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Cell Wall and Autolytic Properties of a Cold-Sensitive Listeria monocytogenes Mutant Deficient in Branched-Chain Fatty Acid Biosynthesis

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CELL WALL AND AUTOLYTIC PROPERTIES OF A COLD-SENSITIVE *LISTERIA MONOCYTOGENES* MUTANT DEFICIENT IN BRANCHED-CHAIN FATTY ACID BIOSYNTHESIS

Anthony M. Martini

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Cellular membranes modulate several critical functions of bacterial physiology, including solute diffusion and nutrient transport. Biophysical properties of phospholipids, as well as environmental pressures such as temperature, can have a profound effect on these membrane functions and the kinetics of associated enzymes. *Listeria monocytogenes* is a facultative intracellular pathogen capable of robust low temperature growth, a characteristic thought to be facilitated by a membrane highly enriched in branched-chain fatty acids (BCFAs). Mutants deficient in the biosynthesis of these fatty acids exhibit severe growth defects under low temperature and extreme pH conditions. Morphological investigations have also revealed these mutants to undergo aberrant cell division and separation, suggesting appropriate membrane fluidity may be integral to these processes. Proper cell division and bacterial morphology requires the coordinated activity of many membrane- and surface-associated proteins. In particular, cell wall structure, biosynthesis, and hydrolysis are integral components of this process, and the relationship between these molecular mechanisms and membrane fatty acid composition is poorly understood. This study seeks to elucidate potential determinants of aberrant cell division in the BCFA

biosynthesis-deficient mutant, MOR401. Properties of interest include autolytic rates, susceptibility to lytic enzymes, peptidoglycan hydrolase profiles, and surface-associated protein profiles. No statistically significant alterations to autolytic rate were observed for whole cells exposed to Triton X-100, mutanolysin, or lysozyme, while EDTA-treated MOR401 was observed to undergo decreased autolysis relative to the wild-type. In contrast, crude cell walls of MOR401 were observed to lyse more rapidly than wild-type. Zymographic analyses revealed decreased extracellular concentrations of certain higher molecular weight peptidoglycan hydrolases in the MOR401 strain, as well as the absence of a ca. 32 kDa enzyme known to hydrolyze *L. monocytogenes* and *Bacillus subtilis*, but not *Micrococcus luteus*, cell walls. Non-covalent surface protein profiles showed several bands of increased intensity in the MOR401 strain, while the covalently-attached surface proteins display a markedly distinct profile compared to the wild-type. Some of these altered properties may suggest alterations to cell wall chemistry, peptidoglycan hydrolase profiles, or reduced access of proteins to the extracellular space. To support future investigations, methods for the purification of cell wall and the extraction of teichoic acid and peptidoglycan have been refined. These procedures will facilitate the analysis of cell wall chemistry, structure, and macromolecular constituents.

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A.M.M.

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CHAPTER I

INTRODUCTION

Listeria monocytogenes is a Gram-positive facultative anaerobe, rod-shaped, non-spore-forming, highly adaptable pathogenic bacterium belonging to the low-GC content Firmicutes phylum (1–3). A ubiquitous organism, *L. monocytogenes* is accustomed to a saprophytic lifestyle associated with soil, plant matter, and animal reservoirs (1, 4, 5). A host of molecular mechanisms contribute to its robust environmental resilience and subsequently inform its pathogenic potential and mode of transmission. *L. monocytogenes* boasts the largest repertoire of carbohydrate transporters and two-component systems of any described pathogenic bacteria (6), shedding light on the organisms resistance to extreme pH and osmotic stress (2, 6). Importantly, *L. monocytogenes* possesses a wide growth temperature range, from an extreme of below zero to upwards of fifty degrees centigrade (1, 2). This array of physiological adaptations permits survival through many food processing techniques, highlighting its role as a predominantly foodborne pathogen associated with contaminated dairy products, raw vegetables and fruits, and processed meats (1, 7, 8). When associated with a mammalian host, *L. monocytogenes* undergoes profound regulatory alterations and adopts an intracellular, parasitic lifestyle (5).

Human infection with *L. monocytogenes* causes listeriosis (1), a potentially severe disease with several distinct etiologies. While infection with *L. monocytogenes* remains

fairly rare, it has a high case fatality rate ranging from 20-30%, routinely placing it behind non-typhoidal Salmonella as one of the leading bacterial causes of death from foodborne illness (3, 9, 10). Healthy, immunocompetent adults exposed to virulent strains of *L. monocytogenes* most often develop a non-invasive listeriosis which manifests as self-limiting gastroenteritis (7, 11). Invasive listeriosis is strongly associated with the immunocompromised (8), and is also prevalent in pregnant women and those at the extremes of age (8, 11). This form of listeriosis results in a persistent bacteremia and often invades the central nervous system (8, 11), resulting in meningoencephalitis (7), though other manifestations have also been observed (11). If infected during pregnancy, complications such as spontaneous abortion, stillbirth, and premature birth may result at fairly high frequency (7, 11). This invasive listeriosis may also pass to the surviving neonate, potentially developing into bacteremia or meningitis and other serious complications (7, 11). The high fatality rates associated with listeriosis persist even when accounting for appropriate antibiotic therapy (8), which typically comprises penicillin and ampicillin (7). The pathogenic potential of *L. monocytogenes* can be attributed to an arsenal of virulence factors promoting its intracellular lifestyle.

