuclease-free water was added to make the total volume in the 0.2 mL PCR tube 20 μ L. The PCR tube was incubated at 25°C for five minutes, then at 42°C for 30 minutes in a thermocycler (MyCycler, Bio-Rad). To inactivate the iScript reverse transcriptase, the sample was heated to 85°C for five minutes. The resulting single-stranded cDNA was kept at -20°C. All reagents were included in the kit. The frozen unpurified samples were submitted to the W. M. Keck Center for Comparative and Functional Genomics for all further analyses.

H1. Variation 1 to cDNA synthesis

Purified total RNA was reverse transcribed using the NimbleGen Array User's Guide to Gene Expression Analysis (Roche Diagnostics, Madison, WI) and/or the Invitrogen SuperScript Double-Stranded cDNA Synthesis Kit (Carlsbad, CA). For first strand synthesis, briefly, ten micrograms total RNA and one microliter random hexamer primer (100 pmol/ μ L; Invitrogen) were combined in a 0.2 mL PCR tube (total volume was 11 μ L using DEPC-treated water). The sample was heated for ten minutes at 70°C in a thermocycler and then briefly placed on ice. To each sample four microliters 5X First Strand Buffer, two microliters 0.1M DTT, and one microliter 10mM dNTP Mix were added. The tube was mixed and incubated at 42°C for two minutes in a thermocycler. Two microliters SuperScript II (Invitrogen) were added and the tube was incubated at 42°C for one hour. For second strand synthesis, 91 μ L DEPC-treated water, 30 μ L 5X Second Strand Buffer, three microliters 10mM dNTP Mix, one microliter 10U/ μ L DNA Ligase, four microliters 10U/ μ L DNA Polymerase I, and one microliter 2U/ μ L RNase H was added to each sample. The tube was incubated at 16°C for two hours in a thermocycler. Two microliters 5U/ μ L T4 DNA Polymerase was added to each sample. The tube was incubated at 16°C for five minutes and then kept at -20°C until RNase clean-up and cDNA precipitation. All

solutions were included in the Invitrogen SuperScript Double-Stranded cDNA Synthesis Kit unless otherwise indicated.

H2. Variation 2 to cDNA synthesis

The same protocol was followed as written in the NimbleGen Array User's Guide to Gene Expression Analysis or in Materials and Methods section H with the exception of starting with 50 µg total RNA.

H3. Variation 3 to cDNA synthesis

The following was performed by the W.M. Keck Center for Comparative and Functional Genomics. The same protocol was followed as written in the NimbleGen Array User's Guide to Gene Expression Analysis or in Materials and Methods section H with the exception of completing only first strand synthesis.

H4. Variation 4 to cDNA synthesis

The following was performed by the W.M. Keck Center for Comparative and Functional Genomics. Five micrograms total RNA was reverse transcribed using SuperScript III RT (Invitrogen). Briefly, 250 ng random primers, one microliter 10mM dNTP mix, and distilled water (to total volume of 13 µL) were added to the total RNA. The tube was heated to 65°C for five minutes and then quick-chilled. Four microliters 5X First-Strand Buffer, one microliter 0.1M DTT, one microliter RNaseOUT, and one microliter SuperScript III RT were added. The mixture was incubated at 25°C for five minutes followed by incubation at 50°C for one hour. The sample was inactivated by incubation at 70°C for 15 minutes. The sample was stored at -20°C until further use.

H5. Variation 5 to cDNA synthesis

The same protocol was followed as written in the iScript Select cDNA Synthesis kit (Bio-Rad) or in Materials and Methods section H with the exception of completing the whole procedure at 5X.

I. RNase clean-up and cDNA precipitation

The following was performed by the W.M. Keck Center for Comparative and Functional Genomics. Total cDNA was purified by adding four microliters 1M NaOH. The mixture was heated to 65°C for 15 minutes. Four microliters 1M HCl and six microliters DEPC-treated water were added. To precipitate the cDNA, 3.4 µL sodium acetate and 100 µL absolute ethanol were added and mixed. It was frozen at -20°C for 30 minutes. The thawed tube was centrifuged for 15 minutes at 12,000 x g at 4°C. After discarding the supernatant, 0.5 mL 75% ethanol was added and vortexed. The tube was centrifuged for five minutes at room temperature. The supernatant was discarded. The tube was centrifuged again. All remaining liquid was carefully pipetted out. The pellet was resuspended in 25 µL DEPC-treated water. Two microliters were analyzed by the Agilent 2100 Bioanalyzer, measuring the quality and quantity. A minimum of one microgram was required to continue labeling.

II. Variation 1 to RNase clean-up and cDNA precipitation

As per the NimbleGen Array User's Guide to Gene Expression Analysis (Roche Diagnostics), briefly, cDNA was purified by adding one microliter 4mg/mL RNase A. After mixing, the sample was incubated at 37°C for ten minutes. To each sample was added 163 µL phenol:chloroform:isoamyl alcohol (25:24:1) (Fisher BioReagents). The total sample was applied to a Phase Lock tube (Fisher Scientific) and centrifuged at 12,000 x g for five minutes. The upper layer was transferred to a sterile 1.5 mL tube. cDNA precipitation was completed by adding 16 µL 7.5M NH₄OAc (Fisher Chemical), seven microliters 5mg/mL glycogen (Fisher

Scientific), and 326 μL absolute ethanol. After mixing, the tube was centrifuged at 12,000 x g for 20 minutes. The supernatant was removed and 500 μL 80% ethanol was added. The tube was centrifuged at 12,000 x g for five minutes. The supernatant was removed and the pellet was dried using a SpeedVac (Thermo Fisher). The pellet was resuspended in 20 μL Nanopure water (Barnstead, Thermo Scientific). Two microliters were analyzed by the Agilent 2100 Bioanalyzer, measuring the quality and quantity. A minimum of one microgram was required to continue labeling.

I2. Variation 2 to RNase clean-up and cDNA precipitation

The unpurified cDNA sample was purified using the suggested protocol in the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen). Briefly, 160 μL phenol:chloroform:isoamyl alcohol (25:24:1) was added and vortexed. The sample was centrifuged at 14,000 x g for five minutes at room temperature. The upper 140 μL was removed and transferred into a fresh 1.5 mL microcentrifuge tube. Seventy microliters 7.5M NH_4OAc and 0.5 mL absolute ethanol were added. The mixture was vortexed and centrifuged at 14,000 x g for 20 minutes at room temperature. After removing the supernatant, the pellet was resuspended in 0.5 mL 70% ethanol. The sample was centrifuged at 14,000 x g for five minutes. The cDNA was dried at 37°C for ten minutes and then resuspended in 20 μL DEPC-treated water. The sample was stored at -20°C until further use. Two microliters were analyzed by the Agilent 2100 Bioanalyzer, measuring the quality and quantity. A minimum of one microgram was required to continue labeling.

I3. Variation 3 to RNase clean-up and cDNA precipitation

Total unpurified cDNA was purified using RNase H (included in Invitrogen SuperScript Double-Stranded cDNA Synthesis Kit) and a QIAquick PCR Purification Kit (Qiagen). Briefly,

one microliter RNase H was added to single-stranded cDNA. The sample was incubated at 37°C for 30 minutes, followed by being placed on ice. Using the QIAquick PCR Purification Kit, 100 µL Buffer PB was added to four microliters cDNA and transferred to a QIAquick spin column. After centrifuging for one minute, the flow-through was discarded and the column was washed with 0.75 mL Buffer PE. The tube was centrifuged for one minute followed by emptying the flow-through. The tube was centrifuged for one minute. The spin column was transferred to a clean collection tube. To elute the sample, 50 µL DEPC-treated water was added and the tube was centrifuged for one minute. Another 50 µL DEPC-treated water was added and again was centrifuged for one minute. The sample was stored at -20°C until further use. Two microliters were analyzed by the Agilent 2100 Bioanalyzer, measuring the quality and quantity. A minimum of one microgram was required continue to labeling.

