Biofilm Forming Properties and Subtype of Wild Type and Rough Strains of *Listeria monocytogenes* in Relation to Hypochlorous Acid Tolerance and Associated Proteins.

by

JAMES P. FOLSOM

(Under the Direction of Joseph F. Frank)

ABSTRACT

This work determines if genetically related strains of *L. monocytogenes* (n=30) exhibit similar biofilm accumulation, and the effect of nutrient concentration on biofilm formation. Selected strains (n=13) were tested for hypochlorous acid tolerance to determine if planktonic hypochlorous acid tolerance or subtype is associated with biofilm hypochlorous acid tolerance. A variant strain (SBS) exhibiting increased hypochlorous acid tolerance and biofilm production was compared to the wild type using fluorescence 2-D difference gel electrophoresis (DIGE). Biofilms were grown on stainless steel in high or low nutrients. Biofilm accumulation was determined using image analysis after staining the cells. The strains were genetically subtyped by repetitive element sequence-based PCR. Planktonic cells were exposed to 20 through 80 ppm of hypochlorous acid, and biofilms were exposed to 60 ppm. We found that nutrient levels influenced biofilm accumulation, but that the nature of this influence differed with strain. Serotype 4b strains produced more biofilm accumulation in high nutrients than serotype 1/2a strains, while serotype 1/2a strains produced more accumulation in low nutrients. Low nutrient media inhibited biofilm accumulation of serotype 4b strains. There was no correlation between genetic subtype and the amount of biofilm accumulation. Some strains were more tolerant of

hypochlorous acid than others were. Biofilm cell density and morphology was not associated with hypochlorous acid tolerance. The 50s ribosomal proteins L7/L12 and L10 were down regulated in biofilm and planktonic SBS respectively. Other proteins down regulated in planktonic SBS were the peroxide resistance protein (Dpr), an unknown protein (LMO1888) and a sugar binding protein (LMO0181). This sugar binding protein was up regulated in biofilm SBS. Regulation of the sugar binding protein indicates that SBS may reserve a carbon source for use during biofilm formation. The results presented show that serotype 1/2a and serotype 4b strains differ in the regulation of their biofilm phenotype, poor biofilm accumulation of serotype 4b strains grown in low nutrient media could be a factor in the predominance of serogroup 1/2 strains in food processing plants. Additionally, hypochlorous acid tolerance mechanisms of planktonic cells and biofilms differ, with planktonic hypochlorous acid tolerance being more affected by inducible traits, and biofilm hypochlorous acid tolerance being more affected by traits not determined in this study.

INDEX WORDS: 2D DIGE, Hypochlorous acid, *L. monocytogenes*, planktonic, biofilm, repPCR

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JAMES P. FOLSOM

B. S. A., The University of Georgia, Athens, GA, 1996

Master of Science, The University of Georgia, Athens, GA, 1999

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JAMES P. FOLSOM

Major Professor: Joseph F. Frank

Committee: Mark A. Harrison
Louise Wicker
Jinru Chen
Gregory R. Siragusa

Electronic Version Approved:

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

This work is dedicated to my fiancée Summer Brooke Smith, who has been my patient and loving companion through the trying times that is graduate student life. It has been hard on both of us and I sincerely thank her for her patience.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Listeria monocytogenes is capable of persisting or growing in wide variety of processing plant conditions. The species is sodium chloride tolerant (20%), and may grow in saline environment as high as 10% sodium chloride *(137)*. *L. monocytogenes* will generally grow at temperatures ranging from 3ºC to 45ºC *(131)*. Use of many common sanitizers results in tolerance and cross protection conferring tolerance to other sanitizers*(84)*. As a result of these characteristics *L. monocytogenes*, is a threat to sanitary conditions for a wide range of processed food products, as is evidenced the great variety of foods implicated in listeriosis outbreaks, including various cheeses, butter, pork tongue product, coleslaw, milk and seafood products *(16, 40, 41, 66, 83, 87, 89, 135)*.

Food contact materials used in food processing plants are vulnerable to colonization by *L. monocytogenes*. A variety of materials can support attachment and even biofilm growth, including Teflon®, stainless steel, various rubber compounds, conveyor belt materials, floor sealants, and wall tile materials *(8, 12, 76, 141, 142)*. *L. monocytogenes* attaches to these surfaces and forms biofilms that confer resistance to killing by commonly used sanitizers *(48, 80, 126)*. Many of the surfaces found in processing plants are complex and difficult to clean, especially conveyor belts *(76)* and complex machinery, leading to spots being missed during sanitation *(85, 86)*. These colonized surfaces are sources of cross contamination leading to product contamination.

L. monocytogenes is a clonal species with little horizontal gene flow occurring *(22, 115, 122, 158)*, as a result there are three clonal groups of this organism *(115, 122)*. These groups are identified consistently by a variety of subtyping methods and have become known as lineage I, II, and III *(103, 158)*. Lineage I is composed of serotypes 4b 1/2b, 3c and 3b, lineage II is composed of serotypes 1/2a, 1/2c, and 3a *(11, 103)*. Lineage II is older than lineage I and the third clonal group, lineage III is the most recent to evolve and contains serotype 4a and 4c *(103, 122)*. Lineage III is closely related to lineage I *(92)*. Other serotypes have not been sufficiently studied to place them in a lineage, because of their relative rarity.

There are indications that *L. monocytogenes* strains differ in their ability to attach *(70)* and grow on surfaces *(144)*, as well as strain specific differences in sanitizer tolerance. The focus of this work is to identify strains exhibiting greater abilities to produce biofilm, and compare the protein expression of these strains to that of poor biofilm producers. In addition, strains will be subtyped, to examine the possibility that biofilm producing strains may be closely related. Isolates representing a range of subtypes and biofilm production will be selected for determination of hypochlorous acid tolerance. It is important to know if closely related strains share a similar tolerance to hypochlorous acid. We will also use this information to select strains for proteomic analysis to identify proteins associated with hypochlorous acid tolerance.

CHAPTER 2

LITERATURE REVIEW

ATTACHMENT OF *L. MONOCYTOGENES* TO FOOD CONTACT SURFACES

L. monocytogenes attaches to polypropylene, polycarbonate, polyurethane, polyethylene terephthalate glycol (PETG), PVC, Teflon (PTFE), Lexan, ethylene propylene diene monomer (EPDM) silicone rubber, buna-N (nitryl rubber), natural white rubber, rubber CNA-70, stainless steel 304, stainless steel 316, stainless steel 430, glass and aluminum *(8, 88)*. Maximum attachment generally occurs in less than 20 min *(88)*. Greater numbers of *L. monocytogenes* attached to stainless steel than buna-N rubber for the first 60 minutes of exposure *(141)*, with attachment to buna-N being similar after 2 hours *(140)*. Attachment to stainless steel also increases with increasing ionic strength of the attachment media *(18)*.

Kim and Frank *(73)* comprehensively studied the effects of various nutrients on the attachment of *L. monocytogenes* Scott A. They found that when grown in defined media, *L. monocytogenes* attached to stainless steel better than when grown in tryptic soy broth (TSB). Increasing the concentration of ammonium chloride or decreasing the iron concentration in the defined media ten fold, reduced attachment of *L. monocytogenes*. Replacing all of the nitrogen sources (ammonium chloride, amino acids, and nitrilotriacetic acid) with soytone or peptone resulted in adherence levels similar to when *L. monocytogenes* were grown in TSB. This decrease in adherence requires growth in the presence of soytone or peptone, as simple exposure prior to attachment had no effect. Substitution of mannose, cellobiose, fructose, or trehalose for

glucose in the defined media had no effect on attachment. Glucose, phosphate, vitamin, and magnesium concentration also had no effect on attachment.

Smoot and Pierson *(140)* studied the effects of growth pH, attachment pH, growth temperature, and temperature shifts on the attachment of *L. monocytogenes* to buna-N rubber in addition, the effect of attachment temperature on attachment to stainless steel and buna-N rubber was studied. The numbers of *L. monocytogenes* that adhere to stainless steel and buna-N rubber increase with increasing growth temperature *(18, 140)*. However for the first ten minutes of exposure, cells grown at 42ºC attached at a rate slower than even cells grown at 10ºC *(140)*. Upward temperature shifts had no effect on attachment but downward shifts resulted in dramatic decreases in attachment. Except for extreme pH, pH range had little effect on attachment.

Other factors such as strain dependant variation, and presence of flagella, affect surface attachment of *L. monocytogenes*. Kalmokoff et al. *(70)* observed that out of 36 strains of *L. monocytogenes*, three produced less attachment and 12 produced more attachment than other strains under standardized conditions. Dickson and Daniels *(30)* found that *L. monocytogenes* grown at 37ºC such that they lacked flagella, attached to several surfaces in lower numbers than cells possessing flagella and grown at 23ºC. They couldn't eliminate growth temperature as a variable but Vatanyoopaisarn et al. *(156)* was able to eliminate this variable and confirm their results. In addition to showing that flagella were important to early adherence, they demonstrated that motility was not necessary for flagella enhanced attachment. The affect of flagella may be due changes in the electronegativity of cells possessing them, as Briandet et al. *(18)* observed that cells grown at 37ºC were more negatively charged than cells grown at 20ºC, and attributed this to the absence of flagella.

The presence of organic soils can affect the ability of *L. monocytogenes* to attach to surfaces. Attachment of *L. monocytogenes* cells is inhibited by the adsorption of milk or milk proteins to surfaces *(7, 57, 63)*, milk soil dried on the surface *(57)* and by growth of the cells in milk *(63)*. However, this effect may be dependant upon the surface properties of the support material. Adsorption of several milk proteins (α-lactalbumin, ß -casein, ß-lactoglobulin) inhibited attachment of *L. monocytogenes* on hydrophobic silicon wafers, but when hydrophilic silicon wafers were employed the proteins facilitated attachment *(1)*. The adsorption of bovine serum albumin (BSA) was also shown to inhibit attachment on silicon wafers *(1)*. It should be noted though, that while organic soil can decrease the number of *L. monocytogenes* that attach, heavy soil will promote growth *(162)* resulting in a biofilm with more cells than otherwise would have been supported.

Other factors have no effect on the attachment of *L. monocytogenes***.** Schwab et al. *(136)* found that the alternative sigma factor, σ^B, is not required for initial adherence, but may be required for further biofilm development. Thickness of the film containing the inocula did not affect attachment of *L. monocytogenes (30).* Presence of lactose has no effect on attachment *(57)*. Sucrose added to milk on the premise that it would enhance polysaccharide production when the milk is used as a growth media for *L. monocytogenes*, did not affect attachment *(63)*.

BIOFILM FORMATION

L. monocytogenes produces a biofilm on a variety of surfaces (floor sealant, stainless steel, aluminum, Teflon (PTFE) and buna-N) *(12, 25, 126, 149)*. Rougher or worn surfaces accumulate greater amounts of biofilm than newer or smoother surfaces *(3, 102)*. While surface roughness measurements of stainless steel are predictive of cleanability, type of polish is not due

to manufacturer variation *(45)*. These biofilms are more resistant to heat and sanitizers than attached and planktonic cells *(48, 80, 102, 162)*.

EFFECTS OF STARVATION ON BIOFILM ACCUMULATION

Authors of *L. monocytogenes* biofilm papers have cited several authors claiming that starvation stimulates the biofilm production of other bacteria. Brown et al. *(19)* found that aquatic bacteria grown in the absence of nitrogen did not grow on surfaces, however when the same bacteria were grown in the absence of glucose, surface growth occurred. Also, Stepanovic et al. *(144)* found that *Salmonella* strains produced more biofilm in microtiter plate wells when 1:20 diluted TSB was used as the growth media compared with TSB and brain heart infusion broth (BHI). This is not inconsistent with Brown et al. *(19)* as glucose in the TSB would be diluted to very low levels. Also cited is Dewanti and Wong *(29)*, who found that *Escherichia coli* 0157:H7 produced biofilms faster and with higher cell density in low nutrient media. In examining the works of the authors, it is readily apparent that starvation is a misnomer, as the media reported are merely reduced nutrient media. This is highlighted by the overlooked fact that Dewanti and Wong *(29)* found biofilm production in defined media required low levels (<1%) of carbohydrate. Further reinforcement of this comes from Hunt et al. *(65)* who reports that biofilms of *Pseudomonas aeruginosa* grown in limited nutrient media begin to detach from the surface when media refreshment ceases. An issue that further complicates an understanding of this matter is that a great many of these studies included only one or a few strains, or consortia from a limited number of sites. When a larger number of *Salmonella* strains were studied it was found that 27.1% did not produce more biofilm in the presence of limited nutrients, and in fact these strains produced more biofilm in rich media *(144)*. Clearly, a more focused study needs to

be done to further clarify the nature of nutrient limitation, starvation, and strain specific responses to these conditions.

Studies of *L. monocytogenes* biofilm production have also produced conflicting results concerning the effect of nutrient limitation on biofilm production. Sasahara and Zottola *(133)* found that a serotype 3a strain of *L. monocytogenes* did not form biofilms on glass cover slips, where the growth media used was tryptic soy broth with added yeast extract (TSBYE) or 0.1% peptone. They assert this as evidence that starvation does not induce biofilm production of *L. monocytogenes*. However Jeong and Frank *(67)*, using a different strain, found that biofilms grown on 2g/l TSB exhibited cell densities 10 fold higher than biofilms grown on 10g/l TSB. Norwood and Gilmour *(107)* speculated that this meant that starvation of *L. monocytogenes* enhances biofilm production. Furthermore, when biofilms of *L. monocytogenes* ScottA were grown for seven days; biofilm cell numbers were superior when grown in TSB for up to four days, and after 7 days biofilms grown in 3g/l TSB produced equivalent biofilm cell numbers *(108)*. The use of defined media for the growth of biofilms on a variety of food plant surfaces has been observed to decrease biofilm production *(12)*. Stepanovic et al. *(144)* found that in general (~80% of strains tested) *L. monocytogenes* produced the best biofilms in BHI, and TSB, in that order, and the worst biofilms in (1:20) TSB and Meat broth, in that order. They also observed that some strains did not produce biofilms in any tested media, and that not all strains produced better biofilms in BHI. In another study, out of 8 strains, two produced more biofilm in TSB than defined media, and five produced more in the defined media, with one strain producing equally well in both *(97)*. The results presented by these authors indicate that studies of the effect of nutrient limitation and starvation should consider strain variation.

Laboratory adaptation may also affect biofilm production. Djordjevic et al. *(31)* found that lineage I strains of *L. monocytogenes* produced more biofilm under nutrient limitation (defined media) while lineage II produced more biofilm in rich media. Borucki et al. *(13)* found that lineage II strains produced more biofilm in the same defined media, and this discrepancy couldn't be rectified using some of the strains from the Djordjevic study. Others found that lineage II (serotype 1/2a) strains produced more biofilm in diluted TSB *(107)*. This could mean the issue is further complicated by some adaptive response *(137)*.

BIOFILM GROWTH KINETICS

When allowed to attach to a surface, *L. monocytogenes* quickly reaches a maximum cell density that remains steady when enumerated by plate counting. These counts of viable cells do not correlate with microscopic observations which show biofilms that continue to exhibit increasing numbers of visible cells and increasing biofilm complexity *(25)*. Biofilm production is temperature and pH dependant. *L. monocytogenes* produces a moderate biofilm at 35ºC, but as growth temperature decreased to 21ºC and 10ºC, the moderate biofilm no longer formed. However, when the growth pH was 8, moderate biofilm formed at 21ºC with fewer cells observed at 35ºC, and at pH 5, biofilm grew poorly at 21ºC and 35ºC *(59)*. The biofilm of strains may also undergo periods of detachment followed by recolonization *(24, 25)*. Amino acids are also important for early biofilm development *(74)*, and mutants unable to initiate biofilm growth were not able to synthesize (p)ppGpp which is required for response to amino acid starvation *(150)*. Additionally growth phase of inoculums have no effect on attachment or biofilm growth *(25)*, and numbers of attached cells do not correlate with amount of biofilm later produced by strains of *L. monocytogenes (70)*.

Several authors have questioned claims that *L. monocytogenes* as a species is a biofilm former *(70, 93, 136)*. All presented results that few or none of the strains tested produced anything more than simple adherence under the tested conditions. Work cited in support of these claims include work of Sasahara and Zottola *(133)* who found that a strain of *L. monocytogenes* did not form biofilms on glass cover slips in either TSBYE or 0.1% peptone. In contrast, Takhistov and George *(149)* observed that strain ScottA completely covered aluminum surfaces, while Chavant et al. *(25)* found that strain LO28 completely covered the surface of both Teflon and stainless steel under certain conditions, while only sparse coverage occurred otherwise. Many researchers have only examined a few strains of *L. monocytogenes* under a limited number of conditions, and it is possible that reports of non-biofilm forming *L. monocytogenes* strains are the result of strain variation. *L. monocytogenes* may or may not be a biofilm producer as a species, but research indicates that some strains do produce biofilm and it is premature to declare that the species as whole does not produce biofilms because so many strains have not been observed to do so.

METHODS FOR THE ENUMERATION OF BIOFILMS AND ATTACHED CELLS

There are several methods of biofilm removal for the enumeration of biofilms, including swabbing, vortexing with or without glass beads, scraping, shaking with glass beads, stomaching and sonication *(49, 67, 82, 97, 107)*. Moltz and Martin *(97)* found that swabbing and vortexing without glass beads yielded similar results when removing biofilms of *L. monocytogenes* for enumeration, however swabbing becomes a less effective means of enumerating cells as the biofilms grow older, and some strains may be more difficult to remove by swabbing *(24)*. Shaking with glass beads, vortexing with glass beads and sonication, are equivalent regarding numbers of cells recovered, but shaking with glass beads yielded a cleaner surface *(82)*.

Stomaching the coupons yielded many more CFU compared to scraping, swabbing and sonicating *(49)*. However vortexing with glass beads injures 8.2% of the cells while removing 97.1% of the cells from stainless steel *(107)*, and scraping with a Teflon policeman removed 97% of the cells from the biofilm *(67)*. Scraping is relatively simple and effective, but may result in contamination and is subject to variability between laboratory researchers *(49)*.

There are also several protocols for enumerating cells in a biofilm after removal or in situ. The extent of biofilm development can be determined and expressed as percent of the surface covered *(25, 74, 156, 159, 161)* or as total cell counts by staining with a DNA stain *(24)*. Viable biofilm cells have been enumerated by direct viable count (DVC) *in situ* or by re-suspending the cells *(48)*. DVC allows researchers to enumerate a larger fraction of viable cells because the method includes injured cells that cannot be revived by traditional enumeration media.

Classic DVC uses nalidixic acid to inhibit DNA replication and metabolically active cells continue to lengthen without dividing, and can be differentiated from dead cells by microscopic observation after staining with acridine orange. Briefly, cells are suspended in yeast extract (0.025%) and nalidixic acid (0.002%) then incubated for 6 h. After filtering the cells and staining with acridine orange, elongated cells are counted *(75)*. Most recently ciprofloxacin was proposed as substitution for nalidixic acid because many gram positive organisms are resistant to nalidixic acid *(6)*. However, both Novobiocin *(47, 124)* and ciprofloxacin *(9, 24)* have been found suitable for use with *L. monocytogenes.* For in situ DVC biofilms are left undisturbed and incubated in the presence of antibiotic and nutrients *(24)*. In situ DVC detects more viable cells than swabbing and plate counting, and DVC of swabbings detects more viable cells than plate counting *(24)*. Comparison of DVC to plate counting on tryptic soy agar with yeast extract

(TSAYE) of injured versus healthy *L. monocytogenes* cells scraped from biofilms found 0.72 log CFU more viable cells for healthy biofilms and 1.03 log CFU for sanitizer injured cells *(124)*.

TWO DIMENSIONAL GEL ELECTROPHORESIS OF *L. MONOCYTOGENES*

Large quantities of cells are required to harvest enough protein for two dimensional protein analyses. Simple flat surfaces that have been used for the study of other aspects of biofilms do not provide enough cells for this task. To address this problem several methods of growing large quantities of biofilm cells for protein extraction have been developed. The major characteristic of these various methods are that they provide greater surface area than hard nonporous surfaces.

The first reported method for preparing immobilized cells for protein extraction, was gel-entrapment *(112)*. For this method, bacteria are suspended in agar 2% w/v tempered to 38ºC, and 10 ml is poured into a Petri dish. After the agar hardens, it is covered with growth media that is changed every 12 hours for several days. The starting bacterial cell count was 1×10^7 CFU. After growth, the cells are harvested from the agar by blending with buffer and the agar is removed by filtration with glass wool filters. The cells are then collected by centrifugation. This method was adapted from other applications involving nutrient diffusion *(95)* and antibiotic susceptibility *(68)*.

Biofilms of *Pseudomonas aeruginosa* and *Bacillus cereus* for protein extraction have been successfully grown on glass wool *(110, 145)*. In this method 0.5-2.5 grams of glass wool is inoculated with 100 ml of bacteria and placed in appropriate media for growth. The starting bacterial cell number was 1×10^6 to 1×10^7, and after 18 hrs of growth, the biofilms are disrupted using vigorous shaking with 45 grams of 6mm glass beads and 10mM Tris-HCl buffer. The disrupted cells are then harvested by centrifugation and suspended in lysis buffer.

A variation on growing biofilm cells on glass wool was developed using glass fiber filters to produce biofilm cells for protein extraction *(153)*. In this method, *L. monocytogenes* cells were immobilized onto a glass wool filters and after washing, the filters are placed on an agar plate. The inoculated filter is placed on top of a sterile glass filter such that cells can only grow on nutrients that diffuse up from the agar surface. After a period of growth, the filters are disrupted by stomaching in filtered stomacher bags and cells are harvested by centrifugation for later protein extraction. This method was adapted from other work involving stress tolerance of biofilms *(152)*.

PROTEOMIC COMPARISONS: PLANKTONIC AND BIOFILM *L. MONOCYTOGENES* CELLS

Three published studies have compared the proteome of planktonic cells to biofilm cells *(56, 58, 153)*. The first was Trémoulet et al. *(153)* who studied a serotype 1/2a strains that had been persistent in a meat processing plant for at least 3yrs. Planktonic cells were grown in TSB, and biofilms were grown using the glass fiber filter method *(152)* using TSB with added agar as the growth medium. Of over 550 protein spots on 31 exhibited differential expression when the strain was grown as a biofilm with 22 proteins up regulated and 9 down regulated.