As a predominantly foodborne pathogen, invasion of the mammalian host by *L. monocytogenes* typically begins in the intestines (8). Following its survival through the intestinal tract, one of two internalins present on the bacterial surface mediates interaction with E-cadherin to facilitate crossing of the intestinal barrier into the bloodstream (8, 12). The other internalin is critical for invasion of other non-phagocytic cells. Macrophages subsequently traffick blood-borne bacteria to the spleen and liver, where innate immunity may quell the infection or permit sustained replication (3, 8). Survival within

macrophages is aided by the production of catalase and superoxide dismutase (1), two enzymes involved in disrupting reactive oxygen species produced by leukocytes. After invasion of the host cell, *L. monocytogenes* escapes the phagosome by the membrane-disruptive activities of listeriolysin O (LLO) and two phospholipases (3, 12). Expression of a hexose phosphate transporter follows escape into the cytosol and scavenges available sugars to facilitate robust intracellular growth (3, 5). The organism is further capable of intracellular propulsion via polar actin polymerization, which utilizes a bacterial surface component and host actin machinery (3). Actin-mediated propulsion allows *L. monocytogenes* to avoid exposure to the humoral immune system by forcibly penetrating the membrane barrier dividing adjacent cells (8, 12).

To appropriately modulate the necessary physiological response to these two distinct lifestyles, *L. monocytogenes* possesses dynamic regulatory machinery. PrfA, an environmentally-sensitive transcriptional regulator, controls the expression of most major virulence factors and many metabolic components (5). Temperature regulates translation of the *prfA* transcript (3, 5), while nutrient availability of the local environment and the metabolism of particular classes of sugars have also been observed to modulate PrfA-dependent gene expression (5, 6). Through these finely-tuned responses to environmental conditions, *L. monocytogenes* is able to adapt and maximize fitness among an array of environments. One of the most intriguing adaptations is a profound ability to modify membrane fatty acid composition, facilitating low temperature growth (2).

Cellular Membrane

Biological membranes serve as a protein anchor for many critical cellular functions in addition to its role as a selectively permeable barrier (13). These membrane

functions can be disrupted by environmental pressures which alter fluidity of the membrane, resulting in severe growth defects (13, 14). Temperature in particular has a well-known impact on membrane fluidity, where a decrease in temperature leads to decreased fluidity (15). Biophysical properties of membrane lipids also contribute to membrane fluidity, defining the temperature at which phase transitions take place (16, 17). The ideal membrane state is typically described as the liquid-crystalline lamellar phase, a state characterized by moderate disorder of fatty acyl chains within the bilayer (13, 18). Temperatures below the transition temperature defined by membrane lipids result in phase transition to a more ordered, gel-like membrane state. Many organisms have developed adaptive strategies to maintain ideal membrane fluidity under variable temperature conditions (19, 20), a phenomenon dubbed “homeoviscous adaptation” (14). In bacteria, modification of membrane fatty acid composition is a common strategy (17, 19).

Odd-numbered, branched-chain fatty acids (BCFAs) comprise an extensive proportion of the *L. monocytogenes* membrane and are thought to contribute to its robust adaptation to low temperature stress (21). In particular, anteiso-C15 fatty acids become the dominant fatty acids at the expense of anteiso-C17 when grown at low temperatures (22, 23). This alteration in membrane fatty acid composition is mediated by two mechanisms of fatty acyl chain modification: chain length shortening and alteration in methyl branching pattern (21). Homologous fatty acids of shorter chain length have lower melting points than their longer counterparts, and the same is true of anteiso- and iso-branched fatty acids (15, 16, 19, 24). Increased unsaturation, a common mechanism of adaptation in other bacteria, is not prominent in *L. monocytogenes* (21). Following a

decrease in growth temperature, these lower melting point fatty acids become incorporated into the membrane in order to lower the phase transition temperature, thereby maintaining appropriate fluidity (15, 17, 21).

Much of the work identifying anteiso-C15 as an integral component of the cold stress adaptation response by *L. monocytogenes* was performed using cold-sensitive transposon mutants. The cld-1 and cld-2 mutants have insertions in the branched-chain α-keto acid dehydrogenase (*bkd*) gene complex, which disrupts BCFA biosynthesis (22). In compensation, the membrane of the mutant strains is enriched in even-numbered, straight chain and iso-branched fatty acids (21). In addition to deficient low temperature growth, these mutants are unable to thrive under acidic and alkaline pH conditions (25) and have a significantly less fluid membrane than the parent strain (22, 26). These mutant strains are further characterized by deficiencies in adhesion (27) and are severely virulence attenuated (28, 29), demonstrating the physiological versatility of anteiso-branched fatty acids. Importantly, membrane fatty acid composition and fluidity can be restored by addition of 2-methybutyric acid (2MB) to the growth medium (21, 22).

The catalyst for this project originated from observations made by PhD student Suranjana Sen during the course of morphological investigations into the cld-2 mutant. Scanning and transmission electron microscopy revealed mutant cells growing in moderately long chains (Figure S2), where wild-type cells grow predominantly as separated rods (Figure S1). The addition of 2MB to the growth medium of MOR401 corrects this phenotype (Figure S3). Further, the polar cell wall material between bacterial cells appeared thickened and to have undergone incomplete separation. These cells also appeared to be slightly elongated. These observations suggest appropriate

membrane fluidity may be essential for the correct assembly and function of the divisome.