J. cDNA labeling, purification, quantification

Purified cDNA was labeled with Cy 3 dye using a NimbleGen One-Color DNA Labeling Kit (Roche Diagnostics) per the NimbleGen Array User's Guide to Gene Expression Analysis. Briefly, one microliter cDNA was added to 40 µL Cy3-Random Nonamers (diluted in β-mercaptoethanol) (included in kit), and Nuclease-free water (to make the total volume 80 µL) in a 0.2 mL thin-walled PCR tube. The tube was placed in a thermocycler for ten minutes at 98°C, then quick chilled on ice for ten minutes. To the tube ten microliters 10mM dNTP Mix (included in kit), eight microliters Nuclease-free water, and two microliters Klenow Fragment (50U/µL) (included in kit) were added. The mixture was pipetted up and down a few times to mix. The tube was incubated for two hours at 37°C in a thermocycler. The reaction was stopped by adding ten microliters 0.5M EDTA (included in kit). Eleven and a half microliters 5M NaCl was added to the tube, followed by brief spinning. This was transferred to a 1.5 mL tube containing 110 µL

isopropanol. The tube was vortexed, incubated at room temperature for ten minutes, and then centrifuged for ten minutes at 12,000 x g. The supernatant was removed. The pellet was rinsed with 500 μ L 80% ethanol, followed by centrifugation for two minutes at 12,000 x g. The contents were dried with a SpeedVac. Labeling efficiency was measured using the Agilent 2100 Bioanalyzer. A minimum of three micrograms labeled cDNA was required to continue to hybridization.

K. Hybridization, wash, and microarray scan

Three point eight micrograms of labeled cDNA were hybridized to a NimbleGen *L. monocytogenes* EGD-e 385K microarray using NimbleGen Hybridization kit (Roche Diagnostics). All procedural steps were repeated as presented in the NimbleGen Array User's Guide to Gene Expression Analysis. Briefly, the dried pellet was resuspended in five microliters water. To each sample, 11.8 μ L 2X Hybridization Buffer (included in kit), 4.7 μ L Hybridization Component A (included in kit), 0.5 μ L Alignment Oligo (included in kit) were added. The tube was vortexed and incubated for five minutes at 95°C. The tubes were placed at 42°C until further use. After preparing the mixers using the NimbleGen Mixer X1 (Roche Applied Science) (included in kit), the mixers were placed in a Maui Hybridization Mixer (BioMicro Systems, Inc., Salt Lake City, UT). Sixteen microliters of sample was loaded into each slide-mixer unit. After the ports were sealed, the samples were allowed to hybridize overnight (17 hours).

The NimbleGen Hybridization Wash Buffer kit (Roche Diagnostics) was used to wash the slides as per the procedure in the NimbleGen Array User's Guide to Gene Expression Analysis. Briefly, the wash solutions were prepared. The mixer was removed from the slide and the slide was washed in Washes I, II, and III for two minutes, two minutes, and 15 seconds, respectively. The slide was allowed to dry on a centrifuge for two minutes (ArrayIt Microarray

High-Speed Centrifuge, Sunnyvale CA). Microarrays were scanned using Axon 4000B scanner (Molecular Devices, Sunnyvale, CA).

NimbleGen microarrays (Roche Diagnostics) were run in triplicate for both planktonic and biofilm cells. The complete genome for *L. monocytogenes* 10403S is incomplete at this time. *L. monocytogenes* EGD-e and 10403S have the same phylogenetic lineage (III) and the same serotype (1/2a). Using cross-hybridization identities (CHI), 2107 EGD-e probes are shown to have CHI value of 100, 2578 probes with CHI value of ≥ 95 , 2695 probes with CHI value of ≥ 90 , and 35 probes with CHI value of < 90 (44) when compared to *L. monocytogenes* 10403S. CHIs represent the percent of sequence identity between strains. Although *L. monocytogenes* 10403S has not been fully sequenced, it is postulated that only false negatives could be present when using a *L. monocytogenes* EGD-e microarray (44).

L. Statistical analysis

The following information was provided by the W. M. Keck Center. The data from the microarray images were analyzed using NimbleScan (Roche Diagnostics) by background data correction and normalization as noted in the NimbleGen Array User's Guide to Gene Expression Analysis. The NimbleGen Array had 13 unique 60-mer probes for each sequence; in the case of *L. monocytogenes* EGD-e, all the probes are replicated in five blocks on the array. The NimbleScan software (version 2.3) was used to calculate RMA Expression values "by container" (i.e., block) for all seven samples using the Robust Multi-Array algorithm¹. RMA includes a model-based background correction, a quantile normalization, and summarization of the 13 probes per sequence into one value per block. The RMA expression values were then imported into R² for statistical analysis using packages from the Bioconductor project³. Testing for significant differences between the three biofilm samples and the three planktonic samples was

done using limma^{4,5}, which accounted for the five replicate measurements per sequence⁶. P-values were adjusting for multiple hypotheses testing using the False Discovery Rate method⁷. Fold changes between the single refrigerated sample and one planktonic sample were computed using the average of the 5 block values per sequence. Statistical analysis for the functional group tables used a stringent cutoff (adjusted $P < 0.0001$; >2.0 -fold change). References for this Statistical analysis are provided below.

¹Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*. 2003 Apr;4(2):249-64.

²R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org>.

³Robert C Gentleman , Vincent J Carey , Douglas M Bates , Ben Bolstad , Marcel Dettling , Sandrine Dudoit , Byron Ellis, Laurent Gautier, Yongchao Ge, Jeff Gentry, Kurt Hornik, Torsten Hothorn, Wolfgang Huber, Stefano Iacus, Rafael Irizarry, Friedrich Leisch, Cheng Li, Martin Maechler, Anthony J Rossini, Gunther Sawitzki, Colin Smith, Gordon Smyth, Luke Tierney, Jean YH Yang and Jianhua Zhang. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biology* 2004, 5:R80. www.bioconductor.org

⁴Smyth, G. K. (2005). Limma: linear models for microarray data. In: *Bioinformatics and Computational Biology Solutions using R and Bioconductor*, R. Gentleman, V. Carey, S. Dudoit, R. Irizarry, W. Huber (eds.), Springer, New York, pages 397-420.

⁵Smyth, G. K. (2004). Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology*, Vol. 3, No. 1, Article 3.

⁶Smyth, G. K., Michaud, J., and Scott, H. (2005). The use of within-array replicate spots for assessing differential expression in microarray experiments. *Bioinformatics* 21(9), 2067-2075. <http://bioinformatics.oxfordjournals.org/cgi/content/short/21/9/2067>

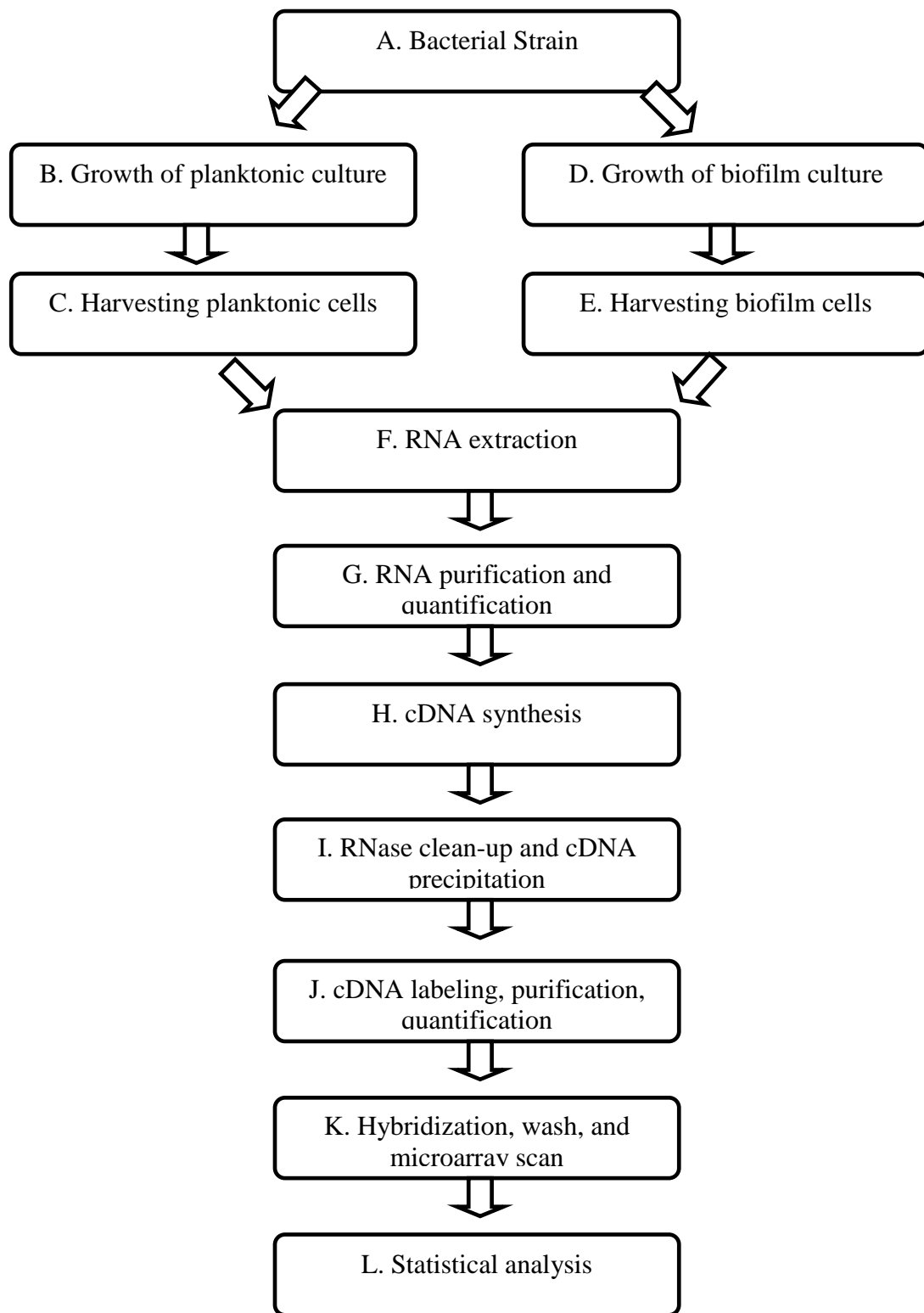


Figure 4: Microarray experiment flow chart.