The second study *(58)* compared the proteome of both starved (no glucose) and unstarved (with glucose) planktonic cells and biofilm cells of a serotype 1/2a strain. Biofilms in this work were grown on stainless steel and removed by sonication. The cells were incubated with radioactive metabolites to label the proteins prior to extraction and autoradiograms of the 2^{nd} dimension gels were made. The proteome of starved planktonic cells contained 680 protein spots compared to 950 protein spots for the unstarved cells, 35 of these proteins were up regulated by starvation. The proteome of unstarved biofilm cells contained 860 protein spot

compared to 548 for starved biofilms. Unstarved biofilms exhibited up regulation for 25 proteins compared with planktonic cells and starved biofilms exhibited 14 up regulated proteins compared to planktonic cells.

Hefford et al. *(56)* studied the protein expression of *L. monocytogenes* biofilms grown on glass slides as compared to planktonic cells. They found 19 proteins were up regulated by growth as biofilms, and identified 8 proteins that were unaffected by growth as a biofilm. Surprisingly they found that flagellin was up regulated by growth as a biofilm. This is interesting, considering Trémoulet et al. *(153)* found flagellin was down regulated by growth as a biofilm at 20ºC, and that flagella are repressed by growth at 37ºC *(111)*, the temperature at which biofilms were grown.

Eight proteins were identified in at least two of these studies, lending a high degree of confidence in their involvement with biofilm formation. One was superoxide dismutase (SOD) which was up regulated in both starved planktonic cells and starved biofilm cells *(58)* as well as in biofilms grown on glass wool *(153)*. This may be because the biofilms grown on glass wool relied on diffusion to obtain glucose from the agar surface resulting in starvation similar to the other study. The other protein the studies had in common was a protein similar to DivIVA which is involved cell division in *Bacillus subtilis*. This protein was up regulated in unstarved biofilms grown on steel *(58)* as well as glass wool *(153)*.

Starvation of planktonic cells results in the up regulation of proteins involved in nutrient scavenging, metabolism and oxidative stress. Proteins up regulated in the biofilm state include metabolism, global regulators and protein repair. When the biofilms are starved, proteins involved with nucleotide metabolism, nutrient uptake, and amino acid metabolism are up regulated *(58)*. Other proteins that were up regulated in biofilms grown on glass wool involved

central metabolism, DNA repair, oxidative stress and global regulators *(153)*. Table 2.1 includes a detailed listing of proteins from these studies.

OTHER PROTEOMIC STUDIES OF *L. MONOCYTOGENES*

There have been several proteomic studies of various aspects of *L. monocytogenes* behavior. Most of these are recent and present reliable protein identifications. Table 2.1 lists all the identified proteins from these studies, and many cryptic ones. Four proteins have been identified in five to six different studies and merit discussion. These proteins are PdhD (subunit E3 of pyruvate dehydrogenase), MptA (Mannose specific PTS IIAB), Flagellin (FlaA), and elongation factor Tu (EF-Tu).

EF-Tu (TufA) transports aminoacyl-tRNA to the site of protein elongation on the ribosome *(20)*. TufA functions in other roles as well; it is associated with the cell membrane *(134, 165)*, functions as chaperonin *(20)* and is de-methylated at onset of starvation *(165)*. Proteomic studies have shown that TufA is up regulated by growth as a biofilm when non-starved *(56, 58)*, and salt stress *(33)*, found associated with the cell wall *(134)* and is unaffected upon entry into stationary phase *(42)*. The finding of TufA in numerous proteomic studies is likely due to a relative over abundance in the cell *(20)*, and involvement in stress responses.

PdhD is a component of the pyruvate dehydrogenase complex which initiates the first step in the Kreb's cycle; conversion of pyruvate, from glycolysis, to acetyl CoA *(64)*. Interestingly PdhD is repressed after 90 minutes of 6% salt stress in BHI *(34)* but expression is enhanced after 1hr salt stress in defined media *(33)*. The protein is up regulated by biofilm growth on glass wool *(153)*, deletion of the gene rpoN *(4)*, and entry into stationary phase *(42)*. PdhD is also found on the cell exterior *(134)*. The presence of PdhD in the cell wall subproteome and its ability to bind plasminogen may indicate that PdhD like EF-Tu, has

additional duties other than central metabolism *(134)*. MptA expression of is also affected by deletion of rpoN and involved in PTS transport of carbohydrates *(4)*. MptA is up regulated by 1 hour of salt stress in defined *(33)* media, down regulated in an rpoN mutant *(4)*, and is absent from strains resistant to class IIa bacteriocins *(52, 120)*. The gene rpoN encodes the alternate sigma factor σ^{54}, and mptA has been previously reported to be regulated in part by σ^{54} *(27)*. Taken with the fact that pdhD is likely regulated by σ^{54} *(4)*, These observations seem to indicate a major role for σ^{54} during salt stress and possibly certain aspects of biofilm accumulation of *L. monocytogenes*.

Interestingly the flaA protein which are the protein subunits of flagella, are reported as both up *(56)* and down *(153)* regulated by growth as a biofilm. FlaA is also reported to be down regulated in a mutant resistant to high hydrostatic pressure (piezotolerance) *(71)*, and unaffected by stationary phase *(42)*, and present in bacteriocin resistant mutants *(35)*. Proteomic studies of biofilms were done at different temperature and conditions of growth, and they suggest that flagella may be important to biofilm development under certain conditions. The piezotolerant mutant was found to have a mutation in ctsR, a regulator involved in heat shock and virulence, thus flaA is indirectly regulated by this regulatory element *(71)*.

ENVIRONMENTAL EPIDEMIOLOGY AND CONTROL OF PERSISTENT STRAINS

L. monocytogenes is often present throughout food processing environments, arriving in many diverse ways: Raw ingredients *(79, 105)*, transport containers *(28)*, and even equipment *(85)*. Epidemiological surveys of food processing areas often find that isolates of certain subtypes are found repeatedly for months or even years *(16, 40, 62, 86, 94, 105, 106, 138, 146)*; and these have become known as persistent strains. In one instance pallets used to transport raw fish into the processing plant were implicated in the spread of *L. monocytogenes* throughout the

environment *(127)*. An outbreak of listeriosis traced to cold smoked trout, was caused by a persistent subtype found in the processing plant packaging machine *(40)*. In another case a subtype was found to persist 7 years in an ice cream packaging machine and other equipment, and was the predominate product contaminant *(94)*. It has been concluded that persistent *L. monocytogenes* often become the dominant subtype contaminating the finished product *(86, 105, 138, 146)*.

The explanation of why certain subtypes colonize so well is elusive, but there is considerable evidence that some subtypes have become specially adapted for survival in certain environmental niches in food processing facilities *(5, 22, 79, 85, 138)*. This may occur due to equipment that is complex and difficult to clean, allowing bacteria to survive sanitation protocols *(85)*. Only 2 of 9 subtypes found in a shrimp processing plant were found in frozen packaged shrimp, indicating these strains have become adapted to freezing conditions *(28)*. In another instance it was believed that a dominate subtype is the source of other closely related types found in the plant that are not persistent *(94)*. Fortunately, once epidemiological studies of the distribution of *L. monocytogenes* subtypes demonstrate the existence of persistent subtypes, targeted cleaning can be utilized to eradicate them from the environment *(94)*. Equipment modifications, and procedural changes were able to eradicate *Listeria* from the environment *(94, 146)*. In another case extreme cleaning (heating in hot water, hot air , or gas flame, and live steam application to walls) measures targeted to the colonized areas eliminated persistent *L. monocytogenes* subtypes *(5)*.

Table 2.1: Proteins reported and characterized in proteomic studies of *Listeria monocytogenes*: Including function/similarity, size (kDa), isoelectric point (pI), and conditions of expression.

Protein Function/Similarity	Gene Name[1]	Size (kDa)	pI	Source	Conditions of expression
Proteins associated with metabolism					
Branched-chain alpha-keto acid dehydrogenase E1 subunit	LMO1372	42.7	4.8	Folio et al. *(42)*	↑Entry into stationery phase
Protein required for pyridoxine synthesis	LMO2101	37.8	5.1	Folio et al. *(42)*	↑Entry into stationery phase
Catabolite control protein	CcpA	40.5	5.12	Duché et al. *(33)*	↑60 minutes of salt stress
	CcpA	42.6	5.45	Folio et al. *(42)*	Unaffected by entry into stationery phase
Enolase	eno	46.5	4.7	Hefford et al. *(56)*	↑Biofilm growth
	eno	47.3	4.7	Folio et al. *(42)*	Unaffected by entry into stationery phase
	eno	43	4.6	Schaumburg et al. *(134)*	Cell wall associated Protein
Phosphoglyceromutase 1	LMO2205	28.2	6.3	Helloin et al. *(58)*	↑Glucose starvation (planktonic cells)
Phosphoglycerate mutase	LMO0907	27	6.3	Helloin et al. *(58)*	↑Biofilms not glucose starved
	pgm	57.3	4.97	Duché et al. *(33)*	↓30 minutes of salt stress
	pgm	56.1	5.1	Hefford et al. *(56)*	↑Biofilm growth
	pgm	62	5.1	Folio et al. *(42)*	↓Entry into stationery phase
	pgm	76	-	Schaumburg et al. *(134)*	Cell wall associated Protein
Triose phosphate isomerase	tpi	26.9	4.8	Hefford et al. *(56)*	↑biofilm growth
	tpi	29.1	4.7	Folio et al. *(42)*	Unaffected upon entry into stationery phase
Phosphoglycerate kinase	pgk	42.1	5.0	Hefford et al. *(56)*	↑by biofilm growth
	pgk	46	4.9	Folio et al. *(42)*	Unaffected by entry into stationery phase
	pgk	42.0	-	Schaumburg et al. *(134)*	Cell wall associated Protein
Glyceraldehyde 3-phosphate dehydrogenase (GADPH)	gap	48.9	5.07	Duché et al. *(33)*	↑30minutes of salt stress
	gap	36.3	5.2	Hefford et al. *(56)*	↑Biofilm growth
	gap	43.8	5.3	Folio et al. *(42)*	Unaffected upon entry into stationery phase
	gap	42.0	5.2	Schaumburg et al. *(134)*	Cell wall associated Protein
Fructose-1,6-bisphosphate aldolase	FbaA	30.1	5.2	Hefford et al. *(56)*	↑Biofilm growth
	FbaA	33.1	5.1	Folio et al. *(42)*	Unaffected by entry into stationery phase
	FbaA	30.0	5.5	Schaumburg et al. *(134)*	Cell wall associated Protein
Transketolase	tkt	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
Transaldolase	LMO2743	28	4.9	Folio et al. *(42)*	↑Entry into stationery phase

[1] Accession numbers when reported are cross referenced to the ListiList gene name (http://genolist.pasteur.fr/ListiList/)

Protein Function/Similarity	Gene Name[1]	Size (kDa)	pI	Source	Conditions of expression
Hypothetical dihydroxyacetone kinase	LMO2696	25.9	5.1	Folio et al. *(42)*	↑Entry into stationery phase
Pyruvate dehydrogenase (E1 β-subunit)	PdhB	34.7	5.7	Helloin et al. *(58)*	↑Glucose starved planktonic cells
	PdhB	49.49	4.51	Arous et al. *(4)*	↑RpoN deletion
	PdhB	41.5	4.8	Folio et al. *(42)*	Unaffected by entry into stationery phase
	PdhB	37.0	4.6	Schaumburg et al. *(134)*	Cell wall associated Protein
Dihydrolipoamide dehydrogenase, E3 subunit of pyruvate dehydrogenase complex	PdhD	57.6	4.9	Trémoulet et al. *(153)*	↑ Biofilm growth (glass fiber filters)
	PdhD	58.8	4.92	Duché et al. *(34)*	↓Salt stress (BHI)
	PdhD	54.9	5.09	Duché et al. *(33)*	↑60 minutes of salt stress
	PdhD	49.48	5.0	Arous et al. *(4)*	↑RpoN deletion
	PdhD	58.4	5.2	Folio et al. *(42)*	↑Entry into stationery phase
	PdhD	54.0	-	Schaumburg et al. *(134)*	Cell wall associated Protein
Glycerol-3-phosphate dehydrogenase	GlpD	66.3	6.4	Folio et al. *(42)*	↑Entry into stationery phase
Phosphotransacetylase	Pta	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
Propanediol utilization protein (PduL)	-	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
Pyruvate phosphate dikinase	LMO1867	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
Tagatose-1,6-diphosphate aldolase	LMO0539	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
Glycine cleavage system protein H	LMO2425	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
Pyruvate dehydrogenase (E1 α-subunit)	pdhA	47	5.8	Duché et al. *(33)*	↑60 minutes of salt stress
	pdhA	45	6.1	Folio et al. *(42)*	Unaffected by entry into stationery phase
Alanine dehydrogenase	LMO1579	49.0	5.09	Duché et al. *(33)*	↑30minutes of salt stress
	LMO1579	39.61	5.08	Arous et al. *(4)*	↑RpoN deletion
	LMO1579	44.7	5.2	Folio et al. *(42)*	Unaffected by entry into stationery phase
NADP-specific glutamate dehydrogenase	LMO0560	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
Acetate kinase	ackA	43.3	5.1	Duché et al. *(34)*	↓Salt stress (defined media)
	ackA	46.5	5.4	Folio et al. *(42)*	Unaffected by entry into stationery phase
Yeast protein-Fatty acid signaling (Frm2p)	LMO2829	21.6	5.5	Helloin et al. *(58)*	↑Glucose starvation (planktonic cells)
	LMO2829	22.2	4.44	Arous et al. *(4)*	↓RpoN deletion
	LMO2829	26.6	4.7	Folio et al. *(42)*	Unaffected by entry into stationery phase

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Protein Function/Similarity	Gene Name[1]	Size (kDa)	pI	Source	Conditions of expression
6-phophpfructokinase	pfk pfk pfk	35.1 34.42 39.4	5.3 5.46 5.6	Trémoulet et al. *(153)* Gravesen et al. *(52)* Folio et al. *(42)*	↑Biofilm growth (glass fiber filters) Class IIa bacteriocin resistance (leucocin) Unaffected by entry into stationery phase
Thioredoxin reductase	BAA08961[2] trxB	19.7 37.3	5.65 4.7	Phan-Thanh et al.*(114)* Folio et al. *(42)*	↑Low pH (3.5 and 5.5) Unaffected by entry into stationery phase
H+-transporting ATP synthase chain β	atpD	53.3	4.7	Folio et al. *(42)*	Unaffected by entry into stationery phase
H+-transporting ATP synthase chain b	atpF	19.4	5.0	Hefford et al. *(56)*	↑Biofilm growth
Flavocytochrome c fumarate reductase subunit a	LMO0355	54.5	5.7	Hefford et al. *(56)*	↑Biofilm growth
Lactate dehydrogenase	ldh ldh	34.19 38	5.05 5.3	Arous et al. *(4)* Folio et al. *(42)*	↑RpoN deletion Unaffected by entry into stationery phase
Aspartate-semialdehyde dehydrogenase	LMO1437			Folio et al. *(42)*	↓Entry into stationery phase
Phage proteins					
Protein gp20 [Bacteriophage A118]	LMO0127	23.5	6.3	Folio et al. *(42)*	↑Entry into stationery phase
Putative tape-measure [Bacteriophage A118]	LMO2287			Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
Coat protein [Bacteriophage SPP1]	LMO2296			Trost et al. *(154)*	Supernatant protein (not found in *L. innocua*)
Putative scaffolding protein [Bacteriophage A118]	LMO2297			Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
Biosynthesis					
Cysteine synthase	cysK cysK cysK cysK	32.2 36 33.1 33.0	5.3 5.3 5.11 5.7	Hefford et al. *(56)* Folio et al. *(42)* Duché et al. *(33)* Schaumburg et al. *(134)*	Unaffected by biofilm growth Unaffected by entry into stationery phase ↑30minutes of salt stress Cell wall associated Protein
Thymidylate kinase	LMO2693	23.1	5.1	Hefford et al. *(56)*	↑Biofilm growth
Phosphomethylpyrimidine kinase	thiD	28.8	5.3	Hefford et al. *(56)*	↑Biofilm growth
Adenylosuccinate lyase	purB	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)

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[2] NCBI accession number

Protein Function/Similarity	Gene Name[1]	Size (kDa)	pI	Source	Conditions of expression
Phosphoribosylaminoimidazole carboxylase I	purE	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
Bifunctional phosphoribosylaminoimidazole carboxy formyl formyltransferase and inosine-monophosphate cyclohydrolase	purH	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
Transport related proteins					
Potassium-transporting ATPase b chain	kdpB	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
ABC transporter	LMO0541	33.9	6	Helloin et al. *(58)*	↑Glucose starvation (planktonic cells)
	LMO1671	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
	LMO1671	39.0	4.8	Schaumburg et al. *(134)*	Cell wall associated protein
	LMO1847	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
	LMO1847	33	5.3	Schaumburg et al. *(134)*	Cell wall associated protein
	LMO2349	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
	LMO2415	31.6	5.5	Helloin et al. *(58)*	↑ Glucose starvation (planktonic cells)
	LMO2415	33.1	4.6	Folio et al. *(42)*	Unaffected by entry into stationery phase
	LMO2415	32.0	6.5	Schaumburg et al. *(134)*	Cell wall associated protein
Fructose-specific PTS enzyme IIB	LMO0399	13.3	7.8	Folio et al. *(42)*	↑Entry into stationery phase
Mannose specific PTS IIB	LMO0783	15.1	6.1	Helloin et al. *(58)*	↑Glucose starvation (planktonic cells)
	LMO0783	19.9	6.2	Folio et al. *(42)*	Unaffected by entry into stationery phase
Mannose specific PTS IIAB (MptA)	LMO0096	34.9	5.11	Duché et al. *(33)*	↑60 minutes of salt stress
	LMO0096	34.99	5.32	Arous et al. *(4)*	↓RpoN deletion
	LMO0096	34.99	5.32	Gravesen et al. *(52)*	Absent class IIa bacteriocins resistant strains
	LMO0096	35	-	Ramnath et al. *(120)*	Absent class IIa bacteriocins resistant strains
	LMO0096	38.9	5.3	Folio et al. *(42)*	Unaffected by entry into stationery phase
Acetate kinase (AppA) oligopeptide transport	LMO0135	55.7	4.73	Duché et al. *(34)*	Higher expression in defined media than BHI
Histidine-containing phosphocarrier protein (HPr/ptsH)	ptsH	9.4	4.8	Hefford et al. *(56)*	Unaffected by biofilm growth
	ptsH	10.9	4.7	Folio et al. *(42)*	Unaffected by entry into stationery phase
Cell division/transport of proteins	ftsE	29	6.2	Helloin et al. *(58)*	↑Glucose Starvation (biofilm)

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Protein Function/Similarity	Gene Name[1]	Size (kDa)	pI	Source	Conditions of expression
Proteins associated with starvation					
ATP dependant protease subunit	clpP	20.4	5.8	Helloin et al. *(58)*	↑Glucose starvation (planktonic)
	clpP	20.4	5.8	Helloin et al. *(58)*	↑Presence of glucose (biofilm)
	clpP	25.9	4.9	Folio et al. *(42)*	Unaffected by entry into stationery phase
	clpP			Karatsaz et al.*(71)*	↑Piezotolerant mutant
	LMO1138	26.4	4.9	Folio et al. *(42)*	Unaffected by entry into stationery phase
Cell division (SpoVG)	LMO0196	13.8	5.3	Helloin et al. *(58)*	↑Glucose starvation (planktonic cells)
	LMO0196	13.2	4.5	Folio et al. *(42)*	Unaffected by entry into stationery phase
	LMO0197	12.5	4.6	Folio et al. *(42)*	Unaffected by entry into stationery phase
	LMO0197	11.4	4.6	Hefford et al. *(56)*	Unaffected by biofilm growth
	LMO0197	20	4.1	Schaumburg et al. *(134)*	Cell wall associated protein
Nucleotide metabolism (Deoxyribose-phosphate aldolase)	dra	25.7	6.1	Helloin et al. *(58)*	↑Glucose starvation (planktonic/biofilm)
	dra	50.4	4.8	Folio et al. *(42)*	Unaffected by entry into stationery phase
Nucleotide metabolism (Uracil phophoribosyl transferase)	upp	25.1	6.4	Helloin et al. *(58)*	↑Glucose starvation (planktonic/biofilm)
Protein associated with various stresses					
Lipase	LMO0950	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
Generic Chaperonin	AAB84724[2]	60.4	4.73	Phan-Thanh et al.*(114)*	↑Low pH (3.5 and 5.5)
Similar to Universal stress protein (UspA)	LMO1580	16.9	5.0	Hefford et al. *(56)*	↑Biofilm growth
	LMO1580	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
Alcohol dehydrogenase (Glycine Betaine synthesis)	CAB15083[3]	43.5	5.08	Phan-Thanh et al.*(114)*	↑Low pH (3.5 and 5.5)
Heat-shock protein (htrA serine protease)	LMO0292	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
	LMO0292	38.0	4.3	Schaumburg et al. *(134)*	Cell wall associated protein
Chaperonin/Heat shock (GroEL)	AAB84724[4]	56.7	4.84	Phan-Thanh et al.*(114)*	↑Low pH (3.5 and 5.5)
	groEL	57.4	4.7	Hefford et al. *(56)*	↑Biofilm growth
	groEL	65.1	4.7	Folio et al. *(42)*	Unaffected by entry into stationery phase
	groEL	52.0	4.6	Schaumburg et al. *(134)*	Cell wall associated protein
Cold Shock protein	cspB	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)

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[2] NCBI accession number
[3] NCBI accession number
[4] NCBI accession number