Cell Division

Bacterial cell division is mediated by an array of canonical proteins which operate under strict spatial and temporal regulation (30, 31), the assembly of which into a contractile ring is termed the divisome (32). Eukaryotic tubulin (FtsZ) and actin (MreB) homologues are both present in rod-shaped bacteria, extensively contributing to maintenance of bacterial shape (33, 34). FtsZ defines the division site with the help of membrane-anchored protein factors (30). Penicillin-binding proteins (PBPs) which synthesize peptidoglycan (PGN) are recruited to the division plane by FtsZ and associated proteins, initiating cell wall biosynthesis at the sidewall (33). MreB participates in elongating the sidewall prior to contraction by FtsZ and also recruits hydrolytic enzymes (33, 34). In Gram-positive rods, constriction culminates in two daughter cells joined by a polar cross-wall (30). Peptidoglycan hydrolases (autolysins) then hydrolyze this wall material to yield fully separate daughter cells. The cytokinetic machinery itself has also been implicated in activation of certain autolytic amidases (35), which are responsible for severing the peptide bridge.

Several additional membrane-associated factors are involved in the division process. PBPs individually play diverse roles in PGN biosynthesis and possess membrane-anchoring domains (36). DivIVA, a membrane protein which self-localizes to areas of increased membrane curvature, has species-specific functions (37). In *L. monocytogenes,* DivIVA is associated with recruitment of the autolysins p60 and MurA for secretion by the auxiliary secretory ATPase, SecA2 (38). SecA2 is itself a peripheral

membrane protein (39, 40). Mutant strains deficient in DivIVA, p60, MurA, and SecA2 all individually produce, among other phenotypes, long-chain cell morphologies (38, 41, 42), demonstrating their integral function in daughter cell separation.

Cell Wall

The cell wall serves several critical functions in bacterial physiology, particularly in Gram-positive organisms which lack an outer membrane (43). In addition to imparting shape and rigidity to the cell, the cell wall provides a barrier to the internal osmotic pressure of the cytoplasm (44, 45). This feature alone makes cell wall integrity a prime concern during bacterial growth and division, as new wall material must be coordinately synthesized and hydrolyzed without compromising cell viability (33). This consideration suggests cell wall turnover and remodeling during division requires precise regulation, and possible spatially coupling (34), of these enzymatic processes.

PGN is a dominant macromolecular component of the cell wall. Structurally, it is composed of canonical disaccharide repeat units of β-1,4-linked N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) (46, 47). Certain chemical modifications to the PGN backbone have been identified to contribute to virulence in *L. monocytogenes*: deacetylation of NAG and O-acetylation of NAM impart resistance to host defense molecules and inhibit recognition by the immune system (48–50). O-acetylation has also been observed to regulate lytic enzymes (51, 52) and is required for appropriate cell separation in *Bacillus subtilis* (53). Peptide bridges extend from the NAM constituent and are cross-linked to provide structural stability (46). Cross-linking may occur directly between peptide moieties of the bridge or be mediated by amino acid cross-bridges (54), and the extent to which PGN is cross-linked has been implicated in pathogenic properties

such as antibiotic resistance (55). The peptide bridges and cross-bridges are observed to undergo considerably more modifications and variability than the backbone structure (46). *L. monocytogenes* is known to possess PGN directly cross-linked through the dibasic amino acid meso-diaminopimelic acid (m-Dpm) (56, 57), a chemotype most prevalent in Gram-negative bacteria (54). This mesh-like structure simultaneously imparts rigidity to the cell and permits flexibility during regular metabolic processes (58).

Aside from its structural role, PGN also serves as an anchor for cell wall-associated proteins and carbohydrate constituents (59). Cell wall proteins provide essential functions by interacting with the outside environment and host (60). These functions are often related to virulence, such as invasion mediated by the internalins, and *L. monocytogenes* is known to express a large repertoire of surface proteins (6, 61). Carbohydrates comprise the largest component of the cell wall in *L. monocytogenes,* of which teichoic acids (TA) are the dominant species (56, 62). TAs are anionic polymers covalently linked to PGN (WTAs) or anchored to the membrane (LTAs) (63). These surface factors have been implicated in a variety of processes and functions including antibiotic susceptibility (64), biofilm development and colonization of natural and artificial surfaces (65–67), horizontal gene transfer (68), and evasion of host immune responses (69). Importantly, these polymers have also been observed to modulate the activity of autolysins (70), peptidoglycan cross-linking, and, subsequently, cell division (70, 71). Modifications of TA, such as through D-alanylation, may also contribute to the control of these processes (72–74). As such, both the chemical structure of PGN and the influence of anionic TAs may influence the activity and localization of autolysins.

L. monocytogenes expresses a number of enzymes with characterized

peptidoglycan hydrolase activity (75). These enzymes cleave covalent bonds within PGN and function in a variety of capacities including cell growth and wall remodeling, separation of daughter cells, and virulence (76, 77). The most well-characterized is p60, a surface-associated autolysin involved in daughter cell separation and virulence (42). Mutant strains lacking p60 exhibit a pronounced chaining morphology and are deficient in actin-based motility. A related extracellular protein, p45, has autolytic activity but unknown function (78). The amidase Ami has been associated with virulence-related motility and adhesion processes (79), while the muramidase MurA functions in cell separation and wall turnover (41). Auto is required for the invasion of certain eukaryotic cells (80). Finally, IspC is a cell wall-anchored enzyme involved in virulence-specific adhesion (81). The hydrolytic nature of these proteins demands their strict regulation to ensure appropriate physiology.