Chapter 4. Results and Discussion

A. Planktonic cell growth

Optimization of each step was required prior to completing the microarray experiment. The variations of growth procedures for planktonic cells were completed to determine optimal growth conditions. All variations were in comparison to the method mentioned in Materials and Methods section B which served as the optimized procedure used for the microarray samples.

Thawing a frozen working stock and creating a ten percent inoculum before incubating the sample overnight resulted in inconsistent optical densities after 12 hours. Although all cultures grew to stationary phase, the overnight cultures had absorbance values at 600 nm ranging from 1.2 to 2.6 (which corresponded to late-log/early-stationary and late-stationary growth phase). The cells growing to different phases can effect gene expression. Additionally, it is a source of unnecessary variability. To correct this issue, Materials and Methods section B completed three successive inoculations before using the sample for experiments. This allowed for the cells to ‘wake up’ and grow at more consistent rates. Growing using method B, the first ten percent inoculation cultures grew to early log phase in 3.0 ± 0.5 hours. The one percent inoculation grew consistently to early log phase within 1.0 ± 0.15 hours. Finally, the third inoculation grew from $OD_{600} = 0.1-0.2$ to early stationary phase within 3.0 ± 0.5 hours. The addition of 25.0 ± 5.0 mL culture from the second inoculum to 75 mL sterile TSB resulted in a starting OD_{600} between 0.1-0.2 for the third inoculation.

Requiring a significant quantity of bacterial cells (5×10^9 cells or 200 μ L by volume aliquot) in a pellet, roughly 100-150 mL of TSB containing cells in late log/early stationary phase were required. This could be obtained in several ways. The method mentioned in Materials and Methods section B2 grew this total quantity in several test tubes in ten milliliter aliquots.

Using a Spec 20 to measure optical density, each individual test tube created greater variation within the sample as a whole. This significantly differed from a sample grown with method mentioned in section B where all absorbance readings were obtained from one flask. Therefore, growing one sample in a single 250 mL Erlenmeyer flask provided a more consistent optical density reading although a ten microliter irreplaceable sample was taken each time to read the optical density using a NanoDrop. Additionally, using newer equipment such as the NanoDrop provides for more accurate readings. Due to the variability of growing planktonic cells in ten milliliter aliquots, this method was not used.

The ultimate goal of this research project was to observe gene expression differences between cells grown as planktonic and biofilm cells. To confirm that the whole procedure was correctly completed, a reference experiment was conducted. Replicating Chan et al. (18) allowed for a minor growth condition change (growing a sample at refrigeration temperature 4°C) and a final relative gene expression comparison. The growth procedure correlating to Chan et al. (18) was mentioned under Materials and Methods section B3. As seen in Figure 5, planktonic cells grown at 37°C only require a few hours (ranging between three and four hours), while planktonic cells grown at 4°C required on average six to nine days to reach early stationary phase.

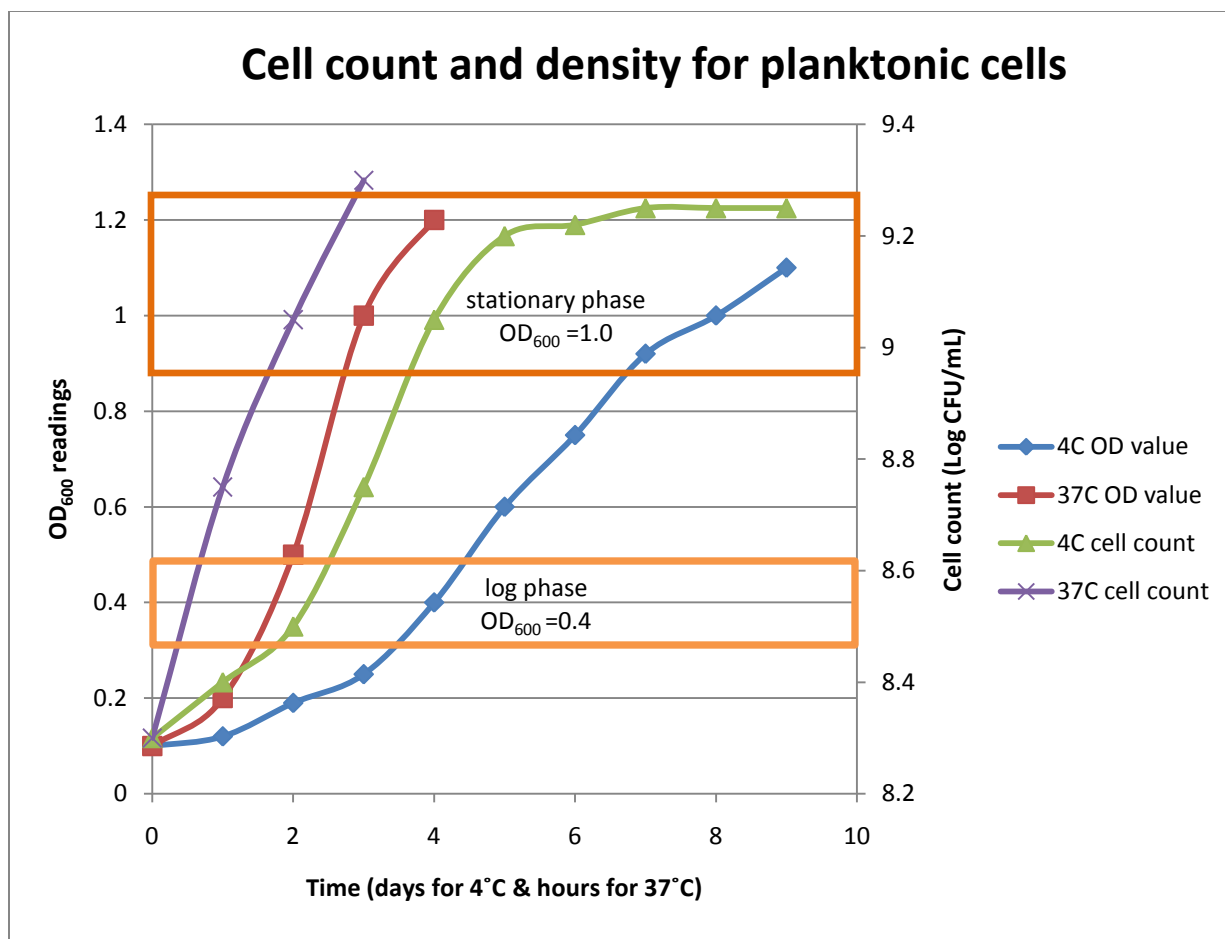


Figure 5: Growth of planktonic cells at 37°C and 4°C as measured by absorbance at 600nm using NanoDrop and cell count.

B. Harvesting planktonic cells

The variation in harvesting planktonic cells experimented with one or two washes with PPB prior to freezing. Completing two washes was copied from previous research (22). Experimental attempts with only one wash proved as efficient (resulted in the same size pellet) and was less time consuming. Conceptually, two washes should ensure that all biofilm cells have detached from the growing surface. After centrifugation, the supernatant for all attempts was clear, indicating all the cells had collected into a pellet at the bottom of the centrifuge tube. Additionally, the TRIzol Reagent (Invitrogen) procedure notes that bacterial cells should not be washed several times due to possible mRNA degradation (45).

C. Biofilm cell growth

Optimizations of biofilm growth lead to changing the surface on which the biofilm was grown. Previous research grew biofilms on stainless steel chips to better represent a typical food processing surface (10, 19, 23, 29, 42, 47, 49, 60). Although this may be more accurate if the surface had an influence on biofilm formation, sufficient cells were not obtained from growing biofilms on 96 stainless steel chips. Several repetitions with the method mentioned in Materials and Methods section D1 did not result in a visible pellet. Altering the procedure to grow the biofilm on a larger surface area, 0.6 mm glass beads were chosen. After optimizing other growth parameters, the quantity of biofilm cells grown on 300-400 g glass beads proved to be a 200 μ L (by volume) pellet or larger. This was sufficient to proceed to RNA extraction.