Protein Function/Similarity	Gene Name[1]	Size (kDa)	pI	Source	Conditions of expression
Sulphatase/phosphatase	LMO0644	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
GCN5-related *N*-acetyltransferase	LMO0395	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
Chaperonin/Heat shock (GroES)	groES	10	4.6	Hefford et al. *(56)*	Unaffected by biofilm growth
	groES	8.9	4.6	Folio et al. *(42)*	↑Entry into stationery phase
Transcription regulator, RpiR family	LMO2795	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
Glycine Betaine transporter	-	43	5.93	Duché et al. *(33)*	↑60 minutes of salt stress
	-	-	-	Duché et al.*(34)*	↑Salt stress in BHI
	gbuA	46.7	6.3	Folio et al. *(42)*	Unaffected by entry into stationery phase
Superoxide dismutase	-	25.9	4.9	Trémoulet et al. *(153)*	↑Biofilm growth (glass fiber filters)
	sod	25.7	5.4	Helloin et al. *(58)*	↑Glucose starvation (planktonic/biofilm)
	sod	26.5	5.2	Folio et al. *(42)*	Unaffected by entry into stationery phase
	sod	23.0	5.6	Schaumburg et al. *(134)*	Cell wall associated protein
Chaperonin/Heat shock (DnaK)	-	66.2	4.52	Duché et al. *(33)*	↑30minutes of salt stress
	dnaK	66.1	4.6	Hefford et al. *(56)*	Unaffected by biofilm growth
	dnaK	69.9	4.6	Folio et al. *(42)*	Unaffected by entry into stationery phase
	dnaK	55.0	4.4	Schaumburg et al. *(134)*	Cell wall associated protein
Ribosomal protein (YvyD)	-	25.1	4.9	Trémoulet et al. *(153)*	↑Biofilm growth (glass fiber filters)
	-	25.8	5.1	Duché et al. *(34)*	Higher expression in defined media than BHI
	LMO2511	25	5.2	Folio et al. *(42)*	Unaffected by entry into stationery phase
	LMO2511	21.0	-	Schaumburg et al. *(134)*	Cell wall associated protein
Transcription regulator, MerR family	LMO0526	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
General stress protein (Ctc)	-	30.9	4.38	Duché et al. *(34)*	Higher expression in defined media than BHI
	ctc	30.7	4.32	Duché et al. *(33)*	30minutes of salt stress
	ctc	30.6	4.5	Folio et al. *(42)*	Unaffected by entry into stationery phase
Ferric uptake regulator (fur)	-	17.8	6.19	Phan-Thanh et al.*(114)*	↑Low pH (5.5)
	fur	16.6	6.6	Helloin et al. *(58)*	↑Glucose starvation (planktonic cells)
	fur	21.5	6.2	Folio et al. *(42)*	Unaffected by entry into stationery phase
Non heme ferritin (dpr)	-	18	5.1	Hébraud et al.*(55)*	↑Cold shock and heat shock
	fri	19.6	4.9	Folio et al. *(42)*	↑Entry into stationery phase
	fri	19.6	4.9	Folio et al. *(42)*	↓Entry into stationery phase

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Protein Function/Similarity	Gene Name[1]	Size (kDa)	pI	Source	Conditions of expression
Inosine-5'-monophosphate dehydrogenase	guaB guaB	54 58.4	5.8 6.1	Duché et al. *(33)* Folio et al. *(42)*	↑60 minutes of salt stress Unaffected by entry into stationery phase
Peptidly-prolyl cis-trans isomerase (protein folding)	LMO2376	24.5	5.5	Helloin et al. *(58)*	↑Presence of glucose (biofilm)
Salt stress protein in *Bacillus subtilis (61)* (yhfK)	LMO2391	25.7	6.6	Helloin et al. *(58)*	↑Glucose starvation (planktonic cells)
Cell Envelope					
Cell division initiation protein	- divIVA divIVA	23.9 30 23.6	4.6 5.6 4.7	Trémoulet et al. *(153)* Helloin et al. *(58)* Folio et al. *(42)*	↑Biofilm growth (glass fiber filters) ↑non-starved biofilm Unaffected by entry into stationery phase
Probable septum formation initiator	LMO0217	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
Cell division	ftsZ ftsZ	41.4 47.7	4.8 4.8	Hefford et al. *(56)* Folio et al. *(42)*	↑Biofilm growth Unaffected by entry into stationery phase
Cell wall shape	mreB	35.5	5.2	Hefford et al. *(56)*	↑Biofilm growth
Flagellin protein	- - - flaA flaA	30.4 31.6 30.4 33.6 -	4.5 4.7 4.9 4.7 -	Duffes et al*(35)* Trémoulet et al. *(153)* Hefford et al. *(56)* Folio et al. *(42)* Karatsaz et al.*(71)*	Present in Divercin V41 resistant strain ↓Biofilm growth (glass fiber filters) ↑Biofilm growth Unaffected by entry into stationery phase ↓Piezotolerant mutant
Flagellum biosynthesis protein (FlhA)	LMO0680	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua)*
Unknown lipoproteins	LMO2416 LMO2417 LMO2636 LMO2637 LMO2637 LMO2637	- 41.0 35.5 30 34	- 5.1 5.8 5.2 4.9	Trost et al. *(154)* Schaumburg et al. *(134)* Schaumburg et al. *(134)* Helloin et al. *(58)* Hefford et al. *(56)* Schaumburg et al. *(134)*	Supernatant protein (not expressed/*L. innocua)* Cell wall associated protein Cell wall associated protein ↑Glucose starvation (planktonic cells) Unaffected by biofilm growth Cell wall associated protein
CD4+ T cell-stimulating antigen, lipoprotein	tcsA tcsA	38.4 39.0	5.0 4.5	Hefford et al. *(56)* Schaumburg et al. *(134)*	Unaffected by biofilm growth Cell wall associated protein
Tetradihydropicolinate succinylase	LMO1011 LMO1011	24.8 30.6	4.6 5.5	Hefford et al. *(56)* Folio et al. *(42)*	↑Biofilm growth Unaffected by entry into stationery phase

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Protein Function/Similarity	Gene Name[1]	Size (kDa)	pI	Source	Conditions of expression
Teichoic acids biosynthesis protein (GgaB)	LMO1080	-	-	Trost et al. *(154)*	Supernatant protein (not found in *L. innocua*)
Autolysin (GW motif)	LMO1076	-	-	Trost et al. *(154)*	Supernatant protein (not found in *L. innocua*)
Penicillin-binding protein (Pbp)	-	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
Peptidoglycan anchored protein (LPXTG-motif)	LMO0880	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
D-alanyl-D-alanine carboxypeptidase (penicillin-binding protein 5)	LMO2754	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua)*
Carboxy-terminal processing proteinase	LMO1851	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua)*
Chitinase	LMO1883	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua)*
Acyltransferase	LMO1291 LMO1291	- 23.0	- 6.0	Trost et al. *(154)* Schaumburg et al. *(134)*	Supernatant protein (not expressed/*L. innocua)* Cell wall associated protein
Glycosyl transferase	LMO2550	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
N-acetylmuramidase (GW motif)	LMO2591	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua)*
Protein synthesis					
Translation elongation factor EF-Tu	tufA - tufA tufA tufA	52.5 45.2 43.3 47.7 45.0	5.3 4.81 4.8 4.9 4.8	Helloin et al. *(58)* Duché et al. *(33)* Hefford et al. *(56)* Folio et al. *(42)* Schaumburg et al. *(134)*	↑Presence of glucose (biofilm) ↑60 minutes of salt stress ↑Biofilm growth Unaffected by entry into stationery phase Cell wall associated protein
Translation elongation factor EF-Ts	tsf tsf tsf	32.6 41.6 37.0	5.1 5.1 -	Hefford et al. *(56)* Folio et al. *(42)* Schaumburg et al. *(134)*	↑Biofilm growth Unaffected by entry into stationery phase Cell wall associated protein
Transcription elongation factor (GreA)	LMO1496 LMO1496	19.5 22.8	5.5 4.6	Helloin et al. *(58)* Folio et al. *(42)*	↑Presence of glucose (biofilm) ↓Entry into stationery phase
30s ribosomal protein S6	- rpsF rpsF	11.8 8.8 -	4.8 5.0 -	Duché et al. *(34)* Folio et al. *(42)* Trost et al. *(154)*	↑Salt stress (BHI) ↓Upon entry into stationery phase Supernatant protein (not expressed/*L. innocua*)
30s ribosomal protein S2	- rpsB	35.2 36.4	5.7 6.0	Trémoulet et al. *(153)* Folio et al. *(42)*	↑ Biofilm growth (glass fiber filters) Unaffected by entry into stationery phase

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Protein Function/Similarity	Gene Name[1]	Size (kDa)	pI	Source	Conditions of expression
50S ribosomal protein L7/L12	rplL rplL	12.5 10.5	4.5 4.5	Hefford et al. *(56)* Folio et al. *(42)*	Unaffected by biofilm growth Unaffected by entry into stationery phase
Ribosomal protein L4	rplD	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
50S ribosomal protein L10	rplJ	22	5.3	Folio et al. *(42)*	↓Entry into stationery phase
50S ribosomal protein L31type B	rpmE	9.7	7.8	Folio et al. *(42)*	↑Entry into stationery phase
Virulence proteins					
Actin-assembly inducing protein	actA			Trost et al. *(154)*	Supernatant protein (not found in *L. innocua*)
Listeriolysin O	hly hly	- 60.3	- 5.9	Trost et al. *(154)* Folio et al. *(42)*	Supernatant protein (not found in *L. innocua*) ↑Upon entry into stationery phase
Internalin A (LPXTG-motif)	inlA			Trost et al. *(154)*	Supernatant protein (not found in *L. innocua*)
Internalin B (GW motif)	inlB inlB	- 70/64	- -	Trost et al. *(154)* Schaumburg et al. *(134)*	Supernatant protein (not found in *L. innocua*) Cell wall associated protein
Internalin C	inlC			Trost et al. *(154)*	Supernatant protein (not found in *L. innocua*)
Zinc metalloproteinase	mpl			Trost et al. *(154)*	Supernatant protein (not found in *L. innocua*)
Phosphatidylinositol-specific phospholipase C	plcA			Trost et al. *(154)*	Supernatant protein (not found in *L. innocua*)
Phospholipase C	plcB			Trost et al. *(154)*	Supernatant protein (not found in *L. innocua*)
Miscellaneous proteins					
Hypothetical protein 3C	S27527[2]	19.2	5.12	Phan-Thanh et al.*(114)*	↑Low pH (3.5 and 5.5)
Similar to arginine repressor	LMO1367			Folio et al. *(42)*	↑Entry into stationery phase
Two-component sensor histidine kinase	LMO1061	-	-	Trost et al. *(154)*	Supernatant protein (not found in *L. innocua*)
Transcription regulator, LytR Family	LMO0443 LMO0443	- 25.0	- 6.2	Trost et al. *(154)* Schaumburg et al. *(134)*	Supernatant protein (not expressed/*L. innocua*) Cell wall associated protein
Pyrimidine operon regulatory protein	pyrR	27.2	6.5	Folio et al. *(42)*	↑Entry into stationery phase
Formylmethionine deformylase and *B. subtilis* YkrB protein	LMO1051	24.0	5.1	Folio et al. *(42)*	↑Entry into stationery phase
Regulation	codY codY codY	18.2 28.7 31.9	5.8 4.9 4.9	Helloin et al. *(58)* Hefford et al. *(56)* Folio et al. *(42)*	↑Presence of glucose (biofilm) ↑Biofilm growth Unaffected by entry into stationery phase

[1] Accession numbers when reported are cross referenced to the ListiList gene name (http://genolist.pasteur.fr/ListiList/)
[2] NCBI accession number

Protein Function/Similarity	Gene Name[1]	Size (kDa)	pI	Source	Conditions of expression
Branched chain AA amino transferase	LMO0978	16.6	6.4	Helloin et al. *(58)*	↑Glucose starvation (biofilm)
yslB (Unknown *Bacillus subtilis* protein)	LMO1236	17	5.7	Helloin et al. *(58)*	↑Presence of glucose (biofilm)
RecO	-	32	5.8	Trémoulet et al. *(153)*	↑Biofilm growth (glass fiber filters)
Phosphpopentomutase (Drm)	- LMO1954	43.74 50.4	4.6 4.8	Arous et al. *(4)* Folio et al. *(42)*	↑RpoN deletion Unaffected by entry into stationery phase
Purine-nucleoside phosphorylase (Pnp/deoD)	- deoD	29.47 27.1	4.59 4.8	Arous et al. *(4)* Folio et al. *(42)*	↑RpoN deletion Unaffected by entry into stationery phase
Hypothetical metalloprotease (ypwA)	P50848[2]	58.2	4.95	Phan-Thanh et al.*(114)*	↑Low pH (3.5)
DNA-directed RNA polymerase omega chain (rpoZ)	LMO1826	7.2	7.8	Folio et al. *(42)*	↑Upon entry into stationery phase
Bacillus Sigma H	-	25.3	5.62	Phan-Thanh et al.*(114)*	↑Low pH (3.5)
Transcriptional regulators	-	16.8 19.7	6.42 4.87	Phan-Thanh et al.*(114)* Phan-Thanh et al.*(114)*	↑Low pH (3.5 and 5.5) ↑Low pH (3.5)
Transcriptional regulator Yfiv	-	18.2	6.45	Phan-Thanh et al.*(114)*	↑Low pH (5.5)
dTDP-4-dehydrorhamnose3,5-epimerase	-	22.1	5.6	Phan-Thanh et al.*(114)*	↑Low pH (3.5 and 5.5)
Peptidoglycan linked protein (LPxTG motif)	LMO1666	-	-	Trost et al. *(154)*	Supernatant protein (not found in *L. innocua*)

[1] Accession numbers when reported are cross referenced to the ListiList gene name (http://genolist.pasteur.fr/ListiList/)
[2] NCBI accession number

Protein Function/Similarity	Gene Name[1]	Size (kDa)	pI	Source	Conditions of expression
Unknown proteins	LMO1830	21.9	6.5	Helloin et al. *(58)*	↑Glucose starvation (planktonic cells)
	LMO1771	17.2	5.6	Helloin et al. *(58)*	↑Glucose starvation (planktonic cells)
	LMO0900	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
	LMO0796	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
	LMO0796	17	5.6	Helloin et al. *(58)*	↑Glucose starvation (planktonic cells)
	LMO0796	22.3	4.7	Folio et al. *(42)*	Unaffected by entry into stationery phase
	LMO2223	11.5	5	Helloin et al. *(58)*	↑Presence of glucose (biofilm)
	LMO2223	11.3	4.3	Folio et al. *(42)*	Unaffected by entry into stationery phase
	LMO1602	16.2	6.2	Helloin et al. *(58)*	↑Glucose starvation (biofilm)
	LMO2256	24.2	5.3	Folio et al. *(42)*	↑/↓Entry into stationery phase
	LMO0775	14.2	7.8	Folio et al. *(42)*	↑Entry into stationery phase
	LMO1125	-	-	Trost et al. *(154)*	Supernatant protein (not found in *L. innocua*)
	LMO2410	-	-	Trost et al. *(154)*	Supernatant protein (not found in *L. innocua*)
	LMO1715	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
	LMO2156	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
	LMO1395	-	-	Trost et al. *(154)*	Supernatant protein (not expressed/*L. innocua*)
		30	6.56	Phan-Thanh et al.*(114)*	↑Low pH (5.5)

[1] Accession numbers when reported are cross referenced to the ListiList gene name (http://genolist.pasteur.fr/ListiList/)

CHAPTER 3

FORMATION OF BIOFILM AT DIFFERENT NUTRIENT LEVELS BY VARIOUS SUBTYPES OF *LISTERIA MONOCYTOGENES*[18]

[18] Folsom, JP and Frank, JF 2005. To be submitted to the Journal of Food Protection

ABSTRACT

Strains of *Listeria monocytogenes* exhibit a range of ability to form biofilms. The objectives of this study were to determine if genetically related strains exhibit similar biofilm-forming capacity, and the effect nutrient concentration has on the ability of different strains to produce biofilm. Biofilms of 30 strains of *L. monocytogenes,* obtained from a variety of sources, were grown on stainless steel in tryptic soy broth (TSB) or a 1:10 dilution of TSB (DTSB) for 24 h at 32ºC. The amount of biofilm formed was determined using image analysis after staining the cells with bisBenzimide H 33258 (Hoechst 33258). The strains were genetically subtyped by repetitive element sequence-based PCR (rep-PCR) using the primer sets rep-PRO_{Dt} and rep-PRO_{G5}. Data were analyzed by using ANOVA and Duncan's multiple range test. Eleven strains produced the same amount of biofilm in the two media. Fourteen strains produced more biofilm in TSB than DTSB. Five strains produced more biofilm in DTSB than TSB. Serotype 4b strains produced more biofilm accumulation in TSB than serotype 1/2a strains, while serotype 1/2a strains produced more in DTSB than did serotype 4b strains. Growth in DTSB resulted in decreased biofilm accumulation for serotype 4b strains. There was no correlation between genetic subtype and the amount of biofilm accumulation. These results indicate that serotype 1/2a and serotype 4b strains differ in the regulation of their biofilm phenotype. The poor biofilm accumulation of serotype 4b isolates when grown in DTSB could be a factor in the predominance of serogroup 1/2 strains in food processing plants, where nutrients may be limited.

INTRODUCTION

Strains of *Listeria monocytogenes* exhibit a diverse capacity to produce biofilm that largely depends on the growth conditions and media as well as strain. Previous research has produced conflicting results regarding the ability of *L. monocytogenes* to produce biofilms, with

reports ranging from none *(133)* to prolific *(25)* biofilm production. *L. monocytogenes* also exhibits strain variation in the amount of biofilm formed when grown on tryptic soy broth or modified Welshimer's broth. Some strains produce more biofilm in one media whereas others produced more biofilm in another media *(97)*. Additionally, one study found that lineage I produced more biofilm than lineage II when grown in modified Welshimer's broth *(31)*, whereas another study found that lineage II strains produced more biofilm than lineage I strains when grown in the same media using a similar procedure *(13)*.

It is apparent that the amount of biofilm produced by strains of *L. monocytogenes* is affected by growth conditions and strain variation; however there is further complexity in biofilm formation behavior. Strains that achieve the same cell density in older biofilms exhibit differing biofilm growth kinetics in the early stages of biofilm growth *(24)*. Even though some strains attach to surfaces more effectively than others, this does not mean they will produce more biofilm after attachment, and even strains from a similar source do not exhibit similar biofilm behavior *(70)*. The biofilms of some strains are more difficult to remove by swabbing than other strains *(24)*, possibly causing reduced estimates of their prevalence by environmental studies. Lower nutrient conditions may stimulate biofilm production by *Listeria (67, 126)*, whereas other data suggests that starvation does not affect biofilm production *(133)*. Blackman and Frank *(12)* found that the use of lower nutrient conditions for biofilm growth reduced the amount of biofilm produced on several surfaces, but growth on Teflon at 21ºC was unaffected, whereas, at 10ºC, growth on Teflon was reduced at low nutrient levels even though biofilm growth on other surfaces was greater.

This currently reported research aims to determine the effect of nutrient level on the biofilm production of different strains of *L. monocytogenes* and to assess whether genetically similar isolates of diverse origin have similar biofilm forming capacity.

MATERIALS AND METHODS

Strains. Thirty strains of *L. monocytogenes* utilized in this project are described in Table 3.1. These strains included food outbreak, processing plant environment, and Yerkes National Primate Center environmental and animal clinical isolates. The cultures were maintained on Microbank™ cryogenic storage beads (Pro-Lab diagnostics, Ontario Canada) at –70°C and were prepared by transferring one bead to five mls of the appropriate media for each experiment. Cultures were subcultured three times before use because Briandet et al. *(17)* found that the surface properties of *L. monocytogenes* became more uniform with successive serial transfers. Each strain was prepared from beads prior to each experiment because additional transfers could lead to phenotypic adaptations associated with laboratory maintenance *(137)* that could affect biofilm formation. Cultures were incubated statically at 32ºC and used or transferred after 20 h.

Biofilm formation. New coupons (2x5 cm) of stainless steel (type 304, finish 4b) were degreased in acetone, sonicated for 60 minutes at 55ºC in alkali detergent (Micro®, International Products Corp., Burlington, N.J.). After an additional 16 to 20 h, they were removed from the detergent solution and rinsed with deionized water, sonicated for 20 min in Zep formula 3586 (30 ml/L; Zep, Atlanta, Ga.) a commercial phosphoric acid-based cleaner, or phosphoric acid (15 ml/L), and rinsed again in deionized water. After cleaning coupons were autoclaved in deionized water.

Sterile coupons were placed into test tubes (1,500 x 25 mm) and submerged in a 20 h culture of *L. monocytogenes* grown on Bacto tryptic soy broth (TSB; Becton, Dickinson and

Company, Sparks, Md.) or diluted (1:10) tryptic soy broth (DTSB). Coupons were incubated 4 h at 32°C, vigorously agitated in 50 ml of sterile phosphate buffer (0.015M KH_2PO_4, pH 7) using sterile tongs and transferred to fresh media. After 24 h of incubation, the coupons are again rinsed in phosphate buffer and then analyzed. Negative controls underwent the same treatment with no inoculation.

Growth Curves. Liquid culture aliquots (50 µl) of each individual strain were used to inoculate 5 ml of culture media. The absorbance at 600 nm was measured hourly with a DU 350 spectrophotometer (Beckman, Fullerton, Calif.) until stationery phase, and then again the next day. Growth curves were obtained using TSB and DTSB with incubation at 32ºC.

Repetitive Element Sequence–Based Rep PCR. Listeria cultures were prepared for rep-PCR after growth on TSB at 37ºC for 18 h and streaking for isolation on tryptic soy agar (TSA; Becton, Dickinson and Company). After incubation at 37ºC for 18 h, isolated colonies were selected, and duplicate lawns on TSA were prepared using the same incubation conditions, the resulting cells were suspended in 2 ml of phosphate buffered saline (PBS; Gibco BRL, Rockville, Md.) and washed twice in PBS. Genomic DNA was extracted using an UltraClean microbial DNA isolation kit according to supplier's instructions (Mo Bio Laboratories, Inc, Solana Beach, Calif.). DNA concentration was determined using GeneQuant spectrophotometer (Amersham Biosciences, Piscataway, N.J.). PCR was performed using RepPRO DNA fingerprinting kits with primers rep-PRO_{G5} [G5] and rep-PRO_{Dt} [Dt] with AmpliTaq® DNA polymerase (Applied Biosystems, Foster City, Calif.) and a MJ Research PTC 225 thermocycler (MJ Research, Watertown, Mass.) according to supplier instructions (Bacterial Barcodes Inc, Houston Tex.). PCR amplicons were separated by electrophoresis in 1.5% (w/v) Seakem LE agarose (Cambrex Corporation, East Rutherford, N.J.) gels with a 1x TAE buffer system.