Membrane Fluidity and Cell Division

The precise roles membrane fluidity and fatty acid composition may play in the cell division processes, as well as cell wall chemistry and structure, is not well understood. In L-form cells which lack a cell wall, membrane fluidity has been observed to play a role in successful cell division (82). Whether this mechanism may play a role in cell wall-dependent cell division is uncertain. Membrane potential, which disturbance of the barrier function of the membrane may compromise (13, 16), has also been observed to modulate the localization of several cell division proteins (83). Finally, induction of the cell wall remodeling pathway in yeast is associated with the response to heat stress, which could be linked to changes in membrane fluidity (18, 84). While these prior investigations may provide insight for future work, we are unaware of any studies which

have looked at cell wall-dependent cell division in decreased membrane fluidity or fatty acid-deficient mutants.

Many factors explicitly involved in the division process are membrane-bound proteins or have membrane-associated components. Perturbations in membrane fluidity which may influence activity or proper localization of these factors could result in aberrant division phenotypes. Alternatively, PGN and TA biosynthesis, which share some cellular machinery, also have several integral components associated with the membrane. Since these two major cell wall constituents play essential roles in the overall division process, their alteration may subsequently result in disturbance of the usual course of division or daughter cell separation. Covalent attachment of proteins to peptidoglycan is mediated by membrane-associated sortases and may also be a source of altered surface protein profiles. Membrane stress may also simply result in compensatory alterations to cell wall properties which interfere with cell division.

To investigate the role membrane fluidity and fatty acid composition may play in cell division, we proposed to identify alterations in autolytic and cell wall properties associated with the BCFA biosynthesis-deficient cld-2 mutant. This mutant assembles a membrane of significantly different fatty acid composition and fluidity than the parent strain, and is subsequently observed to undergo aberrant cell division characterized by incomplete separation and long-chain morphology. This study seeks to document alterations in autolytic rates and peptidoglycan hydrolase profiles associated with the cld-2 mutant. We hypothesized the mutant strain will display significantly disparate rates of autolysis and alter expression of hydrolytic enzymes. We further sought to document changes in cell wall properties and chemistry which may reflect determinants of observed

cell division defects. We hypothesized to observe significant alterations in cell wall chemistry and protein profiles associated with the mutant strain.

The results of this investigation may prove invaluable to our understanding of bacterial physiology and cell division processes. Interplay between membrane fluidity and cell wall-dependent cell division remains underexplored, and this research may elucidate additional factors involved in cell division. Appropriate growth and division is integral to bacterial physiology and pathogenesis, and a more comprehensive appreciation of this process may assist in the study and control of these organisms. Coupling membrane fluidity and composition to cell wall properties may also open the door for synergistic treatments exploiting cell wall alterations following membrane stress.

CHAPTER II

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

L. monocytogenes strains 10403S and its isogenic *bkd* transposon-insertion mutant MOR401 (29) were utilized to assess the effect of BCFA-deficiency on cell wall and autolytic properties. The wild-type 10403S strain is a member of the pathogenic 1/2a serotype of *L. monocytogenes* commonly used in the laboratory setting (85). Both strains were chosen due to their well-established characterization in the literature and readily available nature. Strain MOR401 was kindly provided by Mary O'Riordan. Strains were cultured in brain-heart infusion (BHI) medium (BD) and grown to mid-exponential phase, and experiments analyzed in triplicate.

Whole-Cell Triton X-100 and EDTA-Stimulated Autolysis

Overnight cultures of the respective *L. monocytogenes* strains were grown in BHI medium at 37^{o}C with shaking at 200 rpm, with or without 2MB. Overnight cultures were then diluted 1:50 in fresh BHI at a flask-to-media ratio of 1:4 and allowed to grow until mid-exponential phase. Cells were then harvested by centrifugation at 4,000 g for 10 minutes at 4^{o}C and the pellet washed once in cold, distilled water. Triton X-100-stimulated autolysis was then performed essentially as described by Gustafson et al. (86), with incubation occurring at 37^{o}C and optical density measured at OD_{600}. EDTA-stimulated autolysis was performed essentially as described by Popowska (50),

without shaking. Induction of autolysis was reported as the fractional decrease in initial OD over time.

Whole-Cell Lysozyme and Mutanolysin Cell Lysis

Mid-exponential phase cells were harvested by centrifugation at 4,000 g for 10 minutes at 4^{o}C and the pellet washed once in cold, distilled water. Lysozyme and mutanolysin induced lysis were performed essentially as described by Popowska (50), utilizing room temperature buffer. Cell lysis was reported by the fractional decrease in initial OD over time.

Crude Cell Wall Autolysis

Mid-exponential phase cells were harvested by centrifugation at 4,000 g for 10 minutes at 4°C and the pellet washed once in cold, distilled water. Preparation of crude cell walls was performed according to established methods (87, 88). Briefly, one liter of culture was resuspended in 50 ml cold, distilled water and mixed with 50 ml cold glass beads (0.1 mm diameter, BioSpec) in a BioSpec Small Chamber Assembly. Cells were broken using a BioSpec Bead-Beater, with five two-minutes-on and two-minutes-off operation of the beater. Broken cells were applied to a sintered glass filter draining into a Buchner flask. A vacuum aspirator was applied to facilitate filtration. Following filtration, the filtrate was centrifuged at 14,000 g for 10 minutes at 4°C to recover cell walls, and the deposited cell walls were washed twice in cold, distilled water. A portion of this crude cell wall suspension was taken to analyze crude cell wall autolysis as described by Koehl et al. (89). Cell wall autolysis was reported as the fractional decrease in initial OD over time.