To ensure cells grown on glass beads were only biofilm cells, the liquid medium (containing the planktonic cells) was pipetted out and discarded prior to harvesting. With all the liquid removed from Erlenmeyer flasks, the only visible contents in the flask were the glass beads. Therefore, the remaining cells in the flask were the biofilm cells which were attached to the glass beads.

Upon choosing a new growth surface, an arbitrary quantity of 100 g glass beads was chosen. This formed layers in a 250 mL Erlenmeyer flask. A comparison was made using 33 g glass beads which only formed a monolayer of beads. Conceptually, there should be a significant difference with a larger pellet size when grown on 100 g glass beads. The results show this to be true. There was an eight-fold increase in pellet size when grown on more glass beads. The biofilm forms on and in between the glass beads. Therefore, addition of a partial layer of glass beads created more surface area.

The implication this finding has on food processing material is significant. Two dimensional surfaces such as stainless steel counters provide a sufficient, but not optimal surface for biofilm cell growth. A three dimensional surface with many parts, such as deli slicers, provide more surface area, especially in the areas where two surfaces make contact. In addition, a three dimensional surface can be created by debris and other foreign material such as those found in pipes.

The length of time a biofilm culture is grown significantly affects the quantity of cells in the biofilm. When comparing a three day to a five day biofilm, a pellet three times larger was found for growth after five days. No exact quantification was completed to determine the effect of growing the biofilm for consecutive days. Five days was chosen arbitrarily. A practical example lies in biofilm growth in drinking water pipes. On Monday morning, after a weekend, it may be unsafe to have the first drink from a drinking fountain (for example at a dentist's office) due to possible biofilm formation on the inside of inactive pipes.

When preparing the cultures with the first and second inoculums, the cultures were incubated on a shaker. A variation to the biofilm growth procedure was to place the flask on a shaker for the third incubation. Although it could affect gene expression of oxygen consumption genes, it did not affect the quantity of cells grown. For the continuation of this experiment, the third inoculum was not shaken as to limit variation between growth conditions (since a sample was grown at refrigeration temperature). In addition, food processing surfaces are typically not constantly shaking.

D. Harvesting biofilm cells

Applying PPB to the glass beads and agitating the flask using a platform shaker detaches biofilm cells mechanically. However, manual shaking does not provide enough strength to

detach 100% of the biofilm cells. It is known that these biofilm cells have strong attachment and a resistant coating layer (15). After centrifuging the 2nd wash of the glass beads, a significant pellet did not form on the bottom of the centrifuge tube. A speed of 200 RPM using a platform shaker for three minutes was used to remove biofilm cells. Removal of biofilm cells from the growth surface resulted in clumps of cells as found when biofilm cells slough off from the food processing surface and onto the food product. The method mentioned for biofilm cell removal from stainless steel chips as mentioned in Materials and Methods section E1 was adopted from previous research (22).

E. RNA extraction

Previous techniques for RNA extraction include sonication and French press (22). Other methods include chemical and mechanical means including TRI reagent and disruption beads (bead beater), respectively. Using the TRI reagent method not enough RNA was obtained to continue with this experiment. On average, a concentration of 10 ng/ μ L total RNA was extracted using only a chemical lysing technique. When adding glass disruption beads, the total RNA quantity greatly increased. The optimal ratio of 0.4:1 vol/vol disruption beads to TRI reagent was determined as diagramed in Figure 6. This resulted in total RNA ranges of more than ten times the amount when compared to using a chemical treatment only. Once lysed, the cell contents were released into the supernatant. If a higher ratio of disruption beads was added, there was not enough liquid (supernatant) after centrifugation and the cells would get ‘stuck’ in the disruption beads. Mechanical lysing with disruption beads required repetitions of disruption with a bead beater and resting on ice. Optimal disruption occurred with eight repetitions of 30 seconds each on and off ice. Several variations in RNA extraction were explored to determine which method resulted in the highest concentration and greatest purity.

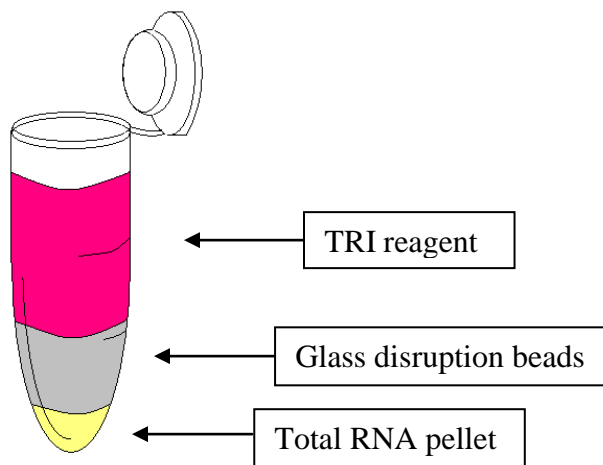


Figure 6. Diagram of 2.0 mL microcentrifuge tube containing 0.4 mL glass disruption beads, 0.2 mL total RNA sample, and 1 mL TRI reagent as used in Materials and Methods section F.

The whole RNA extraction procedure was completed in a two microliter microcentrifuge tube. The total volume of the pellet, glass disruption beads, and TRI reagent did not allow for enough space to leave the supernatant. In addition, leaving a portion of the supernatant after initial centrifugation did not result in a higher total RNA yield. The contents of the initial supernatant was a few more total cells that did not pellet or possibly internal cell contents if the cells were lysed during the harvesting step. Since no difference in total RNA quantity or quality were detected, the supernatant was removed prior to RNA extraction for the microarray procedure. Biofilm cells are known to revert back to planktonic cells if the growth conditions are correct (35, 82). To preserve biofilm cells and to minimize mRNA degradation, RNA extraction must occur within 36 hours of harvesting (90). To test for mRNA degradation, RNA extraction was performed the same day as harvesting and 24 hours after harvesting. No significant differences in quantity or quality were seen between same day and next day RNA extraction as seen in Table 1. Additionally, no total RNA degradation was seen when measuring the absorbance ratios and concentration of the immediate RNA extraction (Sample A) when measured 24 hours later. For the microarray experiment, RNA extraction occurred within 24 hours of harvesting to ensure minimal mRNA degradation.

		Same day as harvesting	24 hours after harvesting
Sample A	A _{260/280}	2.17	2.19
	A _{260/230}	1.66	1.62
	Concentration (ng/μL)	1583.6	1584.0
Sample B	A _{260/280}		2.16
	A _{260/230}		2.43
	Concentration (ng/μL)		1598.4

Table 1. Absorbance ratios and concentration of RNA extractions performed on the same day (Sample A) and 24 hours after harvesting (Sample B).

F. cDNA synthesis and labeling

Converting purified total RNA to cDNA proved to be difficult. All five variations were not successful with little insight of why they failed. The method mentioned in Materials and Methods section H was the only procedure that resulted in a large enough quantity of labeled product as determined by a Bioanalyzer and a Qubit (Invitrogen) to proceed to hybridization. If a large unpurified yield was observed during other trials using the Bioanalyzer, the product was determined to be mostly free nucleotides, left-over RNA and possibly some genomic DNA. The unwanted products should have been removed through the NaOH degradation and ethanol precipitation, although for unknown reasons this did not prove to be true. Following the suggested method by NimbleGen (as mentioned in Materials and Methods section H1), the Invitrogen kit did not reverse transcribe the RNA or it converted only minute amounts that were not detected by several quality assurance methods. Beginning with a larger quantity of RNA (50 μg) (as written in the Invitrogen protocol) did not result in any noticeable cDNA levels. Since the Invitrogen kit utilizes SuperScript II, we attempted using a newer reverse transcriptase SuperScript III. The results showed no cDNA present in the sample. The issue with reverse transcribing *L. monocytogenes* may lie in the initial first strand synthesis or may be due to the presence of a reverse transcriptase inhibitor. Variation 3 (Materials and Methods section H3) completed only first strand synthesis (as approved by NimbleGen support staff). Less than one

microgram DNA was found within the ten microgram RNA sample. Since all the cDNA syntheses utilizing Invitrogen materials were unsuccessful, a change in companies was made. Following the iScript Select cDNA Synthesis kit (Bio-Rad) approximately 1.5 µg unpurified cDNA was produced as indicated by a distinct curve on the Bioanalyzer as shown in Figure 7. Hoping to obtain a larger quantity cDNA, the process was repeated multiplying the whole reaction by five. This proved unsuccessful since undetectable levels were measured by the Bioanalyzer. Therefore, all samples used for microarrays were completed at 1x iScript Select cDNA Synthesis kit.