Electrophoresis was carried out in a Bio-Rad subcell model 192 with circulated buffer (Bio-Rad Laboratories, Hercules, Calif.). Gel images were captured with a FluorChem 8000 (Alpha Innotech, San Leandro, Calif.). Duplicate PCR reactions were done, and amplicons from both reactions were run in separate lanes on the same gel.

Pearson product moment correlation coefficient (Pearson) and cluster analysis were calculated by the unweighted pair-group method by the arithmetic averages (UPGMA) method within the Bionumerics version 2.5 (Applied Maths, Inc, Austin, Tex.) software package. To obtain a dendrogram using fingerprints from both primer sets, the similarity matrices were averaged and a dendrogram was produced by UPGMA. Bionumerics settings required to be set for processing included setting spot removal to 4 and estimating disk size for background subtraction and the least square filtering using the background scale and Wiener cut off value, respectively, of the spectral analysis feature. For the calculation of dendrograms, optimization and band tolerance were estimated using the best cluster separation method, and then adjusted to accommodate variances in the normalizing of the gels.

Measurement of Biofilm Formation. Biofilms were stained by submersion in 0.05mg/ml bisBenzimide H 33258 (Hoechst 33258; Sigma Chemical Co., St. Louis, Mo.) for 20 min. Excess dye was rinsed by dipping coupons in a beaker of water; excess water was wicked away using Kim Wipes (Kimberly Clarke, Neenah, Wis.). After air-drying the stained biofilms were visualized using an epifluorescence microscope equipped with an excitation filter of 330-380 nm, emission filter 435-485 nm and a dichroic mirror of 400 nm. Images of biofilms grown in DTSB were captured using a Nikon Eclipse E600 epifluorescent microscope (Southern Micro instruments, Marietta Ga.) equipped with a Magnafire CCD camera (Southern Micro instruments), and had an area of 593,350 μm^2. Images of biofilms grown in TSB were captured

with a Nikon eclipse TE300 (Southern Micro Instruments) equipped with a Princeton Instruments RTE/CCD-1300-Y/HS CCD (Trenton, N.J.), and had an area of 148,874 μm^2. Ten images were captured on each coupon. Grayscale images were then converted to black and white by thresholding (Adobe Photoshop, Adobe systems, INC, San Jose, Calif.). Each image was thresholded such that the stained cells were white and the background was black. Black and white pixels were counted using Image Tool (University of Texas Health Science Center, San Antonio, Tex.). The amount of biofilm accumulation was reported as percent coverage based on the percent white pixels *(12, 161)*.

Data analysis. Data was analyzed as a 2 by 31 factorial design with 3 replications (30 strains + negative control). ANOVA and Duncan's multiple range test using general linear models (SAS Institute, Cary, N.C.) was used to determine significant effects and interactions using $\alpha=0.05$ for Duncan's Multiple range, and $\alpha<0.05$ for ANOVA. Biofilm accumulation data were transformed [log (percent area +1)], to correct for violations of the normality and constancy of variance assumptions in the ANOVA model.

RESULTS

Planktonic growth. Based on optical density, all strains exhibited similar growth kinetics in TSB and DTSB. When grown in TSB, maximum cell density was achieved in 10 h, whereas the maximum cell density was achieved at 11 h in DTSB. The final optical density of strains grown in DTSB was approximately 10 fold lower than for strains grown in TSB (data not shown).

Subtyping. The rep-PRO_{Dt} primer differentiated the strains into 4 groups at a similarity coefficient of 90% (Fig. 3.1). Groups 1 and 2 contained serotype 1/2a strains, and Group 4 contained serotype 4b strains. Group 3 consisted only of strain #18. The rep-PRO_{G5} primer

differentiated the strains into 6 groups at a similarity coefficient of 90%, with 7 groups after visual inspection (designated a, b, c, d, e, f and g in Fig. 3.2). This primer differentiated the group 1 strains into two groups, a and c. Group 2 strains were further differentiated into groups b and d. The single strain (#18) in group 3 was combined with 4b strains in group g. Strain 12375 was placed into a separate group e. The remaining group 4 strains were divided into groups f and g. Both primers (rep-PRO_{Dt} and rep-PRO_{G5}) differentiated the strains by serotype. This separation occurs at 38% dissimilarity for primer rep-PRO_{Dt}, and 15% dissimilarity for rep-PRO_{G5} (Fig 3.1 and 3.2). Combining the group designations from each primer into an alphanumeric combination yields 8 distinct subtypes. The composite dendrogram with the combined group designations is presented in figure 3.3. This dendrogram correlates roughly with the combined grouping designations. The only notable exception was the separation of strain G3982 from the other members of subtype 4f.

Effect of nutrient level and strains. Biofilm formation data is presented in Table 3.2. The range of biofilm accumulation in TSB was 3.3 to 41.7% area coverage. Biofilms at the upper end of this range exhibited well-developed multi-cell structures, whereas those at the low end of the range consisted of sporadic attachment of single cells or small clumps. The range of biofilm accumulation in DTSB was 0.8 to 19% coverage. Biofilms at the upper end of this range consisted of micro-colonies and attached cells, whereas those at the lower end consisted mostly of attached single cells and occasional small clumps. Biofilm accumulation was decreased for some strains when DTSB is used as the growth media. All strains exhibited some attachment as demonstrated by significant differences from the sterile control. ANOVA indicated that the effects of nutrient level and strain were significant. The mean biofilm accumulation for all strains was greater when they were grown in TSB. The separation of means for biofilm

accumulation in TSB is shown in Figure 3.4. Four strains were placed into two groupings of high biofilm accumulation, whereas the other strains produced a continuum of lower biofilm accumulation. Strains grown in DTSB produced a range of biofilm accumulation with overlapping Duncan's groupings (Figure 3.5). There was also a significant effect of the interaction of nutrient level and strain. Because of this interaction the effect of nutrient level is different for the different strains: 11 strains had the same capacity to produce biofilms at both nutrient levels, fourteen strains produced more biofilm in TSB than DTSB, and five of the strains produced more biofilm in DTSB when compared to TSB (Table 3.2).

Association of biofilm production with subtype and serotype. Subtype and serotype are associated with the decrease in biofilm accumulation caused by DTSB. Table 3.2 shows the Duncan's multiple range test of the biofilm accumulation means by media type and strain. These data indicate if the biofilm accumulation means at each nutrient level differ for a particular strain. Subtypes 4f, 4g and 4e are serotype 4b, and Duncan's multiple range test indicates that all but four of these strains exhibit marked reductions in biofilm accumulation when grown on DTSB. Of these four strains, only strains YM-32, andYm-84 were definitely unaffected by growth in DTSB. While not statistically significant, biofilm accumulation of strain #70 was reduced by almost half by growth on DTSB. Strain 12374 formed no biofilm at either nutrient level, so biofilm accumulation could not be further reduced by growth on DTSB. Subtypes 2d, 2b, 1a, and 1c are all serotype 1/2a. Of these strains, only strain 12443 exhibited reduced biofilm accumulation in the diluted medium.

Subtypes of serotype 1/2a either were stimulated to produce biofilms by DTSB or they produced biofilms equally well in both media, whereas the serotype 4b subtypes generally produced less biofilm accumulation in the diluted medium. Serotype biofilm accumulation

means were compared by ANOVA using Duncan's multiple range test. The biofilm accumulation mean for serotype 4b strains was greater than the mean for serotype 1/2a strains when they were grown in TSB. When the strains are grown in DTSB, the biofilm accumulation mean for serotype 1/2a strains was greater than the serotype 4b strains biofilm accumulation mean.

Neither subtype nor serotype was associated with the amount of biofilm accumulation. Table 3.2 shows the Duncan's multiple range test of the biofilm accumulation data by subtype. This data illustrates that there is significant variation in the amount of biofilm produced for all of the subtypes that have more than one member, for at least one of the nutrient levels tested. Strains SA and SAF are isolates of strain Scott A sourced from different labs. Both of these isolates produce more biofilm when biofilms are grown in TSB; however SAF produced two to four times the amount of biofilm produced by SA.

DISCUSSION

The number *L. monocytogenes* cells present in suspension during attachment does not greatly affect the amount of biofilm that is later formed. Djordjevic et al. *(31)* found only a slight correlation (P=0.03) between initial inoculums and biofilm growth when biofilms were grown for twenty hours, and no significant correlation at 40 h. There is also no relationship between planktonic growth rate and biofilm growth rate *(23, 31)*. Even the number of initial attached cells has no effect on the final amount of biofilm formed *(23, 70)*. In this study all strains exhibited similar growth kinetics, and reach the same optical density at the time of use. In a similar study Djordjevic et al. *(31)* also found that 31 strains of *L. monocytogenes* exhibited similar growth kinetics when grown in modified Welshimer's broth (MWB) *(119)*. Thus precise

standardization of inoculums prior to each experiment is unnecessary. Washing of inocula could change surface characteristics that influence attachment.

This study indicates that rep-PCR subtyping of *L. monocytogenes* produces results that are consistent with other genetic subtyping methods. Piffaretti et al. *(115)* also found that serotype 4b strains of *L. monocytogenes* could be differentiated from serotype 1/2a strains using multi-locus enzyme electrophoresis (MEE). Other studies using a variety of genotyping methods have also observed that serotype 1/2a could be differentiated from serotype 4b *(11, 22, 51, 60, 92, 103, 121, 122, 157, 158)*.

This study further confirms that *L. monocytogenes* strains exhibit variation in biofilm forming behavior. Chae and Schraft observed that the cell density of biofilms differed between strains of *L. monocytogenes (23)*. Others have reported similar variation in biofilm production, with some *L. monocytogenes* strains producing significantly more biofilm than others *(13, 31, 70, 97)*. The rate of biofilm accumulation can be different amongst isolates, even if after a period of time, they reach a similar biofilm cell density *(24)*. When growing as a biofilm, *L. monocytogenes* exhibit cycles of attachment and detachment, the period of which varies with strain *(24, 25, 108)*. Blackman and Frank *(12)* found that the effect of temperature varied according to what surface and media was used. It is clear that *L. monocytogenes* will have differing abilities to produce biofilm according to strain used, growth temperatures, cultural conditions, and growth surface. Researchers cannot assume that relative biofilm production amongst strains will be the same from one condition to another or that the same conditions will yield maximum biofilm cell density for each strain.

The effects of nutrient levels on the development of *L. monocytogenes* strain ScottA biofilms have been previously studied. Our current research indicates that strain ScottA

produces more biofilm in rich media and this observation is corroborated by others who observed ScottA also produces more biofilm in TSB than DTSB for the first four days *(108)*, and modified Welshimer's broth for 24 and 48 h *(97)*. These findings are consistent with those of Kim and Frank *(74)* who reported that amino acids were important for the early development of *L. monocytogenes* ScottA biofilms. While these observations are supported by the results of the current research, Jeong and Frank *(67)* found that biofilms of *L. monocytogenes* Scott A grown at 21°C in a 1:15 dilution of TSB exhibited cell populations 10 fold greater than biofilms grown in 1:3 dilution of TSB. This stimulation of biofilm production has been suggested to be the result of the starvation stress response *(107)*. Experimentally, the reported media used was half the concentration of the DTSB used currently; indicating that nutrient limitation greater than that used in the current research could enhance biofilm production by serotype 4b strains. However, Oh and Marshall (22) found that after 7 days the biofilm growth of *L. monocytogenes* Scott A on DTSB equaled the growth on TSB *(108)*. This raises the possibility that nutrient availability affects biofilms differently according to their age.

Factors other than nutrient level and strain may also influence biofilm production by *L. monocytogenes*. There are three main evolutionary lines of *L. monocytogenes (115, 122)*. These divisions are known as Lineage I, II and III *(103, 158)*. Lineage I is composed of serotypes 4b, 1/2b, 3c and 3b; Lineage II is composed of 1/2a, 1/2c, and 3a *(11, 103)*. Lineage III is the most recently elucidated lineage and contains serotype 4a and 4c *(103, 122)*. In our current work, mean biofilm accumulation of the lineage I group was greater than the lineage II group when grown in TSB, however when grown in DTSB the mean biofilm accumulation of the Lineage II group was greater. Djordjevic et al. *(31)* in a similar study found that division I isolates produced more biofilm than division II isolates when biofilms were grown in modified

Welshimer's broth. However Borucki et al. *(13)* using a modified version of Djordjevic's procedure, found the opposite result. In an attempt to determine the reason for the discrepancy, the two methods in question were compared using a sample of the same isolates used by the former group, again resulting in disagreement *(13)*. In our lab, two isolates of the same strain (SA, and SAF) differed in their ability to produce biofilm. Additionally there was significant variation of biofilm formation amongst members of the same rep-PCR subtype indicating no apparent linkage between rep-PCR subtypes and the multi-step phenotypic process of biofilm formation.

In summary, this study found an association between serotype, subtype and a decrease in biofilm accumulation in low nutrient media (DTSB), indicating that there are differences between the two serotypes regarding their response to their environment, and that serotype 1/2a isolates may also produce biofilm under greater variety of environmental conditions. Such physiological differences could account for the prevalence of serogroup 1/2 *L. monocytogenes* in processing plant environments *(72, 91)* where nutrients may be limited.

ACKNOWLEDGEMENTS

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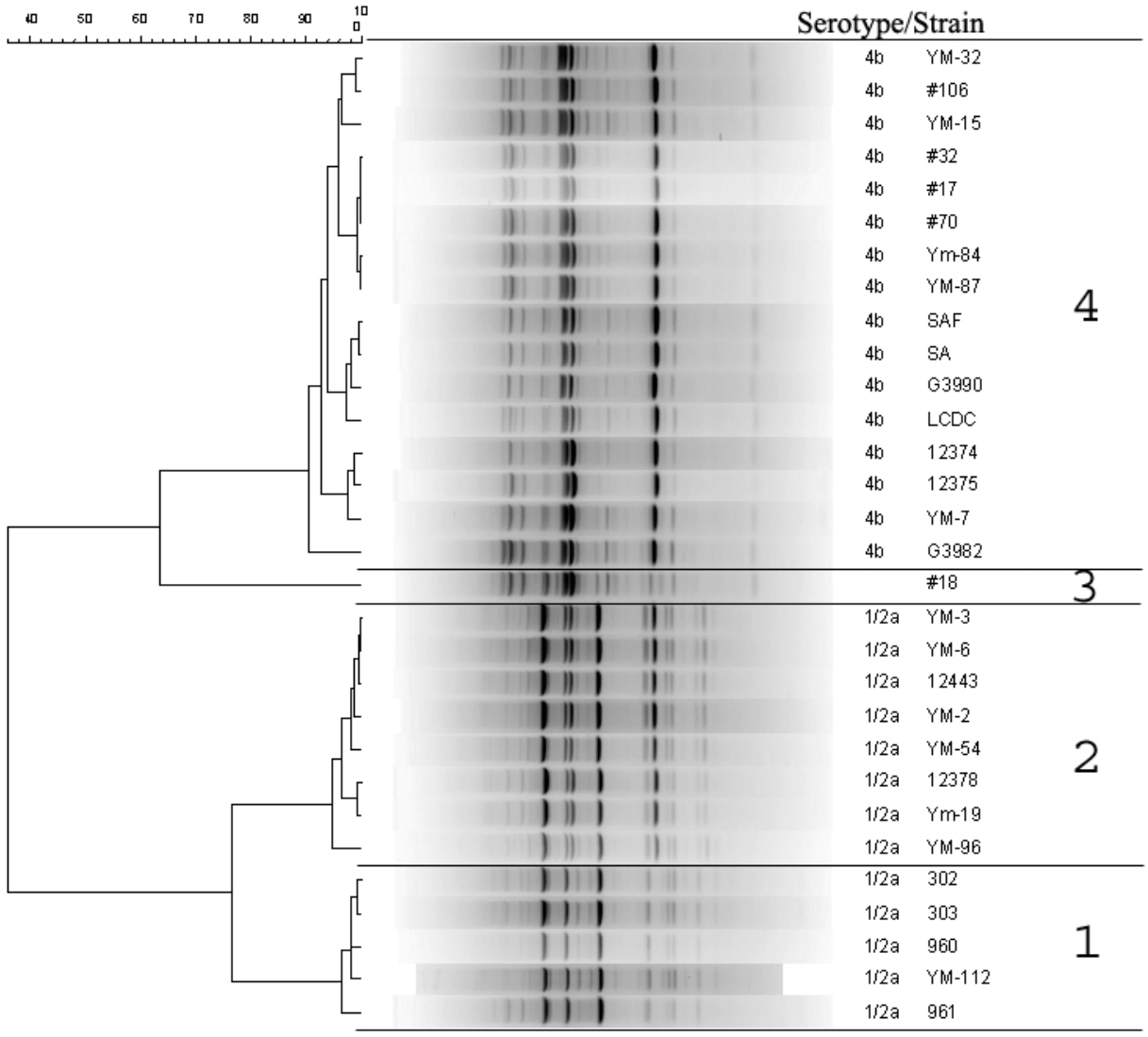

Figure 3.1: Pearson UPGMA analysis of Uprime Dt fingerprints of *Listeria monocytogenes* strains with serotype. Optimization=0.3. Large bold numbers indicate genetic groupings.

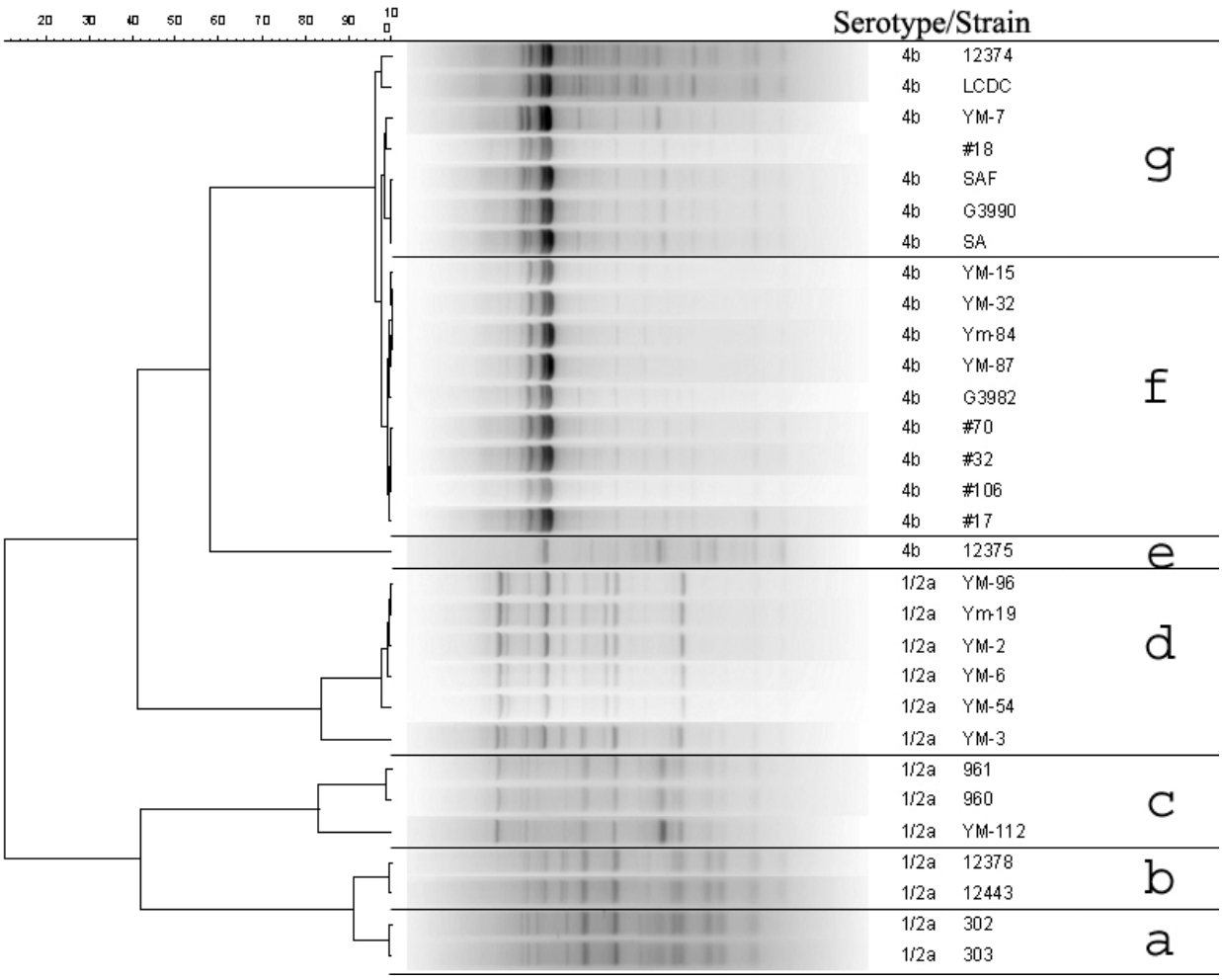

Figure 3.2: Pearson UPGMA analysis of Uprime G5 fingerprints of *Listeria monocytogenes* strains, including serotype. Optimization=0.3. Large bold letters indicate fingerprint type.