Analysis of Peptidoglycan Hydrolase Profiles

Peptidoglycan hydrolase profiles were analyzed by renaturing SDS-PAGE, or zymography, essentially as described by Foster (90). Briefly, autolysin extracts were prepared from 50 ml of mid-exponential phase cells. Cultures were harvested by centrifugation at 4,000 g for 10 minutes at 4°C, and the supernatant decanted into clean centrifuge tubes. The cell pellet fraction was resuspended in 500 ul SDS buffer (1% wt/vol SDS, 1mM EDTA, 10% vol/vol glycerol, 5% vol/vol ß-mercaptoethanol, 50mM pH 7.5 Tris-HCl) lacking bromophenol blue to permit quantification. The suspension was then boiled for 10 minutes at 100°C and centrifuged at 14,000 g for 5 minutes to pellet insoluble material. Supernatant could be stored at -20°C for several days and retained autolytic activity. The supernatant fraction was incubated with trichloroacetic acid (TCA) at a final concentration of 10% at 4°C for 30 minutes. Precipitate was then centrifuged at 20,000 g for 5 minutes at 15°C and washed three times in acetone. The dried protein pellet was then resuspended in 500 ul of SDS buffer and boiled for 3 minutes. Boiled sample was then centrifuged at 14,000 g for 5 minutes and supernatant stored at -20°C. Purified samples were electrophoresed in a polyacrylamide gel containing 0.2% (wt/vol) lyophilized *M. luteus* or *L. monocytogenes* cells, and then incubated overnight in 25 mM Tris-HCl (pH 8.0) containing 1% Triton X-100 to renature proteins. The gel was then stained using a 1% methylene blue, 0.01% KOH stain solution for 5-10 minutes, and destained over the course of several nights in deionized water. Hydrolytic enzymes were visualized as zones of clearing on the opaque background.

Analysis of Cell Surface Proteins by SDS-PAGE

Cell surface protein profiles were assayed by SDS-PAGE. Covalent and non-covalently bound proteins were extracted and analyzed essentially as described by Carvalho et al. (91). Briefly, non-covalent proteins were extracted from 50 ml of mid-exponential phase cells. Bacteria were harvested by centrifugation at 4,000 g for 10 minutes at 4°C, washed once in 20 ml PBS, then washed again in 1ml PBS. Cell were then harvested at 14,000 g for 5 minutes at room temperature, resuspended in 1 ml of PBS with 2% SDS, and incubated for 30 minutes at 37°C. The suspension was centrifuged at 10,000 g for 1 minute and the supernatant passed through a 0.22-um filter. For covalent proteins, 200 ml of mid-exponential phase cells were harvested by centrifugation at 7,000 g for 15 minutes at 4°C. Cells were first washed in 20ml of PBS, then 5 ml of mutanolysin digestion buffer (10 mM Tris-HCl pH 6.9, 10 mM $MgCl_2$, 0.5 M sucrose), and then again in 1.5 ml of mutanolysin digestion buffer. RNase A and mutanolysin were added to a final concentration of 20 ug/ml, and 30ul of 100X EDTA-free protease inhibitor was also added. The solution was then incubated overnight at 37°C with gentle agitation. After centrifugation at 14,000 g for 15 minutes at 4°C, the supernatant was passed through a 0.22-um filter, concentrated with TCA at a final concentration of 15%, and incubated on ice for one hour. Protein was precipitated by centrifugation at 20,000 g for 20 minutes at 4°C, washed three times in cold acetone, and resuspended in 50 ul of PBS.

Optimization of Cell Wall Purification, Teichoic Acid and Peptidoglycan Extraction

In order to facilitate further investigation into the questions posed by this study, the standardization of methods to extract and purify isolated cell wall components may

prove valuable. The methods utilized were modified and synthesized from several sources (87, 92–94). Overnight cultures of *L. monocytogenes* were reinoculated into fresh BHI and allowed to grow until mid-exponential phase and harvested by centrifugation at 7,000 g for 15 minutes at 4°C. Cells were then resuspended in SM buffer (100 mM NaCl, 10 mM $MgSO_4$, 10 mM Tris-HCl pH7.5) and boiled for 30 minutes to kill cells. This buffer is suitable for the downstream analysis of D-alanylation, muropeptides, and inorganic phosphate. If interest is in O-acetylation, a 0.9% NaCl (pH 6.8) buffer should be used (95). Cells were then reharvested under identical conditions and subjected to mechanical breakage by a BeadBeater and 0.1mm glass beads. Breakage was performed by alternating six periods of machine activity and rest, each lasting two minutes. Cell breakage via FrenchPress at 40,000 psi was a viable alternative. Filtrate could then be centrifuged at 1,400 g for 5 minutes to remove much of the cellular debris, and the supernatant transferred to fresh tubes. Cell walls in the supernatant were then pelleted by centrifugation at 30,000 g for 30 minutes at 4°C, washed twice in cold, distilled water and then resuspended in SM buffer. This fraction contains crude cell walls (CCW) which may be used for autolytic assays, provided cells were not heat-killed previously.

To obtain purified cell walls (PCW), the CCW fraction was incubated for 3 hours with 5ug/ml DNase and RNase, and then incubated overnight with 200ug/ml trypsin to degrade proteins. Cell walls were then reharvested under previous centrifugation conditions, resuspended in SM buffer containing 4% SDS and boiled for 30 minutes to further remove enzymes and membrane components. PCWs were then centrifuged at room temperature for 30 minutes at 30,000 g and washed at least five times in room temperature deionize water to remove SDS. Washed cells can be resuspended in a

minimal volume of water and lyophilized, and stored at -20°C until needed.