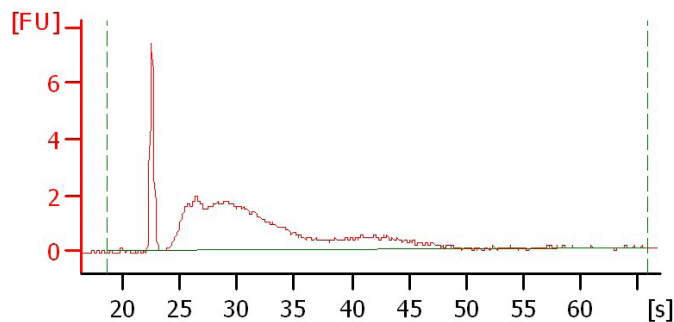


Figure 7. Representative Agilent 2100 Bioanalyzer results for an acceptable cDNA sample (Biofilm sample 2) where [FU] is the quantity of fluorescence units and [s] is seconds.

A typical yield is a 1:1 ratio of total RNA to cDNA. Therefore, in a 100 % efficient conversion, one microgram of RNA would yield one microgram cDNA. The results for this experiment following the method mentioned in Materials and Methods section H showed that reverse transcribing one microgram total RNA yielded on average five micrograms purified and labeled cDNA. This final product must have included cDNA, genomic DNA contamination, or a combination of both. It is unclear why the first attempts yielded too low of cDNA quantity while the final method resulted in too high of product.

The RNase clean-up and cDNA precipitation was completed by the W.M. Keck Center for Comparative and Functional Genomics. According to their reportings, several variations were

attempted as noted in Materials and Methods section I. The first trials followed the suggested protocols according to NimbleGen and Invitrogen and yielded no success. Therefore, NaOH and HCl RNase treatments were completed, followed by an ethanol extraction.

G. Chan microarray analysis:

To ensure that the complete microarray experimental procedure was completed correctly, a microarray experiment by Chan et al. was replicated (18). As reported by Chan et al., 237 genes were up-regulated and 187 genes were down-regulated when grown as stationary phase cells at 4°C (18). The criterion applied for the aforementioned data was a p-value < 0.05 and a minimum of a two-fold change (18). Applying a stricter statistical criterion of a p-value < 0.001 in addition to a two-fold change, 170 genes and 102 genes had higher and lower transcript levels, respectively when grown under the same conditions (18). The results for the current study concluded 80 to be genes up-regulated and 124 genes to be down-regulated in planktonic cells grown at 4°C including only those genes with more than a two-fold change. Appendix 1 lists the up-regulated data collectively obtained from the current study and Chan et al. (18). It lists 289 genes up-regulated in the 4°C stationary phase planktonic cell sample. This list was sorted by largest magnitude fold change with a minimum value of two. A p-value was not determined for the current part of this study because only one refrigerated sample was statistically compared to one planktonic sample grown at 37°C. All gene functions were provided by NimbleGen and were further analyzed using the comprehensive enzyme information system BRENDA and the European Molecular Biology Laboratory- European Bioinformatics Institute (EMBL-EBI) Uniprot.

Although only three genes were identified to be both in the Chan et al. and the current study results lists, several functional similarities were found (18). As presented in Table 2, two

genes present in both studies have unknown functions. Additionally, genes participating in cell membrane invasion and antibiotic resistance, a two-component response regulator, and a transcription antiterminator (BglG family) were located on both lists.

<i>Protein function</i>	<i>Chan et al. (18) genes</i>	<i>Fold change</i>	<i>Dovilas genes</i>	<i>Fold change</i>
Cell membrane Invasion	<i>lmo0582</i>	3.07	<i>lmo0433</i> <i>lmo2396</i> <i>lmo2821</i>	3.15 2.33 2.15
Antibiotic resistance	<i>lmo1250</i>	2.08	<i>lmo0872</i>	2.28
Signal transduction two-component response regulator	<i>lmo0287</i>	3.45	<i>lmo1172</i>	2.05
Replication, Transcription, Translation transcription antiterminator (BglG family)	<i>lmo2668</i>	7.44	<i>lmo0918</i>	2.1
Other (exact comparisons) unknown	<i>lmo0954</i> <i>lmo0955</i>	4.98 3.19	<i>lmo0954</i> <i>lmo0955</i>	2.64 2.88
similar to <i>B. subtilis</i> YvIB protein	<i>lmo2487</i>	3.27	<i>lmo2487</i>	2.1

Table 2. Comparison of general functions of genes up-regulated in planktonic stationary phase cells when grown at 4°C according to the current study and Chan et al. (18).

A compilation of down-regulated genes found in both the Chan et al. and the current study are located in Appendix 2 (18). This provides a list of 285 genes down-regulated in the 4°C stationary phase planktonic cell sample and their corresponding fold change values. Upon comparing the two lists, a gene similar to Flavocytochrome C Fumarate Reductase chain A in anaerobic respiration was found to be down-regulated. Genes that were located on both lists with similar functions are shown in Table 3. Some of the similar gene and protein functions include cell membrane cold-shock proteins, phosphotransferase (PTS) enzymes, enzymes participating in glycolysis and homeostasis, and ATP-binding cassette (ABC) transporter proteins (specifically ATP-binding proteins).

<i>Protein function</i>	<i>Chan et al. (18) genes</i>	<i>Fold change</i>	<i>Dovilas genes</i>	<i>Fold change</i>
Cell membrane cold-shock protein	<i>lmo1879</i>	-2.12	<i>lmo2016</i>	-5.79
Cell metabolic pathways/ Biosynthesis PTS general glycolysis	<i>lmo2259</i> <i>lmo2336</i>	-2.44 -2.1	<i>lmo1003</i> <i>lmo1978</i>	-2.2 -2.27
Other homeostasis ABC transporter (ATP- binding protein)	<i>lmo1018</i> <i>lmo2114</i> <i>lmo0278</i>	-3.39 -2.19 -2.52	<i>lmo1013</i> <i>lmo2139</i> <i>lmo2192</i>	-2.47 -2.89 -2.17
Other (exact comparisons) similar to Flavocytochrome C Fumarate Reductase chain A	<i>lmo0355</i>	-3.91	<i>lmo0355</i>	-2.75

Table 3. Comparison of general functions of genes down-regulated in planktonic stationary phase cells when grown at 4°C according to the current study and Chan et al. (18).

Upon comparing the above data, the numbers of up-regulated and down-regulated genes in both studies are different. Additionally, only four genes were found to be located on both lists and determined to be differentially expressed (either up or down). A few genes were found to have counterparts with similar functions in both studies. This variation can be due to several factors. The 2007 Chan et al. study was completed using a different growth media, different labeling and hybridization methods as well as custom-made microarrays (18). The current study completed the comparison with only one sample. This does not allow for proper statistical analysis and assumes that the single sample is representative of an ideal refrigerated planktonic sample.

H. Biofilm microarray analysis:

The roughly three million base pair *L. monocytogenes* genome was broken down into 192,000 probe sequences and each probe sequence was broken down into 13 target sequences with five replications of each target sequence per microarray. Each NimbleGen microarray was composed of squares where target sequences were attached. If the target sequence was found in the genome, the labeled sequence from the sample hybridized to it. Upon scanning the

microarray, the quantity of labeled sequences per square was recorded as the intensity of brightness. Therefore, the more copies of the target sequence found in the genome, the more brightly the square appeared as seen in Figure 8 top pictures. Although the intensity histogram viewed by the scanning program was atypical for the biofilm samples (not shown), the resulting scans were consistent among the triplicates of the biofilm samples. As seen in the bottom images in Figure 8, each scanned microarray square was aligned and annotated with a gene function. Each square was normalized and background corrected, showing the most intense up-regulated or down-regulated genes as yellow and the least differentiated as purple.