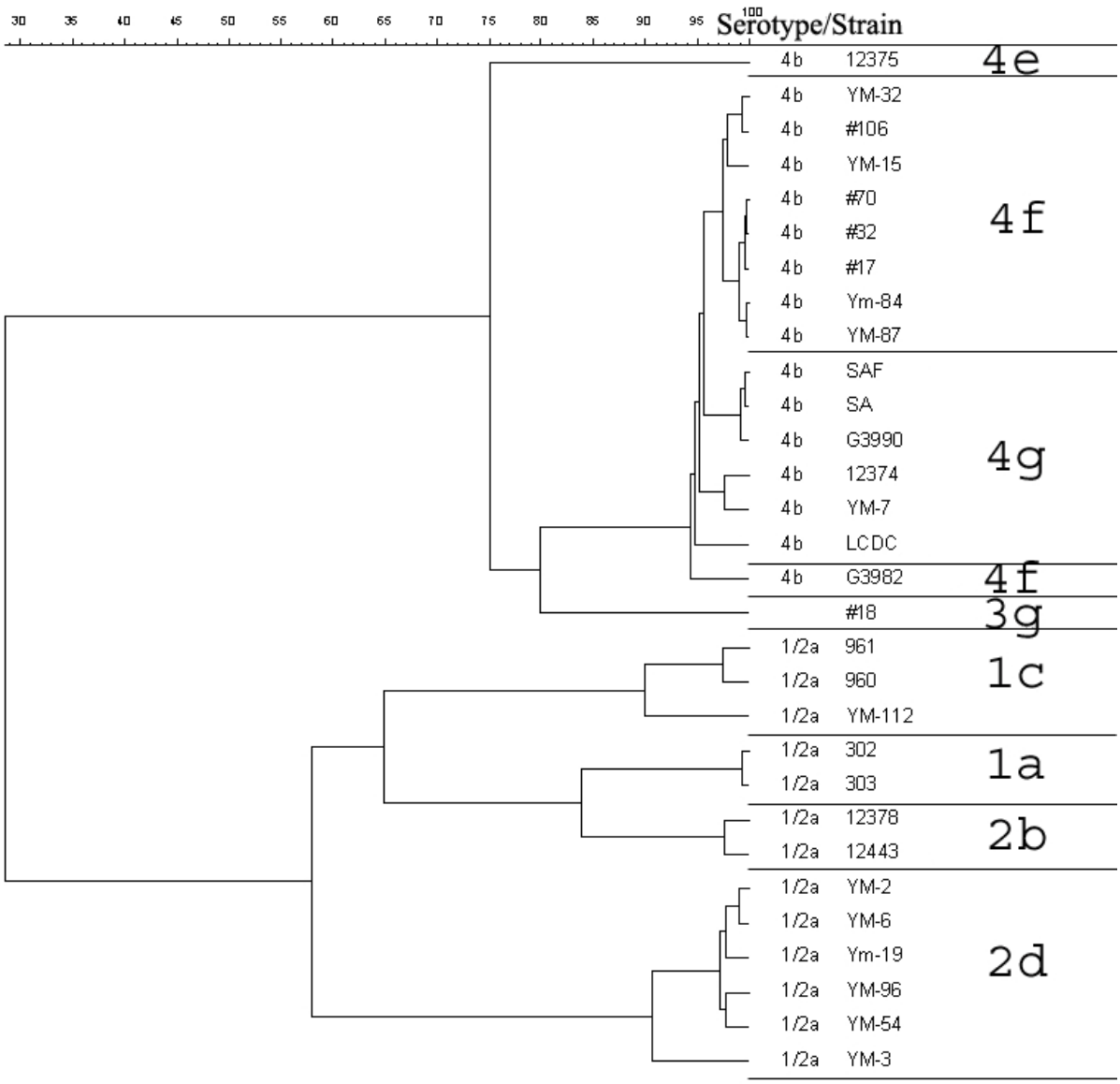

Figure 3.3: Pearson UPGMA analysis of Uprime G5 and Uprime Dt fingerprints of *Listeria monocytogenes* strains, includes serotype. Uprime Dt Optimization=0.3, Uprime G5 optimization=0.3. Large bold alphanumeric designations show the combined group assignments.

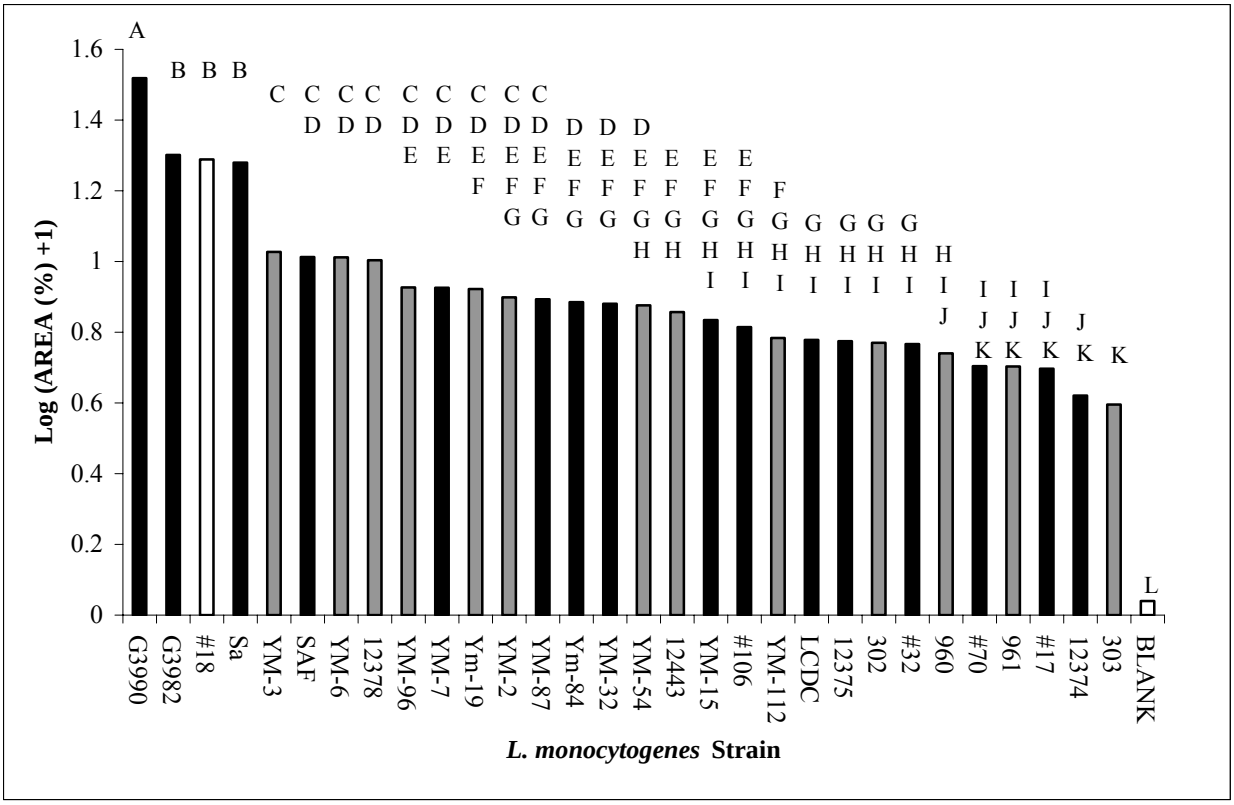

Figure 3.4: Biofilm accumulation (log [percent area +1]) of various strains of *Listeria monocytogenes* on stainless steel in tryptic soy broth after four hours of attachment and 24 hours of incubation at 32 ºC. Letters above each bar indicate the Duncan's multiple range test groupings (α=0.05). Bars with the same letter are not significantly different. Serotype 4b strains are in black, Serotype 1/2a strains are in grey. White bar is serotype not applicable.

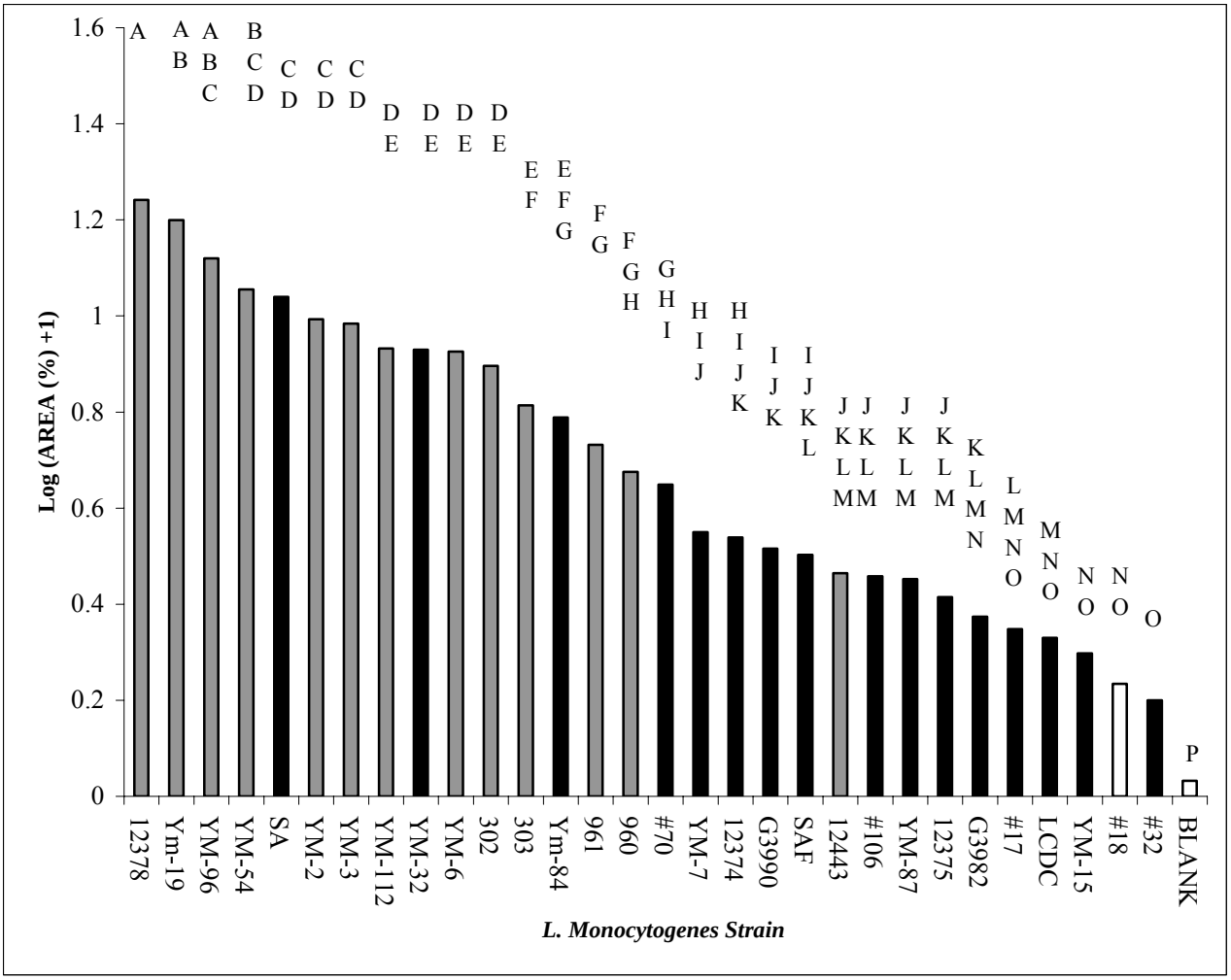

Figure 3.5: Biofilm accumulation (log [percent area +1]) of various strains of *Listeria monocytogenes* on stainless steel in a 1:10 dilution of tryptic soy broth after four hours of attachment and 24 hours of incubation at 32 ºC. Letters above each bar indicate the Duncan's multiple range test groupings (α=0.05). Bars with the same letter are not significantly different. Serotype 4b strains are in black, Serotype 1/2a strains are in grey. White bar is serotype not applicable.

Table 3.1. Strains of *Listeria monocytogenes* used in work.

Strain	Source	Serotype
12375	Monkey (clinical) [a,f,g]	4b
YM-32	Environmental (YNPC[b])	4b
#106	Environmental (FPC[c])	4b
Ym-15	Environmental (YNPC)	4b
#70	Environmental (FPC)	4b
#32	Environmental (FPC)	4b
#17	Environmental (FPC)	4b
Ym-84	Environmental (YNPC)	4b
YM-87	Environmental (YNPC)	4b
SAF	ScottA	4b
SA	ScottA	4b
G3990	Vaucherin cheese outbreak	4b
12374	Monkey (clinical)	4b
YM-7	Environmental (YNPC)	4b
LCDC	Cabbage outbreak	4b
G3982	Jalisco cheese outbreak[g]	4b
#18	Environmental (FPC)[c]	*
961	Monkey (clinical)	1/2a
960	Monkey (clinical)	1/2a
YM-112	Environmental (YNPC)	1/2a
302	Monkey (clinical)	1/2a
303	Monkey (clinical)	1/2a
12378	Monkey (clinical)	1/2a
12443	Monkey (clinical)[e,f,g]	1/2a
YM-2	Environmental (YNPC)	1/2a
YM-6	Environmental (YNPC)	1/2a
Ym-19	Environmental (YNPC)	1/2a
YM-96	Environmental (YNPC)	1/2a
YM-54	Environmental (YNPC)	1/2a
YM-3	Environmental (YNPC)	1/2a

[a] Monkey clinical strains isolated from animals at Yerkes National Primate Center. These strains were obtained from the Center for Food Safety, Griffin, GA.
[b] YNPC: From outdoor animal facilities at Yerkes National Primate Center
[c] FPC: Isolates from various food processing environments obtained from the University of Georgia, Department of Food Science and Technology, Athens, GA
[d] Serotype is undetermined
[e] Research on this strain reported in references *(69)*.
[f] Research on this strain reported in references *(139)*.
[g] Research on this strain reported in references *(148)*

Table 3.2: Biofilm growth (%area covered) of genetic subtypes of *Listeria monocytogenes* in diluted tryptic soy broth (DTSB) and tryptic soy broth (TSB) after a 4 four-hour attachment and 24 hours incubation at 32ºC.

Strains	TSB	[b]Duncan Group	DTSB	[b]Duncan Group	[a]Media for which more biofilm was produced			rep-PRO$_{Dt/G5}$ subtype
					TSB	DTSB	NONE	
YM-3	10.9	A	13.3	BC			*	[1]2d
YM-54	7.4	BC	11.86	ABC		*		
YM-96	8.7	ABC	13.3	AB		*		
Ym-19	8.3	ABC	17	A		*		
YM-6	10.7	AB	10	C			*	
YM-2	8.2	ABC	10	BC			*	
12378	11.8	A	19	A		*		[1]2b
12443	8.9	B	2	B	*			
302	5.7	A	9.9	A			*	[1]1a
303	3.3	B	7.7	A		*		
961	4.3	A	6.6	B			*	[1]1c
960	4.8	A	8.2	B			*	
YM-112	5.6	A	11.6	A			*	
#18	19.5	-	0.8	-	*			3g
G3982	21.3	A	1.47	CD	*			[2]4f
YM-32	7.32	B	8.6	A			*	
#106	6.7	B	2	C	*			
YM-15	6.5	BC	1.3	CD	*			
#70	6.3	C	3.8	B			*	
#32	5.2	BC	0.6	D	*			
#17	4.3	C	1.28	BCD	*			
Ym-84	7.4	B	10	AB			*	
YM-87	7.6	B	2.5	C	*			
SAF	9.7	C	2.74	B	*			[2]4g
SA	21.8	B	11	A	*			
G3990	41.7	A	3.75	B	*			
12374	3.8	E	2.8	B			*	
YM-7	9.9	C	3.8	B	*			
LCDC	5.95	D	1.4	C	*			
12375	5.53	-	1	-	*			[2]4e

[a]The results of Duncan's multiple range test of the biofilm accumulation means by media type and strain (α=0.05).
[b]The results of Duncan's multiple range test of the strain means by genotype (α=0.05). Strains with the same letter are not significantly different.
[1]Serotype 1/2a strains.
[2]Serotype 4b strains

CHAPTER 4

HYPOCHLOROUS ACID TOLERANCE OF *LISTERIA MONOCYTOGENES* BIOFILMS AND RELATIONSHIP TO SUBTYPE, CELL DENSITY, AND PLANKTONIC CELL HYPOCHLOROUS ACID TOLERANCE [19]

[19] Folsom, JP and Frank, JF 2005. To be submitted to the Journal of Food Protection

ABSTRACT

Strains of *Listeria monocytogenes* vary in their ability to produce biofilms. This research determined if cell density, planktonic hypochlorous acid tolerance, or repPCR subtype is associated with the tolerance of *L. monocytogenes* biofilms to hypochlorous acid. Thirteen strains of *L. monocytogenes* were selected for this research based on biofilm accumulation on stainless steel and rep-PCR subtyping. These strains were challenged with hypochlorous acid to determine the tolerance of individual strains of *L. monocytogenes*. Planktonic cells were exposed to 20 through 80 ppm hypochlorous acid in 20 ppm increments for 5 min in triplicate per replication, and the experiment was replicated three times. The number of tubes with surviving *L. monocytogenes* was recorded for each isolate at each level of hypochlorous acid. Biofilms of each strain were grown on stainless steel coupons. The biofilms were immersed in 60 ppm of hypochlorous acid. When in planktonic culture, four strains were able to survive exposure to 40 ppm of hypochlorous acid, while four strains were able to survive 80 ppm of hypochlorous acid in at least one of three tubes. The remaining five strains survived exposure to 60 ppm of hypochlorous acid. Biofilms of 11 strains survived exposure to 60 ppm of hypochlorous acid. No association of biofilm hypochlorous acid tolerance and planktonic hypochlorous acid tolerance was observed, however biofilm hypochlorous acid tolerance was similar for strains of the same subtype. Biofilm cell density was not associated with hypochlorous acid tolerance. In addition, biofilms that survived hypochlorous acid treatment exhibited different biofilm morphologies. These data suggest that hypochlorous acid tolerance mechanisms of planktonic cells and biofilms differ, with planktonic hypochlorous acid tolerance being more affected by inducible traits, and biofilm hypochlorous acid tolerance being more affected by traits not determined in this study.

INTRODUCTION

Various factors affect the tolerance of *Listeria monocytogenes* to hypochlorous acid. In carrier tests *(10, 102)*, and biofilm challenge tests *(80)*, biofilms of *L. monocytogenes* are more resistant to killing by hypochlorous acid than when challenged as a cell suspension or as adherent cells. El Kest and Marth *(39)* observed significant variation in the hypochlorous acid tolerance of 3 strains of *L. monocytogenes.* Lundén et al. *(84)* corroborated this and reported that tolerance to sanitizers including hypochlorous acid can be induced by exposure to hypochlorous acid, quaternary ammonium compounds and other common sanitizers. They also found that strains with lower sanitizer tolerance exhibited greater increases in tolerance, and that all strains studied exhibited essentially the same tolerance after full induction of hypochlorous acid tolerance. The hypochlorous acid tolerance persisted for about a week before the strains returned to base levels.

Tolerance of *L. monocytogenes* to hypochlorous acid is also dependant upon the growth matrix, ion concentration, and temperature. *Listeria* biofilms exhibited maximum tolerance to hypochlorous acid after growth for 48 to 72 h in media containing milk soil, and after 144 h in media containing meat soup *(160)*. El Kest and Marth *(38)* observed that growing *L. monocytogenes* in tryptose broth as opposed to tryptose agar, and then suspending the cells in buffer containing increased amounts of phosphate, resulted in increased tolerance to hypochlorous acid. Lowering the temperature of exposure from 25 or 35 to 5 $^{\circ}$C resulted in greater hypochlorous acid effectiveness against *L. monocytogenes* Scott A *(39)*.

The amount of biofilm produced by *L. monocytogenes*, as measured by determining cell density, varies by strain *(44)*. One objective of this research is to determine the effect of biofilm cell density on tolerance to hypochlorous acid. The morphology of the biofilms was also

characterized. A second objective is to determine if hypochlorous acid tolerance of biofilm cells is associated with hypochlorous acid tolerance of their planktonic counterparts. In addition, the association between genetic subtypes and hypochlorous acid tolerance was investigated.

MATERIALS AND METHODS

***L. monocytogenes* strains.** Thirteen strains of *L. monocytogenes* were used in this study (Table 4.1). In previous research the degree of biofilm accumulation for each strain was determined by microscopic observation and the strains were subtyped *(44)*. Briefly, subtypes were determined using repPCR with RepPRO DNA fingerprinting kits using primers rep-PRO_{G5} and rep-PRO_{Dt} (Bacterial Barcodes Inc, Houston Tex.). In the previous work biofilms were obtained by static growth on 2x5 cm coupons and stained with bisBenzimide H 33258 (Hoechst 33258; Sigma Chemical Co., St. Louis, Mo.) for microscopic observation. Using this information strains were selected to represent a range of biofilm accumulation and subtypes. Strains were maintained at –80 ºC in Microbank™ bead vials (PRO-LAB Diagnostics, Richmond Hill, Ontario, Canada). Tryptic soy broth (TSB; Becton, Dickinson, and Company, Sparks, Md.) was inoculated with one bead and sub cultured two to four times at 32ºC prior to growth of inoculums (20 h at 32ºC without shaking) for testing *(44)*.

Preparation of hypochlorous acid solution. Hypochlorous acid solutions of desired concentration were prepared each day from a sodium hypochlorite stock solution (100 g/l) (Mallinckrodt Baker, Inc., Paris Ky.). The solutions were prepared in water free of organics (deionized 18 million ohms/cm) buffered with 0.015 M KH_2PO_4. Clean glassware were thoroughly cleaned, soaked overnight in strong bleach solution, and rinsed in deionized water. After preparation the hypochlorous acid solution was adjusted to pH 7.00 ± 0.05, and the concentration of hypochlorous acid was verified by UV spectroscopy using the molar absorption

constants OCl^-; 99.6 M^{-1} cm^{-1} at 235 nm and 26.9 M^{-1} cm^{-1} at 290nm, HOCl; 7.8M^{-1} cm^{-1} at 235 nm and 350.4 M^{-1} cm^{-1} at 290 nm *(99)*.

Hypochlorous acid tolerance of cell suspensions. Five milliliters of standardized sodium hypochlorite solution was added to 100 µl of test culture in a test tube (16 mm x 150 mm). After 5 min the hypochlorous acid was quenched with 1 ml of a 10% (w/v) solution of sodium thiosulfate (final conc. 0.07M). Four milliliters of double strength TSB was then added to each tube and the tubes were incubated at 32ºC (sodium thiosulfate conc. was 0.04M). These tubes were observed for 5 days and the presence or absence of growth was recorded. Brackett *(15)* reported that exposure to 0.1M sodium thiosulfate had no effect on viability of *L. monocytogenes*. Each isolate was exposed to 20 through 80 ppm hypochlorous acid in 20 ppm increments in triplicate per replication, and the experiment was replicated three times. The contents of any tubes exhibiting growth were streaked onto RAPID'L.MONO® media (Bio-Rad Labs, Hercules Calif.) to confirm the presence of *L. monocytogenes*. The number of tubes with surviving *L. monocytogenes* was recorded for each isolate at each level of hypochlorous acid. Hypochlorous acid challenge inocula were enumerated on tryptic soy agar with 0.6% yeast extract (TSAYE; Becton, Dickinson, and Company) by spiral plating (Spiral Biotech AP4000, Spiral Biotech, Bethesda Md.) with incubation at 32º C for 18 h.