To extract PGN and TA components, PCWs were resuspended in a 25mM glycine/HCl (pH 2.5) buffer and boiled for 10 minutes. Boiled PCWs were centrifuged at 30,000 g for 30 minutes, and the supernatant fraction containing TAs transferred to a clean 30ml glass Corex tube for storage at 4°C. This extraction was repeated twice and the supernatants pooled. The insoluble cell wall material is PGN from which TA has been extracted, and can be washed several times in cold, deionized water and lyophilized for mass spectrometry analysis. TA was then purified by ethanol precipitation. Ethanol (95%) was added to the supernatant in a 5:1 ratio, and the solution allowed to precipitate overnight at 4°C. Precipitated TA was then centrifuged at 3,000 g for 15 minutes at 4°C, the precipitate resuspended in a reduced volume of buffer, and the precipitation repeated once. Precipitate was then harvested as before, washed three times in acetone, and lyophilized.

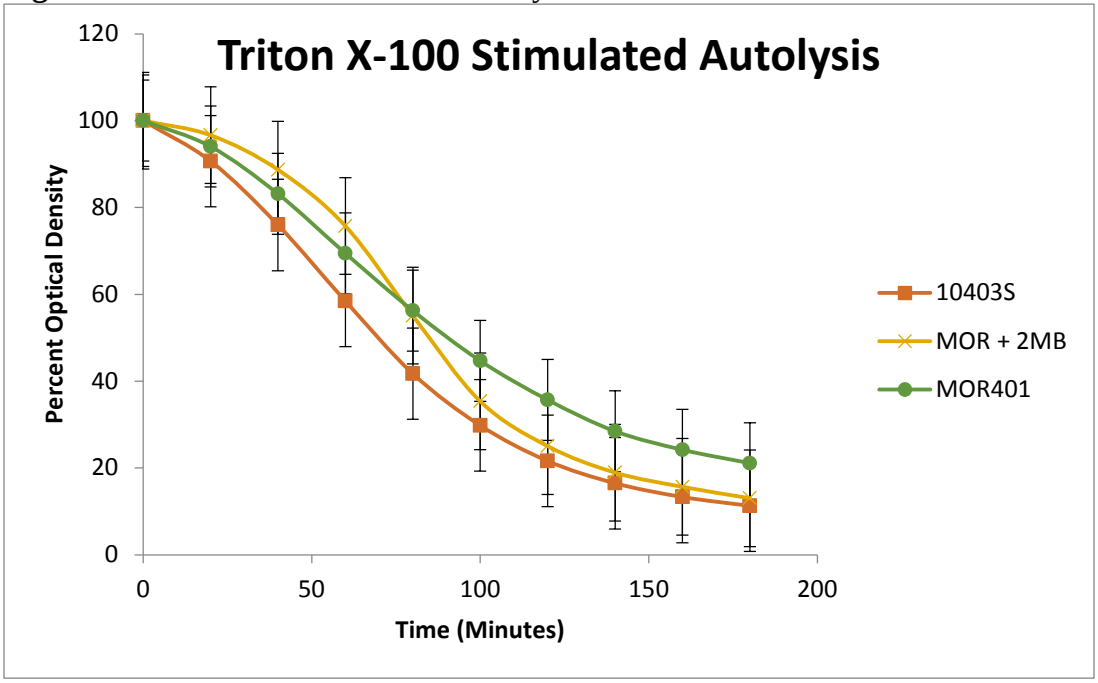
CHAPTER III

RESULTS

Triton X-100 and EDTA-Stimulated Autolysis

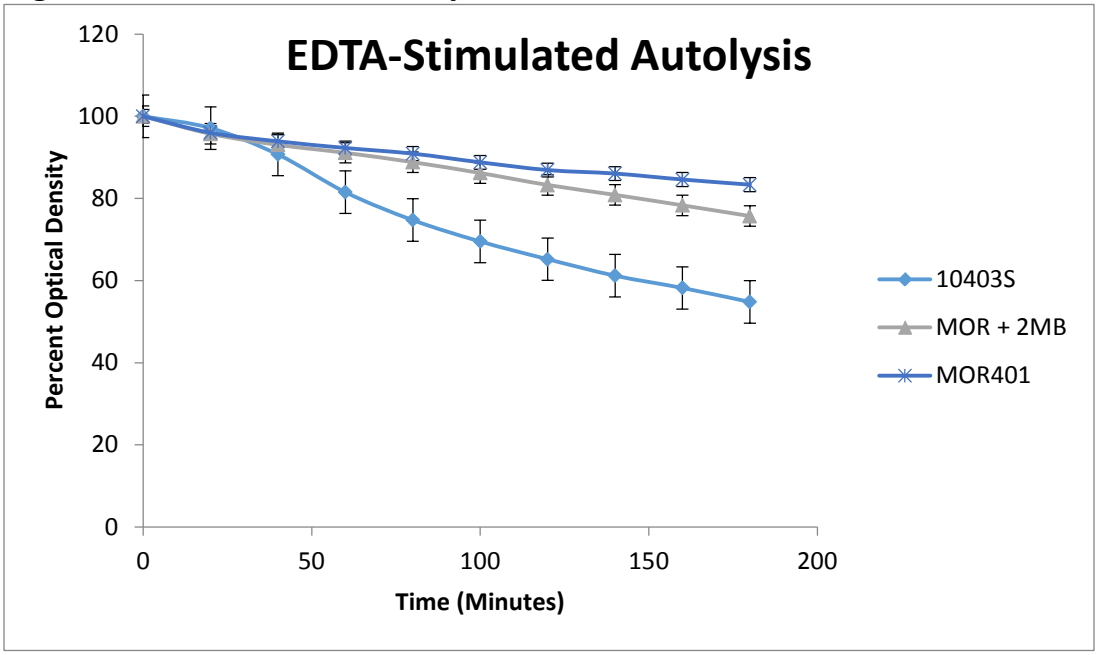
To investigate the hypothesis that altered membrane fatty acid composition may influence autolytic rates, respective *L. monocytogenes* strains were grown, in the presence or absence of 2MB, to mid-exponential phase and harvested by centrifugation. Washed