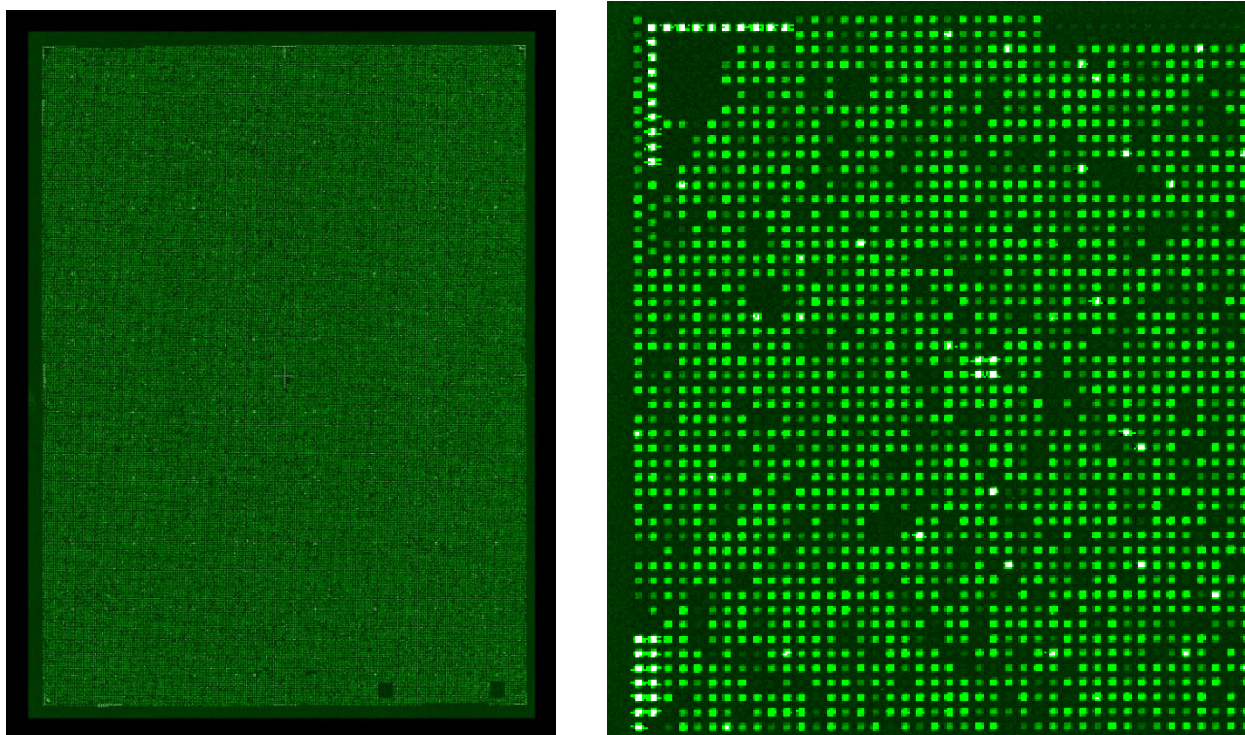


Figure 8. Images of a microarray slide of planktonic sample 3. Image on top left shows whole microarray scan, while image on top right shows zoomed-in top left hand corner. Each square contains a probe to which labeled cDNA has been hybridized. Each probe is an oligo of the *L. monocytogenes* EGD-e genome. The brighter the green color, the more cDNA from a sample has hybridized to that probe. Therefore, the brighter the green color, the more copies of that sequence are present in the genome. The bottom left image shows the NimbleScan program aligning each probe with annotation and probe number. This program also corrects for the background and normalizes prior to statistical analysis. The image on the bottom right is a zoomed-in image of the top left corner of the image to its left.

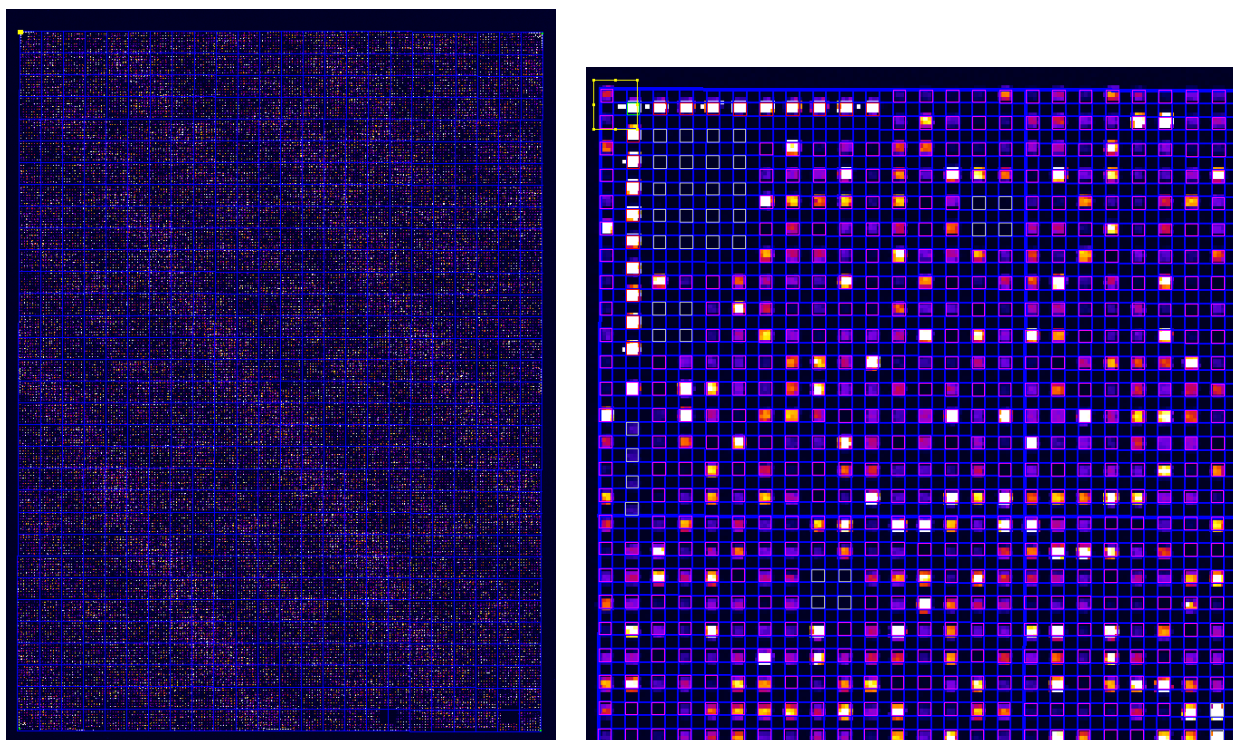


Figure 8 contin.

The results obtained from statistical analysis revealed the Reduced Major Axis (RMA) value distribution as seen in Figure 9. An RMA curve determines how reliable and representative each sample is within and between all samples in the experiment. Briefly, each of the colored lines corresponds to a planktonic or biofilm sample and illustrates the oligo expression values. As seen, the red curve shows the overall consistency of expression density within the three biofilm samples. Therefore, the three biofilm samples grown on different dates had matching data, with little variation in gene expression values. On the other hand, the black curve shows the inconsistency of expression density within the three planktonic samples. This indicates greater expression differences within a growth condition (when grown as planktonic cells) and accounts for less consistent statistical results and greater variability within the results. All further statistical analyses including fold changes accepted planktonic cells as the baseline. Therefore all fold changes were with respect to biofilm cells.

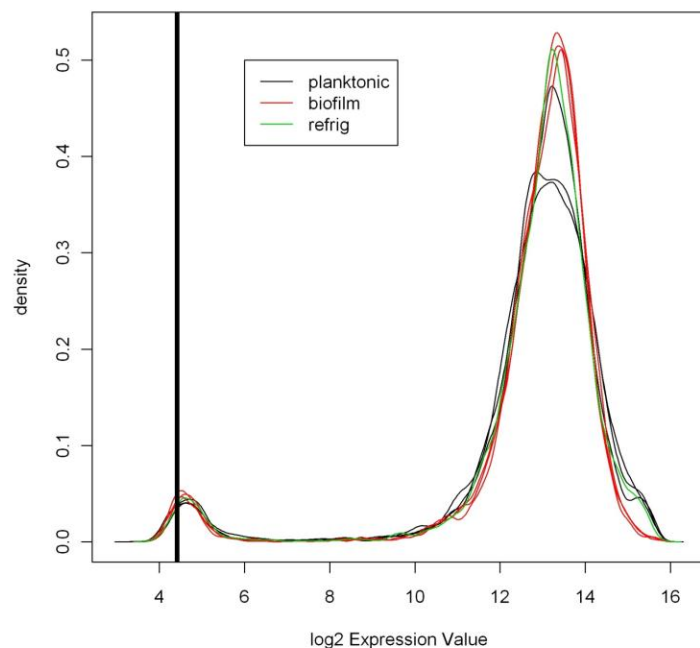


Figure 9. RMA value distributions. Each colored line corresponds to a sample. The thick black line on the figure is actually 7 lines, showing the range of the random oligo expression values.