Tolerance of biofilms to hypochlorous acid. Biofilms for hypochlorous acid tolerance experiments were grown on stainless steel (type 304 4b finish) coupons measuring 11 cm by 7.5 cm. Coupons were submerged in 200 ml of a 20 h culture of *L. monocytogenes* grown in TSB, and incubated at 32ºC for 4 h in sterile plastic pans (Cambro H-Pan 62HP with lid 60HPC, Cambro, Huntington Beach, Calif.). Pans containing coupons were shaken at 70 rpm in a C24 incubator shaker (New Brunswick Scientific, Edison, N.J.). After 4 h, coupons were placed in

sterile sampling bags (Fisher brand 01-815-25, Fisher Scientific, Atlanta, Ga.) with 200 mls of 0.015 M KH_2PO_4 adjusted to pH 7.0 (phosphate buffer) and shaken vigorously by hand for 10 s. After rinsing, the coupons were transferred to sterile plastic pans containing sterile TSB and incubated for 24 h at 32ºC and 70 rpm. After biofilm growth the coupons were washed using a wash bottle (Nalgene #2405-0500, Rochester, N.Y.), directing the stream of sterile phosphate buffer (approximately 50 ml) evenly from top to bottom, front and back to remove the growth media and unattached cells. Each washed coupon was placed into a stainless steel pan that held four other coupons, including a sterile negative control. The pan was filled with 600 ml of phosphate buffer or 600 ml of 60 ppm hypochlorous acid solution.

Coupons treated with phosphate buffer were agitated for 10 min at 70 rpm at room temperature, (Orbit shaker, Lab Line Instruments, INC, Melrose Park, IL) after which they were again rinsed with about 20 ml of buffer using a wash bottle and transferred to a Petri dish. These coupons were scraped with a Teflon policeman (Fisher Scientific) to remove attached cells for enumeration. The coupons were scraped and rinsed with phosphate buffer three times, and a total of 100 ml of buffer was collected. The detached cells were shaken in milk dilution bottles on a wrist action shaker (Burrel Scientific model 75, Pittsburgh, Pa.) at the maximum setting for 10 min to break up cell clumps prior to enumeration. Cell suspensions and dilutions were plated on TSA with incubation at 32 ºC for 24 h. Results were reported as log CFU per 50 cm^2. The cell density of the biofilms was analyzed by using one way ANOVA ($\alpha<0.05$), and by application of the Duncan's multiple range test ($\alpha=0.05$) using SAS (SAS Institute Inc., Cary N.C.) In addition, strains were classified according to biofilm morphology (fully developed, large microcolonies, scattered small microcolonies, numerous small microcolonies, or sparse) using epifluorescent microscopy as previously described *(44)*.

Treated coupons were agitated with hypochlorous acid solution for 5 min at 70 rpm at room temperature (orbit shaker) before the hypochlorous acid was neutralized with 250 ml of 0.15M sodium thiosulfate (final concentration 0.04M). Neutralization of hypochlorous acid was verified using Aquacheck™ total/free hypochlorous acid test strips (Hach Company, Loveland, CO). The coupons were then agitated for another 5 min, rinsed with about 20 ml of buffer and transferred to a Petri dish. *L. monocytogenes* were enumerated by overlaying the coupons with TSA and incubating at 32ºC for 48 h to allow colony formation from the attached survivors *(46)*. TSA was supplemented with 1 g/l added sodium pyruvate to enhance recovery of hypochlorous acid injured cells and 0.03 g/l potassium tellurite to improve visualization of colonies. Survivors were reported as log CFU per 50 cm^2. This experiment was replicated four times. Data was transformed (number of CFU + 0.01)$^{1/10}$ to normalize, and analyzed by using one way ANOVA (α<0.05) and Duncan's multiple range test (α=0.05) (SAS, SAS Institute, Inc)

RESULTS

Hypochlorous acid tolerance of planktonic *L. monocytogenes*. Data on the hypochlorous acid tolerance of planktonic cells are presented in Table 4.2. Four strains (18, 303, 961, and 12374) survived exposure to 40 ppm of hypochlorous acid but not at higher concentrations. Four strains (12378, G3982, YM3 and 302) survived 80 ppm of hypochlorous acid in at least 1 of 9 tubes. The remaining five strains (17, YM6, 960, G3490, and SA) survived 60 ppm of hypochlorous acid. The observed hypochlorous acid tolerance was not affected by the cell density of the inocula, as there was an insignificant (α=0.15) difference in the inocula densities between strains. The strain with lowest average plate count and the strain with highest average plate count both exhibit the lowest level of hypochlorous acid tolerance in the planktonic form. There was also no apparent association of hypochlorous acid tolerance with genetic

relatedness. All subtype groups with more than one member show significant intragroup differences for planktonic cell hypochlorous acid tolerance.

Hypochlorous acid tolerance of *L. monocytogenes* biofilms. One-way ANOVA of unchlorinated biofilm cell counts indicated that the strains differed in their ability to produce biofilms as was expected based on previous research *(44)*. The results of the Duncan's multiple range test are presented in Figure 4.1. Three strains produced biofilms containing more than 6 log cfu/50 sq cm, 9 strains produced biofilms of 5-6 log cfu/50 sq. cm and 1 strain produced biofilm having less than 5 log cfu/50 sq. cm.

One-way ANOVA indicated that strains differed in hypochlorous acid tolerance of their biofilms. The Duncan's multiple range test indicated that 12378 was more resistant than other strains (Fig.4.1). Biofilms of 11 strains (G3982, SA, G3990, YM-3, YM-6, 960, 12378, 12374, 302, 303 and 961) survived exposure to 60 ppm of hypochlorous acid. Hypochlorous acid tolerance of the biofilms could be grouped into 3 levels, with three strains surviving at a level greater than 10 CFU/50 cm^2 (12378, 960 and 961), eight surviving with an average less than 10 CFU/50 cm^2. (G3982, SA, G3990, YM-3, YM-6, 12374, 302, and 303) and two (17 and 18) that were reduced to undetectable levels (Fig. 4.1). In contrast to planktonic cells, hypochlorous acid tolerance of the biofilms was associated with subtype. Strains of subtypes 1c (960 and 961), 4g (SA, G3990, and 12374), 2d (YM-3 and YM-6) and 1a (302 and 303) behaved similarly regarding biofilm hypochlorous acid tolerance. However subtype 4f (G3982 and 17) contained both tolerant and non tolerant strains (Fig. 4.1).

Cell density of the biofilms did not correlate with hypochlorous acid tolerance, as some of the more hypochlorous acid tolerant strains produced biofilms containing lower numbers of cells. In addition, strains that were the most sensitive to hypochlorous acid in planktonic form were not

the same strains that were most sensitive to hypochlorous acid when grown as biofilms. Of the four strains that were least tolerant to hypochlorous acid in suspension, one strain was tolerant (961), one strain was sensitive (18) and two strains exhibited slight tolerance (303 and 12374) when grown as biofilms. However, hypochlorous acid tolerance of the planktonic cell suspensions is more closely associated with hypochlorous acid tolerance of the biofilm. Of the four strains that were most tolerant in suspension, one strain was the most tolerant (12378) and three were slightly tolerant (G3982, YM-3, and 302) (Fig. 4.1) when grown as biofilms. This could indicate that there are at least two mechanisms of hypochlorous acid tolerance and that planktonic hypochlorous acid tolerance and biofilm hypochlorous acid tolerance are related.

There was no apparent association of hypochlorous acid tolerance with biofilm morphology. Five morphology patterns were observed. These are illustrated by representative micrographs presented in Fig. 4.2. G3990 and SA produced well-developed biofilms that cover large portions of the surface. Strains G3982, 303, 18, 12374, YM-6 and YM-3 produced localized accumulations of large microcolonies. Strains 17, 961 produced small microcolonies, and strains 960, 302 exhibited sparse biofilm accumulation with microcolony production rare. Strain 12378 was the only strain to produce numerous small microcolonies that produce nearly as much surface area coverage as the well-developed biofilms. This strain also produced the biofilm that was most tolerant to hypochlorous acid (Fig 4.1). Strains 960 and 961 produced moderately tolerant biofilms that consisted of small microcolonies or sparse accumulation. Strains 17 and 18 produced biofilms that did not survive hypochlorous acid treatment, and these strains produced biofilms of either small or large microcolonies, The remaining strains were only slightly chorine tolerant and produced either fully developed biofilms or biofilm of large microcolonies.

DISCUSSION

Results of this study confirm the findings of others *(26, 39, 84)* that strains of *L. monocytogenes* exhibit different degrees of hypochlorous acid tolerance when challenged in suspension. The lack of association of hypochlorous acid tolerance with subtype is consistent with the observation of Lundén et al. *(84)* that hypochlorous acid tolerance is induced in planktonic cells, as this may account for the strain variability of planktonic cell hypochlorous acid tolerance seen in this work. In addition, induction of hypochlorous acid tolerance prior to biofilm growth may result in biofilms with greater hypochlorous acid tolerance than that observed in this study. Such a response of biofilm cells would underscore the need to ensure that hypochlorous acid sanitizers are used in an effective manner.

An agar overlay method was used to analyze treated coupons for survivors because it is more sensitive than scraping or swabbing. This method does not dilute cells as with scraping. In addition, scraping or swabbing does not release all viable cells from the surface, which is a significant factor when there are few survivors, as in this study. The limitation of the overlay method is that each colony may represent multiple survivors in a microcolony, rather than individual viable cells, since microcolonies are not disrupted. Therefore, the overlay method will underestimate surviving CFU when counts are high, but be more sensitive and accurate than swabbing methods when there are low numbers of survivors. This tendency to underestimate numbers when surviving counts are high should be considered when interpreting the data presented in Figure 4.1.

While this study highlights strain variation in hypochlorous acid tolerance of planktonic and biofilm *Listeria*, others have observed this strain variation behavior with other sanitizers when testing cell suspensions. Earnshaw and Lawrence *(37)* observed strain variation for *L.*

monocytogenes in their tolerance to three commercial cleaning agents (quaternary ammonia based, alkaline foam with alkaline metal hydroxide and hypochlorous acid, and a blend of alkali metal hydroxide) when the chemicals were used at levels reduced from manufacturers recommended concentration. Sallam and Donnelly *(132)* reported differing maximum sublethal concentrations for two quaternary ammonia sanitizing agents for suspensions of *L. monocytogenes*. Lundén et al. *(84)* also reported that strains of *L. monocytogenes* exhibited differing MICs for two different quaternary ammonia sanitizers. Tuncan *(155)* found that planktonic cells of strain V7 exhibited increased tolerance to quaternary ammonia when exposure was 2ºC.

This study is the first to describe strain variability in hypochlorous acid tolerance of *L. monocytogenes* grown as biofilms, and provides evidence that hypochlorous acid tolerance of biofilms possibly associated with genetic subtype, whereas no evidence for the association of hypochlorous acid tolerance with planktonic cells subtype was observed. This observation implies that hypochlorous acid tolerance of the biofilms may be controlled by different mechanisms than is hypochlorous acid tolerance of planktonic cells. This conclusion is supported by work of Robbins et al *(125)* who found that 1 strain tested was very tolerant of ozone but only in planktonic form and not the biofilm state. Also supportive of our current work is data obtained by Arizcun et al. *(2)* showing strain variation in the tolerance of 12 strains grown as biofilms and treated sequentially with NaOH and acetic acid.

It is of interest that the strain exhibiting the most hypochlorous acid tolerance in biofilm form exhibited unique biofilm morphology, but the significance of this observation is unclear, as additional strains that produce similar biofilm would need to be tested to determine if this specific morphology is associated with hypochlorous acid tolerance. Data from the other strains

tested indicate that the traits primarily responsible for hypochlorous acid tolerance of the biofilms are unrelated to biofilm cell density or morphology. Other characteristics, such as excreted polymers should be studied. Data of Wirtanen and Mattila-Sandholm *(160)* indicates that maximum chorine tolerance of *L. monocytogenes* biofilm manifests about the same time as maximum polysaccharide production. They also observed that the amount of exocellular polysaccharide produced by the biofilm is influenced by previous hypochlorous acid exposure and the type of added soil. The ability of *L. monocytogenes* strains to survive hypochlorous acid exposure may be more closely associated with the amount of exocellular polysaccharide produced by the biofilm cells than the number of cells contained within the biofilm.

ACKNOWLEDGEMENTS

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TABLE 4.1. *Listeria monocytogenes* isolates used in this work.

Isolate	Source	Serotype	Subtype[b]
17	Environmental (FPC)[a]	4b	4f
G3982	Jalisco Cheese outbreak	4b	4f
SA	ScottA	4b	4g
G3990	Vaucherin Cheese outbreak	4b	4g
12374	Monkey (clinical)[d]	4b	4g
18	Environmental (FPC)[a]	ND[c]	3g
961	Monkey (clinical)[d]	1/2a	1c
960	Monkey (clinical)[d]	1/2a	1c
302	Monkey (clinical)[d]	1/2a	1a
303	Monkey (clinical)[d]	1/2a	1a
12378	Monkey (clinical)[d]	1/2a	2b
YM-6	Environmental (Monkey housing)[d]	1/2a	2d
YM-3	Environmental (Monkey housing)[d]	1/2a	2d

[a]FPC: Isolates from various food processing environments obtained from the University of Georgia, Department of Food Science and Technology, Athens, Ga.
[b]Strains were subtyped in previous work *(44)*
[c]not determined
[d]Isolated from Yerkes National Primate Center and obtained from the Center for Food Safety, Griffin, Ga.

TABLE 4.2. Hypochlorous acid tolerance of planktonic *Listeria monocytogenes* cells grown in tryptic soy broth at 32ºC for 20 h.

Isolate	No. of tubes (of 9) with growth Hypochlorous acid conc. (ppm) 20	40	60	80	Initial Log CFU/ml	SD	Subtype[a]
12378	9	9	7	1	9.30	0.08	2b
YM-6	9	6	6	0	9.31	0.03	2d
YM-3	9	9	7	1	9.20	0.05	2d
302	9	9	8	2	9.26	0.23	1a
303	9	9	0	0	9.26	0.02	1a
961	9	9	0	0	9.04	0.33	1c
960	9	6	8	0	9.25	0.06	1c
18	9	9	0	0	9.43	0.46	3g
G3990	9	8	7	0	9.31	0.07	4g
SA	9	9	8	0	9.37	0.09	4g
12374	9	9	0	0	9.29	0.03	4g
17	9	9	9	0	9.16	0.17	4f
G3982	9	9	5	1	9.32	0.10	4f

[a]Strains were subtyped in previous work *(44)*

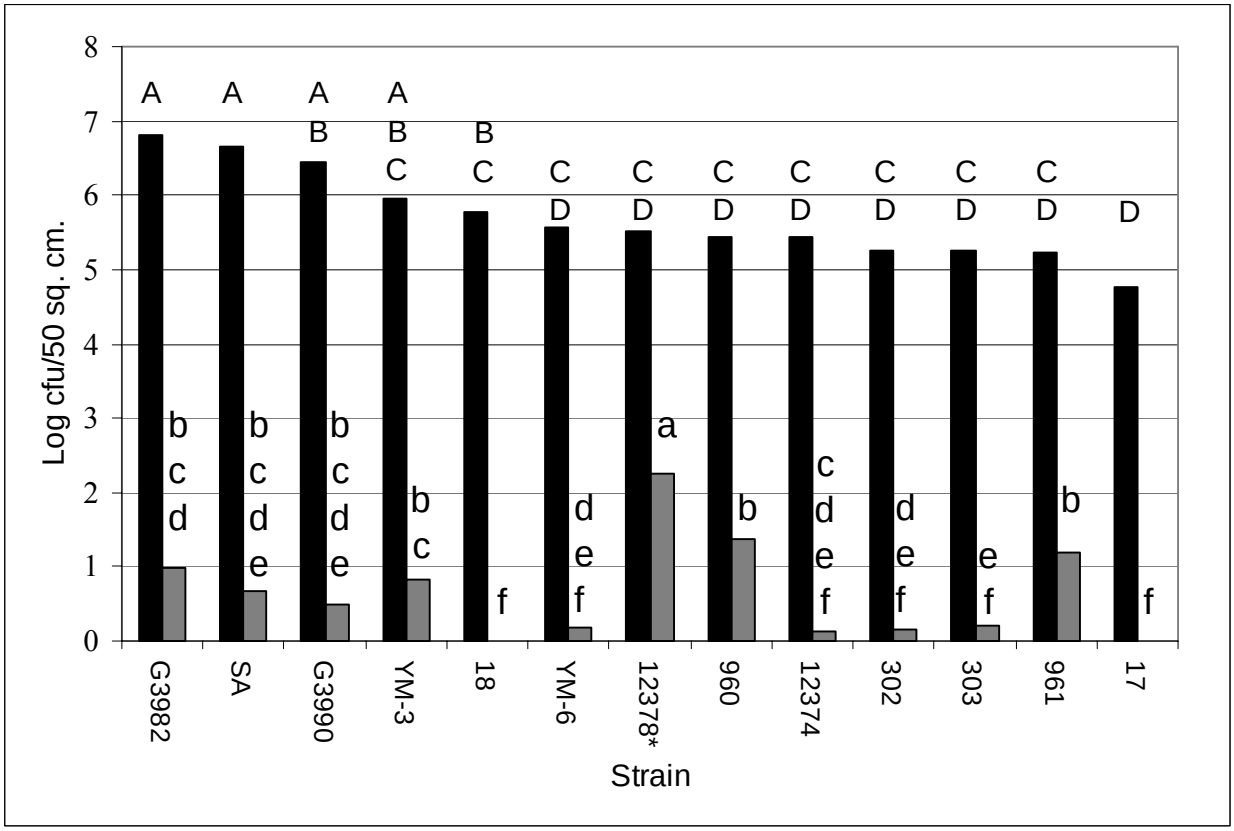
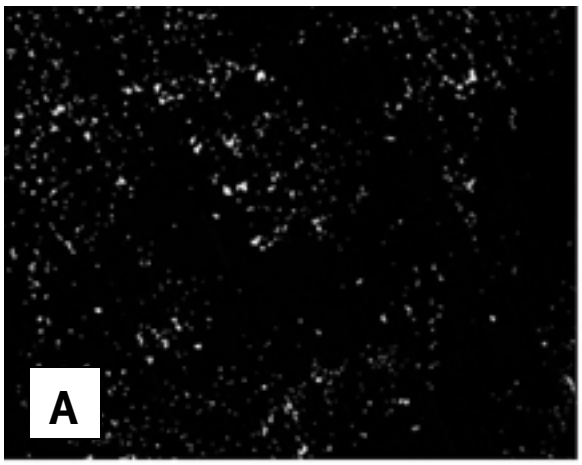


FIGURE 4.1. Viable *Listeria monocytogenes* (strain and subtype) in biofilms (log CFU per 50 cm^2) before (black) and after (grey) treatment for 5 min with 60 ppm hypochlorous acid. Biofilms were grown at 32 ºC for 24 h in TSB. CFU on untreated biofilms were determined by plating detached cells on TSAYE with incubation at 32ºC for 48 h. CFU on treated biofilms were determined by overlaying the treated surface with TSAYE containing 1 g/l sodium pyruvate and 0.5 g/l potassium tellurite with incubation at 32ºC for 48 h. Letters above the bars indicate means (n = 4) separated using the Duncan's multiple range test (α=0.05). *, average depicted for isolate includes estimates from two trials in which colonies were too numerous to count (>500).