cells were resuspended in buffer containing 0.05% Triton X-100 or 1mM EDTA, and autolysis rates determined by the percent decrease in OD_{600} over time. Both agents serve to increase the permeability of the membrane, thereby inducing lysis of cells by natural hydrolytic enzymes. Triton X-100 is a nonionic detergent, while EDTA is a metal chelator. In Triton X-100-stimulated cells, no statistically significant differences in the rate of autolysis could be observed between the mutant strain grown in the presence or absence of 2MB and the wild-type (Figure 1). The MOR401 strain without addition of 2MB exhibited a slightly decreased rate of autolysis compared to when it was grown with 2MB or the wild-type, although this difference could not be shown to be statistically significant. In contrast, wild-type EDTA-stimulated cells were observed to lyse significantly faster than MOR401, with or without the addition of 2MB (Figure 2).

Figure 1. Triton X-100 Stimulated Autolysis



Percent decrease in OD of parent and mutant strains grown with and without 2MB when incubated in 0.05M Tris-HCl pH 7.5, with and without 0.05% Triton X-100.

Figure 2. EDTA-Stimulated Autolysis

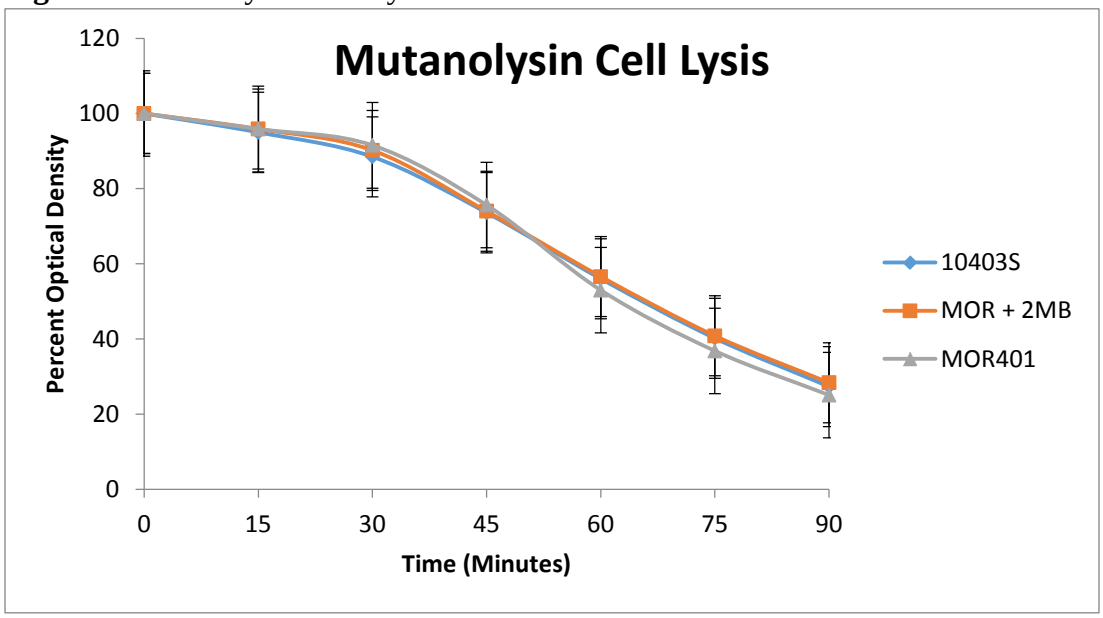


Percent decrease in OD of parent and mutant strains grown with and without 2MB when incubated in 0.05M Tris-HCl pH 7.5 with 1 mM EDTA.