For categorization of gene and protein functions, applying a criterion of FDR p-value < 0.05 and at least a two-fold change resulted in a list of 646 genes up-regulated and 789 genes down-regulated (lists not attached). Appendix 3 lists the 398 genes that were up-regulated in biofilm cells with a strict criterion of FDR $p < 0.0001$ and at least a two-fold change. The list was arranged by lowest FDR p-value with their respective gene name and function annotation as provided by NimbleGen. Data analysis was completed using the online databanks BRENDA and EMBL-EBI Unipro. Table 4 lists general functional categories and possible subcategories into which the up-regulated genes were placed. For data mining, functional categories were formed if more than one gene fell into a category. Therefore no single gene was analyzed unless the specific function was known to be significant to biofilm growth prior to this study.

Genes up-regulated in biofilm cells fell into various protein function categories. Three genes (*lmo1548*, *lmo1547*, and *lmo1546*) are known to participate in cell shape determination.

Knowing that biofilm cells have an EPS and are more resistant to antibiotics, the aforementioned genes may encode proteins which provide structural support and protection needed for a biofilm to withstand stress from chemical and mechanical cleaning agents. Other biofilm studies confirm that cell envelope synthesis, adaptation and protection genes have been up-regulated (73, 82). In the current study, three genes were up-regulated and two genes were down-regulated which play a role in peptidoglycan synthesis. This up-regulation has also been mentioned in *P. aeruginosa* biofilm studies (82). Aiding in fatty acid biosynthesis, six genes were identified to be up-regulated. These fatty acids can participate in membrane production. As mentioned before, biofilm initiation is postulated to be a stress response (42). For protection, the bacteria can up-regulate genes which help it adapt for survival in certain environments as found in other *L. monocytogenes* biofilm studies (42). Genes such as *lmo2016* and *lmo1364* are known to be cold-shock proteins which are indicative of a stress response.

To transport proteins and sugars across a membrane to be metabolized, *L. monocytogenes* uses a phosphotransferase system (PTS). While various proteins involved in the PTS for mannose, fructose, beta-glucoside, glucitol/sorbitol, cellobiose, and lichenan are found in both the up-regulated and down-regulated lists, a gene for the PTS for histidine (*lmo1102*) is only up-regulated in biofilm cells. According to EMBL-EBI, histidine transporters also have substrate specificity to asparagine and glutamine. In addition, PTS is known to aide in chemotaxis, allowing for the bacteria to come into closer proximity to sugars. Four non-PTS genes involved in chemotaxis were also detected. *L. monocytogenes* biofilm research by Kumar determined proteins involved in two-component regulatory system and chemotaxis which were up-regulated (50). The ABC genes function in transporting metabolic products, lipids, drugs, and sterols across a membrane as well as in RNA translation. Fourteen ABC transporter genes were

identified to be up-regulated in biofilm cells. Membrane transport proteins have been shown in other biofilm studies to be up-regulated (77, 82).

Proteins involved in biosynthesis have been identified to be up-regulated in *L. monocytogenes* biofilm cells from previous studies (42). Five genes relevant to purine biosynthesis and four genes used in menaquinone biosynthesis were significantly expressed at higher levels in biofilm cells. While purines are necessary for RNA and DNA synthesis, menaquinone can be a redox signal for a two-component regulatory system as determined by EMBL-EBI.

ATP synthase is known create a proton gradient by hydrolyzing ATP, which provides energy for flagella and transport of nutrients into the cell. Nine ATP-synthase genes were found to be up-regulated and two were down-regulated in biofilm cells. Energy generation genes have been found to be up-regulated in other *L. monocytogenes* biofilm studies (42). As previously mentioned, biofilms form strata where the inner cells survive in anaerobic conditions. Four genes listed in Table 4 are specific to anaerobic respiration and anaerobic glucose metabolism. Schembri et al. determined that *E. coli* biofilms up-regulate genes related to low oxygen survival (77).

Several transcriptional and translational factors such as expression of ribosomal proteins, initiation and elongation factors, and tRNA synthases/ protein biosynthesis enzymes were up-regulated in biofilm cells. This is supported by Hefford et al. noticing up-regulation of regulatory proteins in *L. monocytogenes* biofilms (42). Additionally, three genes encoding flagellar proteins were up-regulated in biofilm cells. Although the inner biofilm cells lack flagella, the outer cells maintain their motility function.

As expected, several genes were similar to those found in *B. subtilis*. Of the 66 hypothetical proteins or those genes with unknown functions, some may be directly involved in biofilm formation and survival, yet it is unknown at this time.

<i>Protein function and genes</i>	<i>Fold change</i>	<i>FDR p-value</i>
Cell membrane		
Cell-shape		
<i>lmo1548</i>	3.127	3.14E-10
<i>lmo1547</i>	3.253	3.63E-10
<i>lmo1546</i>	3.233	4.49E-08
Cold-shock		
<i>lmo2016</i>	4.461	5.96E-12
<i>lmo1364</i>	3.212	3.63E-10
Cell metabolic pathways/Biosynthesis		
PTS- histidine		
<i>lmo1002</i>	3.364	2.87E-08
Purine biosynthesis		
<i>lmo1766</i>	4.74	4.00E-07
<i>lmo1773</i>	3.342	2.54E-06
<i>lmo1764</i>	3.922	1.19E-05
<i>lmo1856</i>	1.872	3.37E-05
<i>lmo1524</i>	1.782	0.00032
Menaquinone biosynthesis		
<i>lmo1676</i>	2.829	4.46E-07
<i>lmo2635</i>	2.481	5.28E-06
<i>lmo1930</i>	1.89	0.000253
<i>lmo1677</i>	1.862	0.000289
ATP synthase		
<i>lmo2536</i>	4.723	7.24E-12
<i>lmo2535</i>	3.84	3.63E-10
<i>lmo2532</i>	3.182	1.15E-08
<i>lmo2530</i>	3.519	3.96E-08
<i>lmo2534</i>	2.86	6.05E-08
<i>lmo2528</i>	1.967	2.21E-05
<i>lmo2529</i>	2.153	2.48E-05
<i>lmo2531</i>	2.629	7.00E-05
Fatty acid biosynthesis		
<i>lmo1806</i>	4.769	2.72E-11
<i>lmo1807</i>	2.581	2.08E-07
<i>lmo1572</i>	3.234	1.77E-05
<i>lmo2201</i>	2.08	0.000205
<i>lmo1394</i>	2.027	0.000364
<i>lmo0970</i>	2.28	0.00085

Table 4. Functions and subfunctions of genes up-regulated when grown as biofilm cells.

<i>Protein function and genes</i>	<i>Fold change</i>	<i>FDR p-value</i>
Anaerobic enzymes <i>lmo1917</i> <i>lmo1406</i> <i>lmo1407</i> <i>lmo0355</i>	2.824 3.685 2.209 2.664	3.02E-07 1.98E-06 2.88E-05 9.32E-05
Peptidoglycan synthesis <i>lmo1464</i> <i>lmo1521</i> <i>lmo2036</i>	2.625 2.317 1.677	2.08E-08 7.35E-06 0.000549
Replication, Transcription, Translation Elongation factor <i>lmo1657</i> <i>lmo2654</i> <i>lmo1355</i> <i>lmo2653</i>	4.86 2.744 2.461 2.455	1.15E-10 2.19E-07 1.72E-06 1.65E-05
Initiation factor <i>lmo2610</i> <i>lmo1785</i> <i>lmo1325</i> tRNA synthase/ Protein Biosynthesis <i>lmo1552</i> <i>lmo1756</i> <i>lmo1607</i> <i>lmo1522</i> <i>lmo0228</i> <i>lmo1458</i> <i>lmo0237</i> <i>lmo1559</i> <i>lmo1459</i> <i>lmo1531</i> <i>lmo1754</i> <i>lmo1504</i> <i>lmo1519</i> Ribosomal proteins (50 genes)	4.861 3.081 2.411 2.335 2.402 2.817 1.722 1.996 2.354 1.82 1.791 1.814 2.062 2.677 2.253 1.783	1.15E-11 1.15E-08 3.35E-06 4.79E-07 2.97E-06 6.47E-06 0.000424 0.00049 0.000502 0.000579 0.000628 0.000889 0.000117 0.00012 0.000169 0.000207
Motility Flagella <i>lmo0690</i> <i>lmo0708</i> <i>lmo0700</i>	3.54 2.823 2.243	4.83E-10 9.87E-07 3.81E-05
Signal Chemotaxis <i>lmo0691</i> <i>lmo2011</i> <i>lmo0692</i> <i>lmo1699</i>	2.004 1.86 1.873 1.82	1.45E-05 9.48E-05 0.0006 0.000842

Table 4. contin.

<i>Protein function and genes</i>	<i>Fold change</i>	<i>FDR p-value</i>
Other		
Similar to <i>B. subtilis</i> (22 genes)		
Hypothetical/unknown proteins (66 genes)		

Table 4 contin.