FIGURE 4.2. Representative micrographs of biofilms associated with strains used in this study. Biofilms were grown in trypticase soy broth with incubation at 32ºC for 24 h. (A) Sparse biofilm typical of strains 960 and 302. (B) Small microcolonies typical of strains 17 and 961. (C) Localized accumulation of large microcolonies typical of strains G3982, 303, 18, 12374, YM-6 and YM-3. (D) Well-developed biofilm typical of strains G3990 and SA. (E) Numerous small microcolonies of strain 12378.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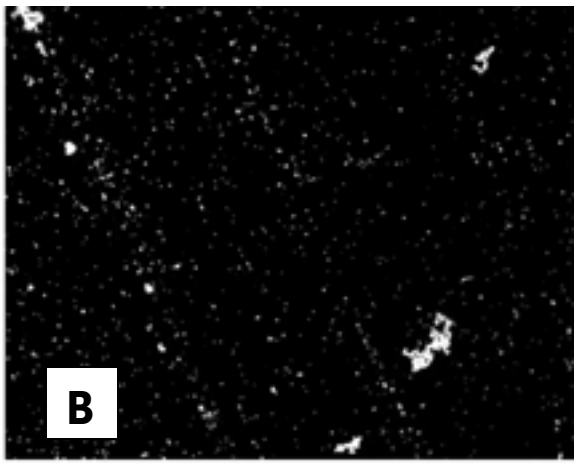
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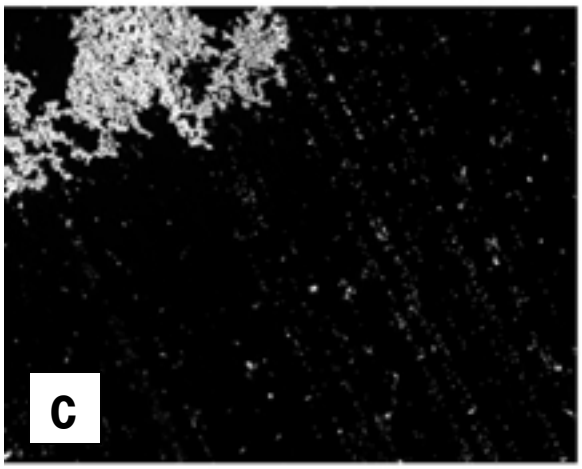
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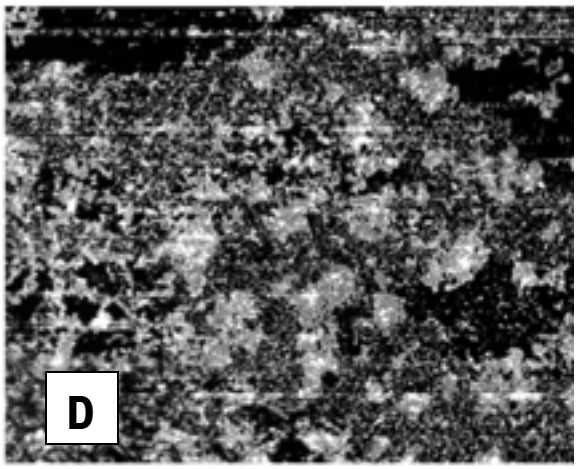
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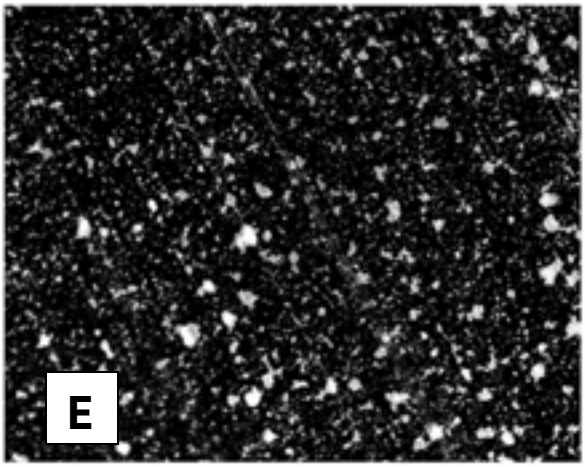
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CHAPTER 5

PROTEOMIC ANALYSIS OF A HYPOCHLOROUS ACID TOLERANT *LISTERIA MONOCYTOGENES* CULTURAL VARIANT EXHIBITING ENHANCED BIOFILM PRODUCTION[20]

[20] Folsom, JP and Frank, JF 2006. To be submitted to the Journal of Food Protection

ABSTRACT

L monocytogenes cultural variants exhibiting increased biofilm production and hypochlorous acid tolerance were isolated from cell suspensions of SA exposed to hypochlorous acid. The objective of this study was to compare the protein expression of a selected cultural variant to the S-form, SA, and identify proteins associated with biofilm production and/or chlorine tolerance. The cultural variant will be selected from a group based on biofilm production and chlorine tolerance. Suspension chlorine tolerance for each cultural variant was determined by exposure to 60 to 120 ppm hypochlorous acid for 5 min. Biofilm hypochlorous acid tolerance of SA and the cultural variants was determined by growing biofilms on stainless steel and subsequent exposure to 200 ppm hypochlorous acid for 5 min. All cultural variant strains were able to survive 120 ppm of hypochlorous acid in suspension, which is twice that of the S-form. There was little difference in the hypochlorous acid tolerance of the cultural variant planktonic cells. The cultural variants produced greater amounts of biofilm than the S-form, and were more hypochlorous acid tolerant. Strain SBS was selected for proteomic comparison because it produced the most biofilm and was most tolerant of hypochlorous acid when grown as a biofilm. Two dimensional difference gel electrophoresis was used to compare protein expression of planktonic and biofilm cells of SBS to SA. The 50s ribosomal protein, L10 was down regulated in biofilm SBS. Other proteins down regulated in planktonic SBS were the peroxide resistance protein (Dpr), and a sugar binding protein (LMO0181). This sugar binding protein was also up regulated in biofilm SBS. One spot down regulated in planktonic SBS contained both 50s ribosomal protein L7/L12 and an unknown protein (LMO1888).

INTRODUCTION

The usual form of *L. monocytogenes* is known as the S-form, and produces smooth, circular colonies with a glistening surface. The S-form dissociates to form a variety of cultural variants. Rough cultural variants (R-form) of *Listeria monocytogenes* produce rough colonies on agar and flaky growth with a surface pellicle in broth *(137)*. R-forms of *L. monocytogenes* do not exhibit blue sheen with Henry illumination, and lack the translucence normally exhibited by the S-form. While the R-form does not revert to the S-form *(137)*, they may develop into further cultural variants that produce colonies with smooth edges *(98)*.

Several conditions promote the development of R-form *L. monocytogenes*. Colonies of *L. monocytogenes* incubated for several days on plates often dissociate to produce the R-forms *(53, 81)*, and lowering the pH increases the conversion rate *(53)*. Rowan and Anderson *(128)* isolated R-forms from milk heated after inoculation with *L. monocytogenes*. R-forms have also been isolated from infected mice *(118)*, and result from growth as a biofilm *(98)*. Evidence indicates that this variation in morphology is associated with stress response, as R-forms exhibit increased adherence and greater heat resistance than the S-form *(128)*.

There are several classes of R-form *L. monocytogenes*. Kuhn and Goebel *(77)* studied a small number of R-forms and found that 3 (n=4) of them produced lower amounts of a protein they named p60 (iap or cwhA), in the supernatant. These R-forms (type I or MCR) grow as filaments with regular septation *(129)* without separation of cells, and exhibit reduced virulence *(54, 77, 118)*. However, it is not clear if p60 plays a role in the R-form, as Wuenscher et al. *(163)* found that complementation of an R-form with p60 resulted in conversion to S-form. However it was later found that p60 null mutants produce smooth colonies on agar *(116)*, and that similar strains contain mutations in a gene (secA2) responsible to for the secretion of p60

and other proteins *(81)*. This recent observation is consistent with another observation of Wuenscher et al. *(163)* that complementation with p60 did not restore full expression of p60 in the supernatant. Another class of R-forms that produce normal amounts of p60, are virulent, and produce filaments with variable septation with no separation of cells (Type II or FR) have also been found *(81, 129, 130)* and this class does not exhibit mutations in secA2 *(81)*. Still other R-forms of *L. monocytogenes* exist. Monk et al. *(98)* described R-forms that produce low levels of p60 without exhibiting mutations in *secA2,* and also produced filaments of unseparated cells. No genes have been identified to account for these R-form cultural variants.

This research describes the isolation of R-form and smooth colony cultural variants of *L. monocytogenes*, which exhibit increased biofilm production and hypochlorous acid tolerance compared to the S-form. Our objective is to select one of these cultural variants for proteomic study to identify proteins whose expression is affected, and to identify proteins that may be related to increased biofilm production and hypochlorous acid tolerance.

MATERIALS AND METHODS

Rough *L. monocytogenes* strains. During previous work chlorination of a cell suspension of *Listeria monocytogenes* SA resulted in an R-form cultural variants of *L. monocytogenes (43)*. Preliminary testing of this cultural variant (SAR) in suspension indicated it was more tolerant of hypochlorous acid than SA. After chlorination of SAR, a colony was isolated (SAR5). SAR5 exhibits morphological characteristics similar to SAR. During the enumeration of R-form inocula prior to chlorination, a smooth cultural variant was found (SBS). A similar occurrence was described Monk et al. *(98)* SBS produces smooth colonies on TSA, that differ from the colonies of SA in color and had slight surface texturing not present on SA

colonies. All strains were maintained at -80ºC in Microbank™ bead vials (PRO-LAB Diagnostics, Richmond Hill, Ontario, Canada).

Characterization of rough mutants. Cultural variants were characterized by the CAMP reaction, presence β-hemolysis, motility, oxidase reaction and the API *Listeria* system. β-Hemolysis and CAMP reaction using β-lysin disks were determined as previously described *(90)*. Motility was determined by stabbing isolates into motility agar (Remel, Lenexa Kans.). The oxidase reaction was determined using oxidase reagent droppers (Becton Dickinson Microbiology systems, Sparks, Md.) according to the manufacturer's instructions. Isolates were speciated using the API Listeria system (BioMérieux, Hazelwood, Mo.). Eighteen-hour growth of each strain was taken from TSA, and wet mounts in phosphate buffered saline were prepared for phase contrast microscopy (Nikon Eclipse E600 epifluorescent microscope, Southern Micro instruments, Marietta Ga.) and micrographs (Magnafire CCD camera, Southern Micro instruments) of the cells were made. Macroscopic images of the colonial morphology on tryptic soy agar (TSA; Becton Dickinson Microbiology systems)and Listeria selective agar (LSA; Becton Dickinson Microbiology systems) were made at 30x (American Optical, model Forty, Buffalo, N.Y.).

Preparation of hypochlorous acid solution. Hypochlorous acid solutions of desired concentration were prepared each day from a sodium hypochlorite stock solution (100 g/l) (Mallinckrodt Baker, Inc., Paris Ky.). The solutions were prepared in water free of organics (deionized 18 million ohms/cm) buffered with 0.015 M KH_2PO_4. Glassware were thoroughly cleaned, soaked overnight in strong bleach solution, and rinsed in deionized water. After preparation the hypochlorous acid solution was adjusted to pH 7.00 ± 0.05, and the concentration of hypochlorous acid was verified by UV spectroscopy using the molar absorption constants

OCl^-; 99.6 M^{-1} cm^{-1} at 235 nm and 26.9 M^{-1} cm^{-1} at 290nm, HOCl; 7.8M^{-1} cm^{-1} at 235 nm and 350.4 M^{-1} cm^{-1} at 290 nm *(99)*.

Planktonic hypochlorous acid tolerance. The hypochlorous acid tolerance of plankonic cells of strains SAR, SAR5, and SBS was determined by exposing cell suspensions to 60 to 120 ppm hypochlorous acid in 20 ppm increments. Each was inoculated from beads into 5 ml tryptic soy broth (TSB; Becton Dickinson Microbiology systems) and grown 20h statically at 32ºC in screw cap test tubes prior to their use for hypochlorous acid challenge. Hypochlorous acid challenge inoculums were enumerated on tryptic soy agar with 6 g/l yeast extract (TSAYE; Becton Dickinson Microbiology systems) using a Spiral Biotech AP4000 (Advanced Instruments, Inc., Norwood, Mass.). After growth, aliquots of culture (100 µl) were placed into a test tube, and five ml of standardized sodium hypochlorite solution was added to each tube and vortexed. After five min the hypochlorous acid was quenched with 1 ml of 0.63M sodium thiosulfate. Four ml of double strength TSB was then added to each tube and the tubes were incubated at 32ºC. These tubes were observed for five days and any turbidity was noted. Exposures to hypochlorous acid were in triplicate per replication, and the experiment was replicated three times. The contents of any tubes exhibiting growth were streaked onto RAPID'L.MONO® media (Bio-Rad Labs, Hercules Calif.) to confirm *L. monocytogenes* growth. The number of tubes with surviving *L. monocytogenes* was recorded for each isolate at each level of hypochlorous acid.

Biofilm hypochlorous acid tolerance. Biofilms of SA, SAR, SAR5 and SBS were grown on stainless steel (type 304, finish 4b) coupons measuring 11 cm by 7.5 cm. For this experiment each isolate was inoculated from beads into 5 ml TSB and grown 20h statically at 32ºC in screw cap test tubes prior to sub-culturing for an additional 20h at 32ºC to yield 200 ml of culture.

Two coupons per strain were submerged in 200 ml of each culture of *L. monocytogenes*, and incubated at 32ºC for 4h in sterile plastic pans (Cambro H-Pan 62HP with lid 60HPC, Cambro, Huntington Beach, Calif.). Pans containing coupons were shaken at 70 rpm in a C24 incubator shaker (New Brunswick Scientific, Edison, N.J.). After 4h coupons were removed and placed in sterile sampling bags (Fisher brand 01-815-25) with 200 mls of 0.015 M KH_2PO_4 adjusted to pH 7.0 (phosphate buffer) and hand shaken vigorously for 10 s. After rinsing, the coupons were transferred to sterile plastic pans containing 200 ml of fresh TSB and incubated for and additional 24h at 32ºC and 70 rpm. The coupons were washed using a wash bottle (Nalgene #2405-0500, Rochester, N.Y.), with nozzle widened by trimming (Bore shape/size, oval/3.4mm X 2.2 mm). Phosphate buffer (50 ml) was only applied to the top front of the coupon, and the back was washed by directing the stream of buffer evenly from top to bottom. After washing coupons were placed biofilm side up into separate stainless steel pans (Ollrath, Super Pan II NO. 3062-2/L33, Sheboygan Wis.). The prepared biofilms of each strain were treated with 200 ml of 200 ppm hypochlorous acid or phosphate buffer. A sterile stir bar was placed underneath the coupon during hypochlorous acid treatment to prevent the entrapment of cells, and release of viable cells after the hypochlorous acid was neutralized.

Biofilms prepared and treated with phosphate buffer (untreated controls) were agitated for 10 min at 70 rpm (Orbit shaker, Lab Line Instruments, INC, Melrose Park, Ill.), after which they were again rinsed with about 20 ml of phosphate buffer using a wash bottle and transferred to a Petri dish. Attached cells were enumerated by scraping with a Teflon policeman (VWR #13197-416, Bel-Art, Pequannock, N.J.) and rinsing with phosphate buffer three times, such that cells were collected with a total of 100 ml of phosphate buffer. The detached cells were shaken in milk dilution bottles for 10 min on a wrist action shaker (Burrel Scientific model 75, Pittsburgh,

Pa.) at maximum for 10 min, to break up cell clumps prior to enumeration. The number of detached biofilm cells was reported as log CFU/50 cm^2.

Biofilms prepared for treatment with hypochlorous acid were agitated for 5 min before the hypochlorous acid was neutralized with 175 ml of 0.2M sodium thiosulfate (final concentration 0.1 M). Neutralization of hypochlorous acid was verified using Aquacheck™ total/free Hypochlorous acid test strips (Hach Company, Loveland, Colo.). The coupons were then agitated for another 5 min, rinsed with about 20 ml of buffer and transferred to a Petri dish. It was necessary to determine surviving numbers of *L. monocytogenes* by overlaying the coupons with TSAYE, as scraping and enumeration was not sufficiently sensitive to detect survivors. TSAYE was supplemented with 1 g/liter added sodium pyruvate to enhance recovery of hypochlorous acid injured cells and 0.03 g/liter potassium tellurite to improve visualization of colonies. Survivors were reported as log CFU/50 cm^2.

Preparation of cells for protein extraction. Planktonic cells of SA and SBS were inoculated from beads into 5 ml TSB and grown 20h statically at 32ºC in screw cap test tubes prior to sub-culturing for an additional 20h at 32ºC to yield 250 ml. Cells were harvested by centrifugation (5000g, 5 min, 20ºC) and washed three times in 40mM tri buffer (pH=8.5) and suspended in 1ml of lysis buffer (Tris 40mM, Urea 8M, 4% CHAPS; pH=8) before protein extraction. Biofilm cells of SA and SBS were prepared similar to the method of Tremoulet et al. *(152)*. Planktonic cells of SA and SBS were inoculated from beads into 5 ml TSB and grown 20h statically at 32ºC in screw cap test tubes prior to sub-culturing for an additional 20h at 32ºC to yield 10 ml, and resuspended in 13 ml of phosphate buffer. Ten sterile Glass fiber filters (extra thick glass fiber filter, Pall #66084, Pall Life Sciences, Ann Arbor Mich.) were placed in 150 mm Petri dishes (1 filter per dish) and the inocula was dispensed onto the filter. Filters were

incubated at room temperature for 5 min to allow attachment. The unattached cells were washed from the filter by swirling in 200 ml of phosphate buffer on an orbital shaker at 60 rpm for one min. Individual filters were washed in a sterile 150 X 75 mm Pyrex dish. After washing the filters were transferred to a 150 mm Petri dish containing TSAYE. A sterile glass fiber filter was placed between the agar surface and the inoculated filter, to provide for diffusion of nutrients from the agar surface to the inoculated filter. Filters were incubated at 32ºC for 72 h; washed in ice cold phosphate buffer in the manner described previously, and placed in filtered stomacher bags (Nasco Whirl-Pak B01318, Fisher Scientific, Atlanta, Ga.). The filters were kept refrigerated (≈5ºC,<1 h) until 100 ml of ice cold 40mM Tris (pH=8.5) was added after which the filters were stomached for 5 min. The bacterial suspension was harvested and kept on ice. The contents of the stomacher bag were allowed to drain through another filtered stomacher bag, in order to remove more of the glass wool (Nasco Whirl-Pak B01318). The filtrate was then centrifuged at 10,000 rpm for 30 min, and the pellet resuspended in 40 ml of cold Tris (pH=8.5), and filtered using 100 micron Steriflip filters (Millipore, Billerica, Mass.). The biofilm cells were washed two more times with cold Tris (pH=8.5) and suspended in 1ml of lysis buffer. The cells were stored at -80ºC until protein extraction.

Protein extraction and purification. Protein extraction was accomplished by sonication of planktonic cells and biofilm cells on ice (50% power/50% pulse/2 min increments for 10 min, Omni Ruptor 400, Omni International INC, Marietta, Ga.). The lysate was centrifuged at 9500 rpm for 10 min at 4ºC, and the pellets were extracted using a ReadyPrep sequential extraction kit according to manufacturer's instructions for extraction 2 and 3 (Bio-Rad Laboratories, Hercules, Calif.). The resulting protein was further purified using an Ettan 2-D clean up kit (GE Healthcare, Piscataway, NJ), and resuspended in reagent 3 (ReadyPrep sequential extraction kit,

Bio-Rad Laboratories) and frozen at -80ºC. The three fractions were combined and protein concentration was determined by Bradford's method using the Bio-Rad protein assay (Bio-Rad Laboratories), according to manufacturer's instructions.

Fluorescence 2-D Difference Gel Electrophoresis (DIGE). 50 or 100 µg of protein was labeled using Cyanine dye DIGE Flours (cy3 or cy5, GE Healthcare). Briefly, the protein suspension was mixed with 1 or 2 µl of cy dye (200 pmol in dimethylformamide (DMF)) and incubated on ice for 30 min. The labeling reaction was stopped by the addition 1 µl of lysine (10mM), and the samples were incubated on ice for 10 min. After labeling, the volume was brought up to 150 µl with reagent 3. The cy3 and cy5 labeled proteins were multiplexed onto one 17 cm Readystrip IPG strip (IPG strip; Bio-Rad Laboratories) for isoelectric focusing.

All 17 cm IPG strips were rehydrated (50V for 12hours) with the multiplexed samples. Isoelectric focusing (Protean IEF cell, Bio-Rad Laboratories) was performed according to the programming recommended by the manufacturer for each pH range. Proteins were focused on IPG strips at three different pH ranges (3-10NL, 4-7 and 4.7-5.9, Bio-Rad Laboratories). After isoelectric focusing, the IPG strips were equilibrated for SDS PAGE with equilibration buffer I and II (Bio-Rad Laboratories) according to manufacturer's instructions. IPG strips were loaded onto 1 mM Tris-glycine gradient (8% to 15%) gels (Jule, Inc, Milford, Conn.). Electrophoresis of gels was performed using an Ettan Dalt II (GE Healthcare) at 5 watts per gel for 1h and 2 watts per gel overnight. Electrophoresis was completed at 45 mA per gel. For each pH range, protein from planktonic cells of SBS and SA were compared on a single gel, and proteins from biofilms of those strains were compared. For the 3-10NL and 4-7 strips 100 µg of protein was loaded, and for the 4.7-5.9 strips 50 µg was loaded.

Gel imaging. Labeled proteins were imaged using a Typhoon 9400 imager (GE Healthcare) resulting in 2 images. Gel images were analyzed with Decyder DIA software (GE Healthcare) and proteins spots were chosen if expression changed more than 2-fold. Gels were then stained with sypro ruby (Invitrogen, Carlsbad, Calif.) and DeCyder BVA was used to align the picked spots from the cy dye images with the Sypro ruby images.

Peptide sequencing. 2.0 mm gel plugs were picked, and automatically prepared for mass spectrometry using a Spot Handling Workstation (GE Healthcare). The plugs were washed twice with 50 mM ammonium bicarbonate/50% methanol for 20 min at room temperature, followed by washing with 75% acetonitrile for 20 min at room temperature. The plugs were dried (40ºC for 10 min). The plugs were then incubated in 10 mM dithiothreitol (DTT)/20 mM ammonium bicarbonate at 37^{o}C for 1h, followed by 100 mM iodoacetamide/20 mM ammonium bicarbonate (room temperature 30 min). Protein in the plug was digested with trypsin (200 ng trypsin, 37ºC for 2h), and peptides were extracted twice with 50% acetonitrile/0.1% trifluoroacetic acid (20 min room temperature) and solvents removed by evaporation at 40ºC for 30 min. (SpeedVac, Jouan, Winchester, Va.). Approximately 25% of the resulting peptides were applied to the mass spectrometry target plate with partially saturated α-cyano-4-hydroxy-cinnamic acid (Sigma-Aldrich, St. Louis, Mo.).

Mass spectrometry data were acquired using the 4700 Proteomics Analyzer (Applied Biosystems, Foster City, Calif.) calibrated using two trypsin autolysis peaks (1045.45 and 2211.096 m/z), using standard acquisition methods. Mass lists were submitted to a sub-database of NCBInr (*Listeria sp*.) using Mascot v. 1.9.05 (http://www.matrixscience.com/), considering fixed cysteine, carbamidomethylation and methionine oxidation modifications, 1 missed tryptic

cleavage, and 35 ppm mass accuracy. Identifications were cross-examined using mass accuracy, molecular weight, and isoelectric point (pI).

RESULTS

SAR and SAR5 produced large, rough, light beige colonies that are opaque on TSA, while SBS produced smooth, beige, opaque colonies on TSA that are slightly smaller than the S-form. These morphologies are unlike the white or bluish translucent colonies of typical *L. monocytogenes* strains (fig. 5.1). When grown on LSA all cultural variants were smooth, and difficult to discern from the S-form (not shown). R-form cultural variants of *L. monocytogenes* with similar colony morphology and growth characteristics have been previously described *(53, 54, 77, 78, 81, 128, 137)*. Growth of all three cultural variants in TSB was flaky with a surface pellicle as described by Seeliger *(137)*, however our cultural variants did not produce filaments like other reported R-forms (fig. 5.1). Cells of these cultural variants tended clump (fig. 5.1) and when challenged with hypochlorous acid, the survivors grow only as colonies attached to the glass surface of the test tubes for many hours before resuming planktonic growth. This behavior was also observed by To et al. *(151)*, when challenging *L. monocytogenes* strains with benzalkonium chloride. Results of testing the identity of the cultural variants indicated all were *L. monocytogenes*.

All cultural variants (SAR, SAR5, and SBS) were able to survive 120 ppm of hypochlorous acid when challenged in suspension (table 5.1), and this is two times higher than the concentration the parent strain SA survived *(43)*. There was no difference amongst the strains in regards to suspension hypochlorous acid tolerance was (table 5.1). Biofilms produced by the cultural variants survived treatment with 200 ppm hypochlorous acid (table 5.2). In contrast to results obtained on cell suspensions, biofilms of cultural variant SBS were

significantly more tolerant of hypochlorous acid than the other cultural variants. SBS also produced a biofilm that contained 10-fold more cells than the others did. While all cultural variants survived a much higher levels of hypochlorous acid than the S-form, they also produced over 1000-fold more biofilm cells than SA when grown as a biofilm (table 5.2). SBS was chosen for proteomic comparison because of it's superior biofilm production, and biofilm chlorine tolerance.

Five of these proteins were found on the 3-10 range gel, eight in the 4-7 range gel and 2 on the 4.7-5.9 range gel. Four proteins were up regulated in planktonic SBS, but none could be identified; 2 were found on the 4-7 range gel and 2 found on the 4.7-5.9 range gel. Fifteen proteins were down regulated in biofilm grown SBS cells of which one was identified. Two were found on the 3-10 range gel, nine on the 4-7 range gel, and 4 on the 4.7-5.9 range gel. Seven proteins were up regulated in biofilm grown SBS cells of which one was identified; with four of those found on the 4-7 range gel, and the other 3 on the 4.7-5.9 range gel. Details of the identified proteins are listed in Table 5.3.

DISCUSSION

The procedures used to assess the hypochlorous acid tolerance in our previous work *(43)* were modified to accommodate the increased biofilm produced by the cultural variants. Despite agitation of the medium during growth, the biofilms of SBS were easily removed by the washing procedures used in our previous work *(43)*. This necessitated modifying the washing procedure as described in the methodology section. Monk et al. *(98)* also experienced difficulty washing the biofilms of cultural variants grown in a bioreactor, noting that some of the biofilm structure would be lost to washing despite growth in turbulent flow.

Culture preparation was changed because surface pellicle, flakiness, and biofilm production diminished with subculturing. However, changes in colony morphology were not observed, and the variants did not revert to the S-form. Drenkard and Ausubel *(32)* observed a similar phenomenon with *Pseudomonas aeruginosa* PA14. They found that Pseudomonas aeruginosa PA14 cultured in the presence of antibiotics produced atypical variants that were multi-drug resistant and produced rough or smooth small colonies. These *P. aeruginosa* variants were more hydrophobic than the wild type, and exhibited enhanced attachment and biofilm production. The biofilms that were formed were unlike those produced by the wild type, exhibited increased cell mass and were multi drug resistant. The incidence of this phenotypic variation was affected by environmental stimuli such as NaCl concentration, growth temperature, and nutrient availability. The authors found that the emergence of the variants was regulated by a putative two component regulatory system. However, these variants would completely revert to the wild type after several days' cultivation in the absence of antibiotic.

L. monocytogenes studied in this work were cultural variants of the same strain, thus the small number of proteins (19-22) found to be altered in expression was expected. Because there are no protein expression changes due to strain difference, we expect that proteins found in this research are involved with the cultural variation and may include proteins associated with increased biofilm production or hypochlorous acid tolerance. However, it is possible that many more proteins exhibited altered expression, as much of the protein extracted was not properly resolved, due to the preponderance of proteins with an isoelectric point between pH 4 to 6 *(42)*. The pH 3-10 and pH 4 to 7 IPG strips became overloaded (Figure 5.2) resulting in a large area on the 2nd dimension gels where protein spots overlap. This area was better resolved by narrowing

the pH range and lowering the protein load, but some proteins outside the pH range of 4 to 6 may not have been detected, since overloading may impede proper isoelectric focusing.

The peroxide resistance protein (Dpr/fri/flp) down regulated in the SBS cultural variant is an iron binding ferritin similar to the DNA binding protein Dps of *Escherichia coli*; however Dpr does not bind DNA *(14)*. The mRNA of Dpr is transcribed by either σ^A or σ^B *(117)* and the gene is also under the regulatory control of the peroxide resistance regulon *(109)*. The regulatory element of this regulon is PerR, a metal binding protein whose negative regulation is relieved during oxidative stress, allowing transcription of a wide variety of peroxide resistance genes, including Dpr. Mutants lacking functional PerR are extremely resistant to hydrogen peroxide *(123)*. Dpr is also linked to oxygen tolerance in other organisms *(109, 164)*. This is a result of the iron sequestering function of Dpr; which inhibits the formation of hydroxyl radicals formed via the Fenton reaction *(164)*. While one might expect that decreased expression of Dpr in planktonic SBS would result in decreased tolerance to hypochlorous acid, the observation that SBS is more tolerant of hypochlorous acid indicates that the peroxide resistance regulon does not have an important role in hypochlorous acid tolerance as is the case with the *E. coli* homologue, the oxyR regulon *(36)*. However, the flaky growth, surface pellicle and petite colony characteristics may be associated with reduced oxygen tolerance due to decreased expression of Dpr.

Sugar binding protein components of gram positive ABC transporters are anchored in the cell membrane by attachment of a lipid to an n-terminal cysteine. The first twenty amino acids of LMO0181 precede a cysteine and are consistent with other lipodation signals *(147)*. The LMO0181 gene coding for this protein is a member of an operon that includes seven proteins in total (LMO0178-LMO0184). These genes encode a repressor of xylose metabolism, and other

genes related to the breakdown and transport of carbohydrates *(50, 96)*. The regulation of this operon is uncertain, but evidence indicates that it maybe negatively regulated by the virulence regulator prfA *(96)*. The reduced expression of this operon in planktonic SBS, and increased expression in biofilm grown SBS may indicate that the protein expression changes found in SBS result in additional carbon sources being available for biofilm formation. Thus, these changes in regulation could be responsible for increased biofilm production of SBS.

The organization of the genes encoding L7/L12 and L10 of *L. monocytogenes* is the same as found in *E. coli (50)*. Ribosomal protein L7/L12 (RpiL) is one of many proteins that are associated with the 50s ribosomal subunit. Four L7/L12 proteins form a protein complex with ribosomal protein L10, and are required for protein elongation. Ribosomal protein L7 is formed from L12 by acylation of the n-terminus, and is functionally identical to L12 *(104, 113)*. While the ratio of L7 to L12 varies by growth conditions, it is not known why *(21)*. Since growth of SBS is not impaired compared SA, it is unlikely that protein synthesis is decreased by the decreased expression of L10 in biofilm grown SBS and L7/L12 in planktonic grown SBS. Clearly, there must be an alternative function for these proteins. Research indicates the possibility that L7/L12 may be altered to perform other cellular roles *(100, 101)*, and a protein structurally similar to L7/L12 whose gene is located in a similar position in the genome as L7/L12 functions as surface located virulence factor in *N. gonorrhoeae (143)*. These observations are consistent with the results of others who found that ribosomal protein L7/L12 expression is unaffected by growth as a biofilm *(56)*, and transition into stationery phase *(42)*. Since amino acid starvation reduces ribosome production *(104)*, one would expect ribosomal proteins to be down regulated under those conditions. Ribosomal protein L10 however is down

regulated on entry into stationery phase *(42)*, casting doubt on an alternative function, and there is no evidence for other cellular roles.

DivIVA is involved with the initiation of cell division and LMO1888 shares 51% homology with the Bacillus cereus DivIVA (NCBI ACCESSION AAP08541). While another protein has been ascribed the function of DivIVA in *Listeria* spp. (NCBI ACCESSION CAD00098), it seems likely that this protein plays some role in cell division because of this homology.

We have observed a change in expression of at least five proteins by cultural variants of *L. monocytogenes* isolated after exposure to hypochlorous acid. These patterns of expression were concomitant with either hypochlorous acid tolerance, or increased biofilm production. The proteins affected are diverse in function and likely regulated by several systems. The cultural variants described in this work occurred with the use of a common food processing plant sanitizer. There is a need to understand the frequency with which such cultural variants arise in the food processing environment. If they are common, then it will be important to determine how virulent these strains may be, in addition to their survival characteristics.

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TABLE 5.1. Survival of planktonic cultural variant strains of *Listeria monocytogenes* exposed to hypochlorous acid (60-120ppm) for 5 min.

	# Tubes out of 9 with growth				
Strain	60 PPM	80 PPM	100 PPM	120 PPM	Inoculum log (cfu/ml)
SAR[a]	9	9	8	1	9.44
SBS[b]	9	9	5	2	9.42
SAR5[a]	9	8	8	2	9.39

[a]Cultural variant exhibiting rough colony morphology
[b]Cultural variant exhibiting smooth colony morphology

TABLE 5.2. Biofilm growth and survival of biofilms exposed to 200 ppm hypochlorous acid for 5 min.

		Hypochlorous acid survivors (cfu/50 cm^2)		
Strain	Adherent cells Log(cfu/50 cm^2)	Rep 1	Rep 2	Rep 3
SA	6.71	1	0	0
SAR	10.11	16	1	6
SBS	10.98	>1000	>1000	>1000
SAR5	10.03	5	5	882

TABLE 5.3. Changes in the expression of proteins identified from peptides sequenced: Proteins are listed with size, isoelectric point (pI), protein coverage by the peptides (%), and the isoelectric focusing (IEF) range of the strip used.

Protein Name	IEF Range	Gene Name[a]	Size (KDa)[c]	pI[c]	(%) of Protein covered	X-Fold Expression change
Protein expression changes in biofilm grown SBS						
Ribosomal protein L10	4-7	RplJ	17.8	5.4	44	-2.2
ABC Transport: Sugar binding	4-7	LMO0181	46.8	4.6	45	+2.4
ABC Transport: Sugar binding	4.7-5.9	LMO0181	46.8	4.6	26	+2.0
Protein expression changes in planktonic SBS						
ABC Transport: Sugar binding	4-7	LMO0181	46.8	4.6	43	-2.74
Peroxide resistance protein (Dpr)	4-7	fri	18.2	4.9	49	-2.2
Hypothetical protein	4-7	LMO1888	12.9	4.6	48	-2.1[b]
Ribosomal protein L7/L12	4-7	RplL	12.5	4.6	20	

[a]ListiList gene name *(50)*
[b]Protein spot yielded two proteins
[c]Isoelectric points (pI) and sizes presented are the calculated results.

Figure 5.1. Images of colonies illustrating colony and cell morphology of *L. monocytogenes* strains SA (a), SBS (b), SAR. (c) SAR5 (d). Images on left phase contrast images (1000X, bar=10μm). Images on right stereoscope image (30X, bar=1 mm).

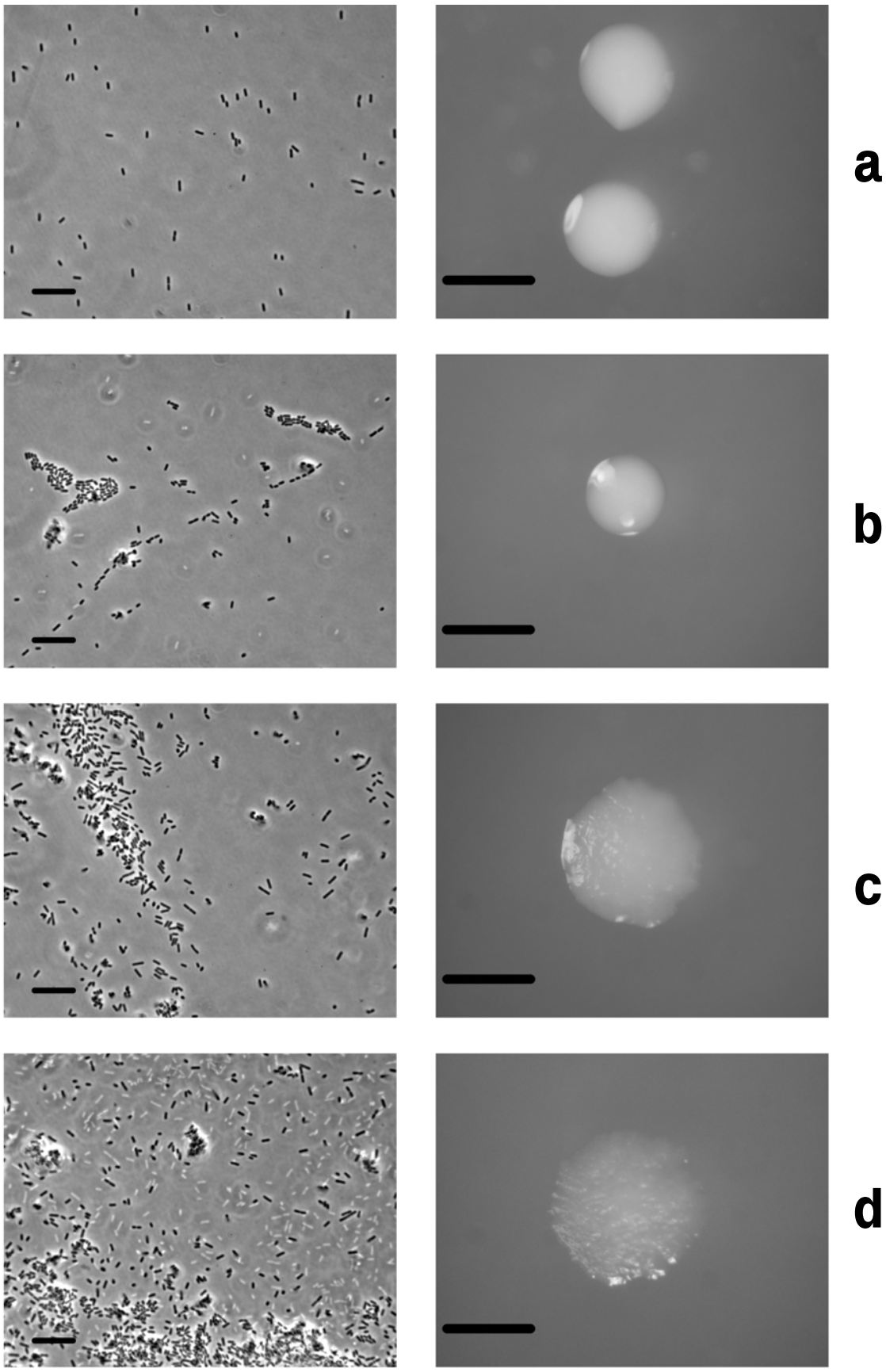
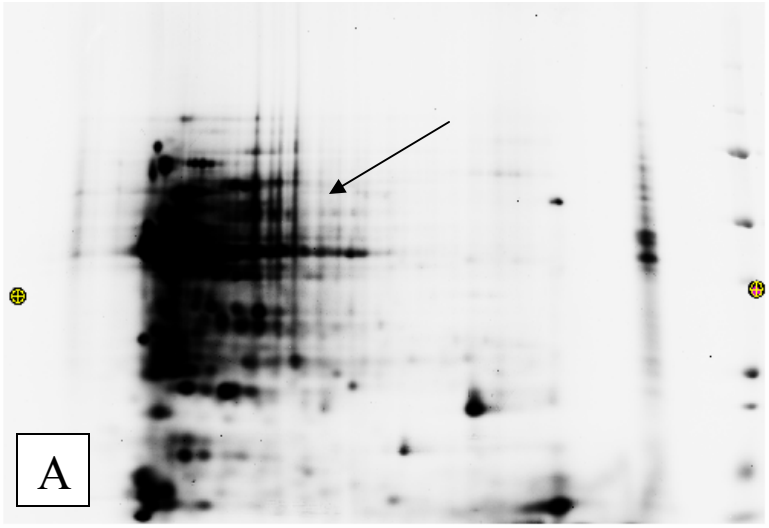
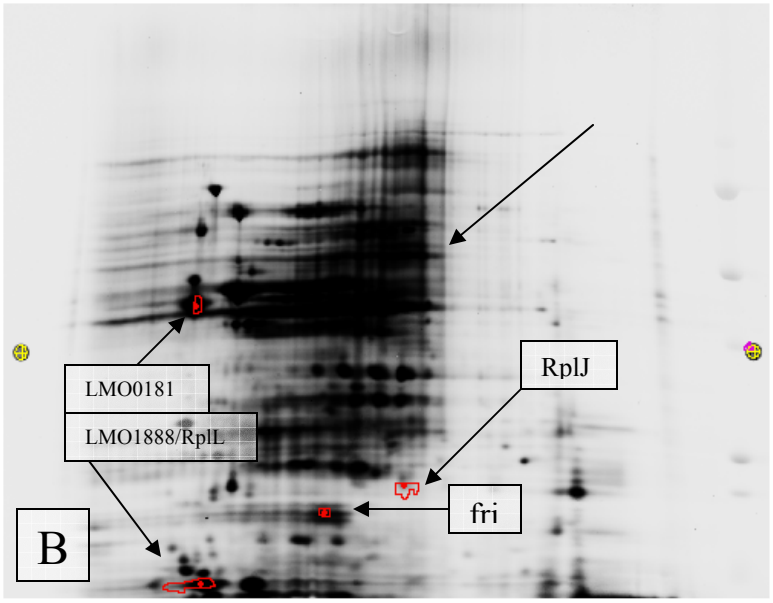
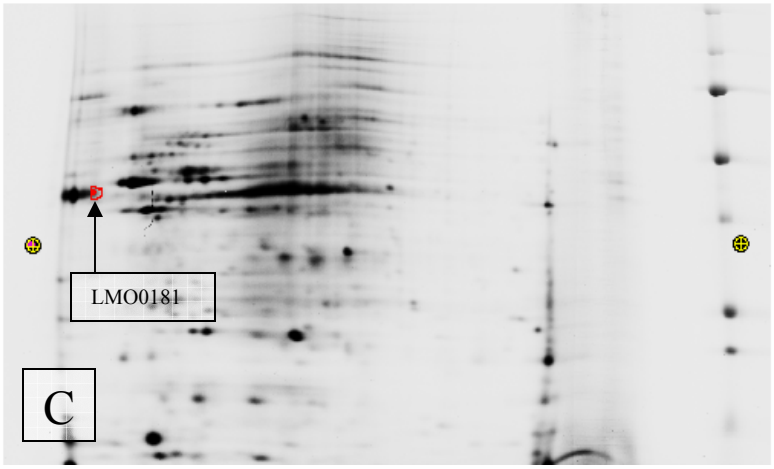

a
b
c
d

FIGURE 5.2. Images of representative 2D gels with identified proteins indicated by ListiList gene name*(50)*. A. pH range 3 to 10. B. pH range 4 to 7. C. pH range 4.7 to 5.9. Regions of the gel where proteins were poorly resolved are indicated by arrows.


A


LMO0181
LMO1888/RplL
RplJ
fri
B


LMO0181
C

CHAPTER 6

SUMMARY

Strains of *Listeria monocytogenes* produce differing amounts of biofilm accumulation according to serotype and growth media. Certain strains accumulate more biofilm than others. Serotype 4b strains produce more biofilm when grown in tryptic soy broth (TSB), and serotype 1/2a strains produce more biofilm when grown in 1:10 diluted TSB. Growth in TSB represses the biofilm accumulation of serotype 4b strains.

Strains of *Listeria monocytogenes* exhibit differing degrees of hypochlorous acid tolerance when challenged in suspension or as a biofilm. While suspension hypochlorous acid tolerance is not similar for closely related strains, many strains with similar biofilm hypochlorous acid tolerance are also closely related. Biofilm hypochlorous acid tolerance was not associated with biofilm cell density or biofilm morphology. The hypochlorous acid tolerance mechanisms for suspension hypochlorous acid tolerance and biofilm hypochlorous acid tolerance are different.

Cultural variants of *Listeria monocytogenes* may result from exposure of the S-form to stressful conditions. Cultural variants found in this work may exhibit rough or smooth colony morphology, are significantly more hypochlorous acid tolerant than the S-form, and they produce biofilms that contain more viable cells. Proteomic analysis of a smooth cultural variant indicates that expression of several proteins is altered during planktonic and biofilm growth when compared to the S-form.

Proteins involved with oxidative stress, sugar transport, protein translation, and unknown cell division functions were found. The peroxide resistance protein (Dpr/fri/flp) is down regulated by the SBS cultural variant and is an iron binding ferritin similar to the protein Dps of *Escherichia coli*; however Dpr does not bind DNA *(14)*. Dpr plays a role in oxygen tolerance *(109, 164)*, because of it's iron sequestering function; which inhibits the formation of hydroxyl radicals formed via the Fenton reaction *(164)*. Thus, the flaky growth, surface pellicle and petite colony characteristics may be associated with reduced oxygen tolerance due to decreased expression of Dpr. Expression LMO0181 is reduced planktonic SBS, and is increased in biofilm grown SBS. LMO0181 is a sugar binding protein and part of an operon that include a repressor of xylose metabolism, and other genes related to the breakdown and transport of carbohydrates *(50, 96)*. The reduced expression of this operon in planktonic SBS, and increased expression in biofilm grown SBS may indicate that the protein expression changes found in SBS result in additional carbon sources being available for biofilm formation. LMO1888 is a protein of uncertain function though it shares 51% homology with the Bacillus cereus DivIVA (NCBI ACCESSION AAP08541), another protein has been ascribed this function in *Listeria* spp. (NCBI ACCESSION CAD00098). Ribosomal protein L7/L12 may be decreased in planktonic SBS, and ribosomal protein L10 is down regulated in biofilm SBS. Ribosomal protein L7/L12 (RpiL) is one of many proteins that are associated with the 50s ribosomal subunit. Ribosomal protein L7 is formed from L12 by acylation of the n-terminus, and is functionally identical to L12. Growth of SBS is not impaired compared SA, and it is unlikely that protein synthesis is decreased as a result of these expression changes.

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