A complete list of down-regulated genes in biofilm cells can be found in Appendix 4. It provides the list of 306 genes with strict criterion of FDR p-value < 0.0001 and at least a two-fold change. Categorizing them into general functional groups, Table 5 indicates the gene names, fold changes and FDR p-values for a few selected genes. Other genes not mentioned in this list may play a significant role in biofilm survival, yet it is unknown at this time.

Surprisingly, seven proteins associated with the peptidoglycan and internalins were found to be down-regulated in biofilm cells. Peptidoglycan proteins provide structural support and internalins aid in attachment and invasion. Therefore conceptually, these proteins should be up-regulated in biofilm cells, thus helping them to survive by combating environmental stresses and by obtaining nutrients in the host cells.

As mentioned before, the PTS aids in sugar and other metabolic product transport across a membrane. The mannitol and galactitol PTS are both down-regulated in biofilm cells. In addition, ascorbate and aldarate metabolism is down-regulated as indicated by two genes (*lmo0735* and *lmo0499*). Five genes involved in cobalamin biosynthesis were down-regulated in biofilm cells. This may play a role in iron-related metabolism as mentioned in Unipro. Metabolism of porphyrin and chlorophyll involves three genes that were down-regulated and one gene that was up-regulated in biofilm cells. The significance of this metabolic pathway to biofilm cultures is unknown. Twenty two genes involved in transcription regulation were also found to be down-regulated. Surprisingly, the only virulence gene to be differentially expressed was that of Phospholipase C which was down-regulated in biofilm cells. Fifteen more genes

encoding ABC transporters were found to be down-regulated in comparison to being up-regulated.

<i>Protein function and genes</i>	<i>Fold change</i>	<i>FDR p-value</i>
Cell membrane Internalin/ Peptidoglycan bound		
<i>lmo0732</i>	-2.32	1.45E-06
<i>lmo0835</i>	-2.69	3.66E-06
<i>lmo2179</i>	-2.53	6.17E-06
<i>lmo2396</i>	-2.64	1.29E-05
<i>lmo0627</i>	-2.15	6.35E-05
<i>lmo0514</i>	-2.43	8.18E-05
<i>lmo0262</i>	-2.00	00.00010707
<i>lmo0264</i>	-2.11	0.00011523
<i>lmo2470</i>	-2.19	0.00023565
<i>lmo2027</i>	-2.4	0.00025578
<i>lmo0175</i>	-2.09	0.00038036
<i>lmo1136</i>	-1.68	0.00078047
<i>lmo0159</i>	-1.81	0.00086714
Cell metabolic pathways/Biosynthesis		
PTS- mannitol		
<i>lmo2729</i>	-2.55	2.71E-06
PTS- galactitol		
<i>lmo2097</i>	-2.26	4.26E-05
<i>lmo0507</i>	-1.96	0.00012279
<i>lmo2096</i>	-1.91	0.0001872
<i>lmo0508</i>	-1.82	0.00023119
<i>lmo2665</i>	-1.65	0.00060892
Ascorbate and aldarate metabolism		
<i>lmo0735</i>	-6.14	6.43E-09
<i>lmo0499</i>	-2.2	0.00029856
Cobalamin biosynthesis		
<i>lmo1148</i>	-2.24	0.00010475
<i>lmo1141</i>	-1.96	0.00021033
<i>lmo1147</i>	-2.19	0.00027338
<i>lmo1197</i>	-1.92	0.0003058
<i>lmo1202</i>	-1.91	0.00063594
Porphyrin and chlorophyll metabolism		
<i>lmo1195</i>	-1.92	0.00016909
<i>lmo1203</i>	-2.01	0.00030108
<i>lmo1193</i>	-1.79	0.00065192
Replication, Transcription, Translation		
Transcriptional regulators (22 genes)		

Table 5. Functions and subfunctions of genes down-regulated when grown as biofilm cells.

<i>Protein function and genes</i>	<i>Fold change</i>	<i>FDR p-value</i>
Virulence factor Phospholipase C <i>lmo0205</i>	-2.04	0.00097528
Other Similar to <i>B. subtilis</i> (9 genes) Sugar ABC transporter (29 genes) Unknown/hypothetical (76 genes)		

Table 5 contin.

In addition to observing global gene expression, LIPI-1 virulence gene expression was noted. As shown in Table 6, all genes in the Pathogenicity Island are clustered together in the genome as indicated by successive gene ID numbers. As Stoodley et al. stated, biofilm cells are known to be more pathogenic than planktonic cells (82). Through this experiment, five of the six genes directly associated with the pathogenicity of *L. monocytogenes* were down-regulated in cells grown as biofilms. Therefore, it can be concluded that this increased virulence of biofilm cells is not due to up-regulation of virulence genes. With the exception of PlcB (*lmo0205*), all of the genes listed in the table below did not show a minimum of a two-fold change and therefore were not listed in the appendices.

<i>Listeria Pathogenicity Island I (LIPI-1) Virulence genes and identities</i>	<i>gene ID</i>	<i>up-regulated fold change</i>	<i>down- regulated fold change</i>	<i>p-value</i>
PrfA- Virulence regulatory factor PrfA	<i>lmo0200</i>		-1.337	0.06315
PlcA- Phosphatidyl-specific phospholipase C	<i>lmo0201</i>		-1.287	0.1718
LLO- Listeriolysin O	<i>lmo0202</i>	1.510		0.00704
Mpl- zinc metalloproteinase precursor	<i>lmo0203</i>		-1.491	0.01187
ActA- Actin-assembly inducing protein	<i>lmo0204</i>		-1.158	0.37716
PlcB- phospholipase C	<i>lmo0205</i>		-2.038	0.00023

Table 6. List of virulence genes located on LIPI-1 and their differential gene expression.

Chapter 5. Conclusions

Before proceeding with the differential gene expression analysis of biofilm cells, optimization of the growth procedure, RNA extraction, and cDNA synthesis were completed. Although the conversion of RNA to cDNA proved to be difficult, the experiment was completed. When comparing the results of the side experiment (growth at 4°C) to that of Chan et al. (18), a quantitative assessment did not show equal numbers, yet some genes were functionally similar.

For biofilm cells, a significant number of genes were found to be up-regulated and down-regulated. General functions of genes found to be up-regulated include those participating in cell shape, cold-shock proteins, histidine PTS, and peptidoglycan synthesis. Also genes involved in purine and menaquinone biosynthesis, ATP synthase, fatty acid biosynthesis, flagella motility, chemotaxis, and survival in anaerobic conditions were found to be up-regulated. The functions of down-regulated genes include encoding internalins and peptidoglycan bound proteins, those involved in galactitol PTS, ascorbate and aldarate metabolism, cobalamin biosynthesis, and porphyrin and chlorophyll metabolism. Several transcriptional regulator genes were also down-regulated. Several genes were identified to be either up-regulated or down-regulated but are currently not assigned a function. Other genes have close similarities with *B. subtilis* which phylogenetically is accurate. Surprisingly, only one virulence factor (PlcC) was identified to be differentially expressed. Therefore, increased pathogenicity of biofilms is not due to differential gene expression.

Future studies can verify this data using qRT-PCR as well as complete a further analysis of the genes and proteins listed in the appendices for a more in depth study. Additionally, many genes were not assigned functions. Further research can determine protein functions and their significance to biofilm formation. This can also be explored through comparison across other bacterial species biofilms. Upon identifying and categorizing the hypothetical proteins, it can be determined whether multispecies biofilms are more pathogenic than single species biofilms. Perhaps the unknown proteins encode a biofilm pathogenicity island or can be horizontally transferred to other species.

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Appendix A.

This appendix provides a complete list of genes up-regulated at 4°C stationary phase planktonic cells. It is found as supplemental information named **Appendix A.pdf**.

Appendix B.

This appendix provides a complete list of genes down-regulated at 4°C stationary phase planktonic cells. It is found as supplemental information named **Appendix B.pdf**.

Appendix C.

This appendix provides a complete list of genes up-regulated when grown as stationary phase biofilm cells. It is found as supplemental information named **Appendix C.pdf**.

Appendix D.

This appendix provides a complete list of genes down-regulated when grown as stationary phase biofilm cells. It is found as supplemental information named **Appendix D.pdf**.

Vita.

Lina Angelika Dovilas was born in the western suburbs of Chicago, IL in June 1986. She graduated from Nazareth Academy in LaGrange Park, IL in 2004. After high school, she attended the University of Illinois at Urbana-Champaign where she received her Bachelor's of Science in Molecular and Cellular Biology with a minor in Chemistry. She started her Master of Science degree in August 2008 at University of Illinois at Urbana-Champaign and earned her degree in August 2010.