Lysozyme and Mutanolysin Cell Lysis

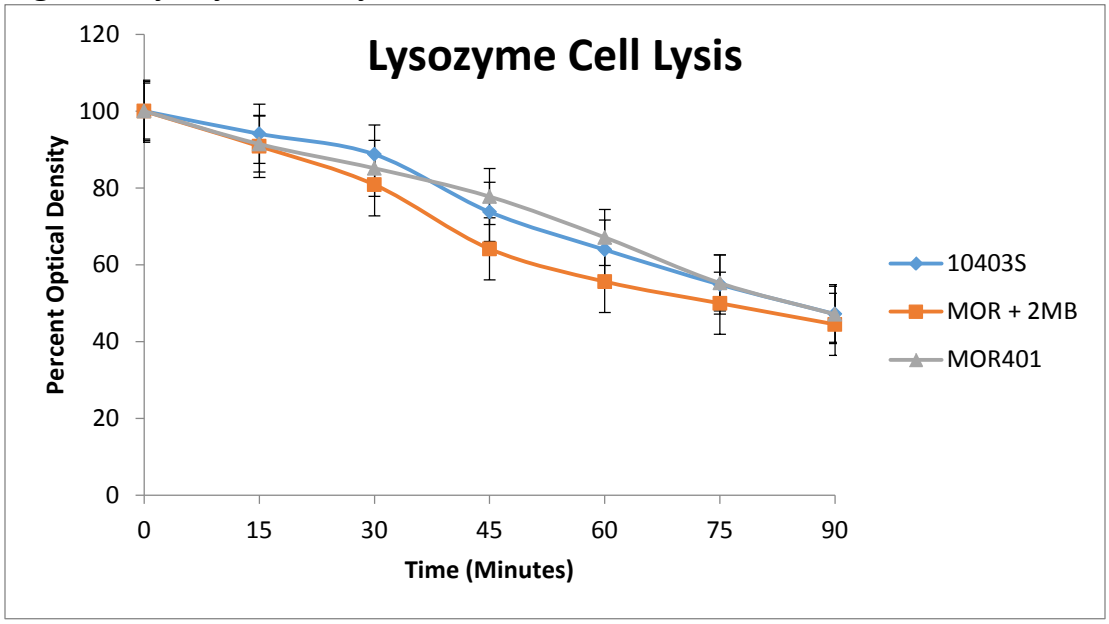
To assess whether cell wall chemistry or structure could be altered upon disruption of wild-type membrane fatty acid composition, susceptibility to cell wall lytic enzymes was assayed. Mutanolysin and lysozyme are both muramidases capable of hydrolyzing the ß-1,4-linkage between NAM and NAG constituents of PGN, though they are known to possess certain unique activities against some bacterial species. In addition, lysozyme possesses cationic antimicrobial peptide (CAMP) activity independent of its function as a muramidase. Washed cells were resuspended in buffer containing either 40ug/ml mutanolysin or 10ug/ml of human lysozyme. Treatment of cells with mutanolysin yielded no significant differences in cell lysis rates among *L. monocytogenes* strains (Figure 3); in fact, observed lysis rates were nearly identical. Likewise, lysozyme treatment resulted in very similar rates of lysis among strains (Figure 4).

Figure 3. Mutanolysin Cell Lysis



Cell lysis induced by incubation with mutanolysin as measured by the percent decrease in OD over time.

Figure 4. Lysozyme Cell Lysis

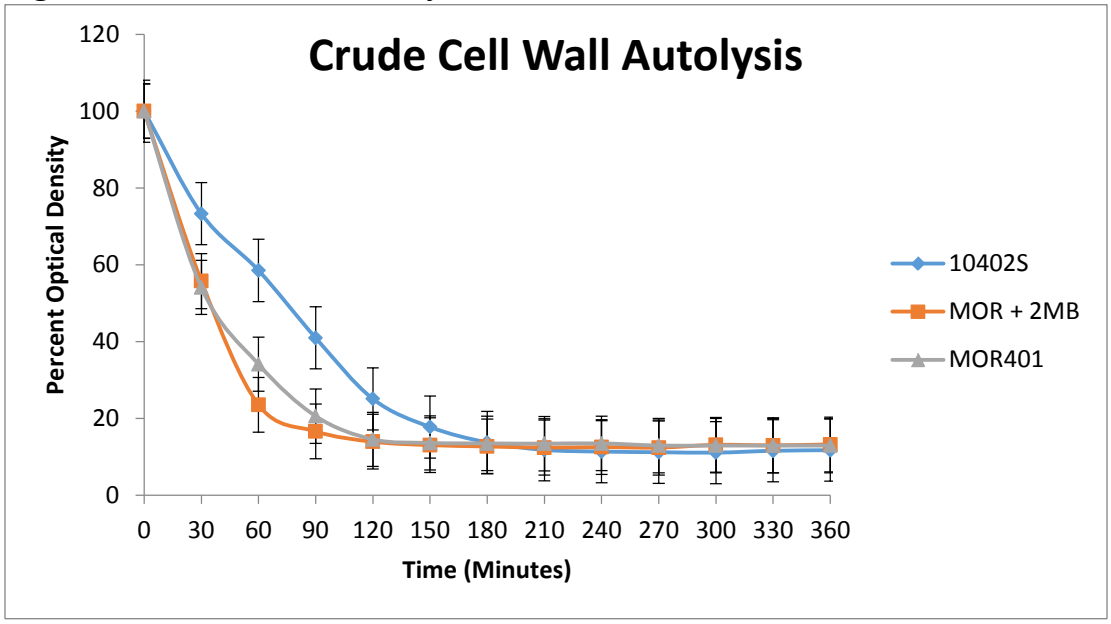


Cell lysis induced by incubation with lysozyme as measured by the percent decrease in OD over time.

Crude Cell Wall Autolysis

The autolytic rate of isolated cell walls retaining functional hydrolase activity was also assayed to probe for potential cell wall alterations. Mid-exponential phase cells were subjected to mechanical breaking procedures to isolate crude cell wall material retaining autolytic activity. This cell wall fraction was resuspended in buffer and autolysis monitored by the percent decrease in OD_{580} over time. In comparison to the wild-type strain, MOR401 grown in the presence or absence of 2MB exhibited significantly increased autolysis rates (Figure 5).

Figure 5. Crude Cell Wall Autolysis



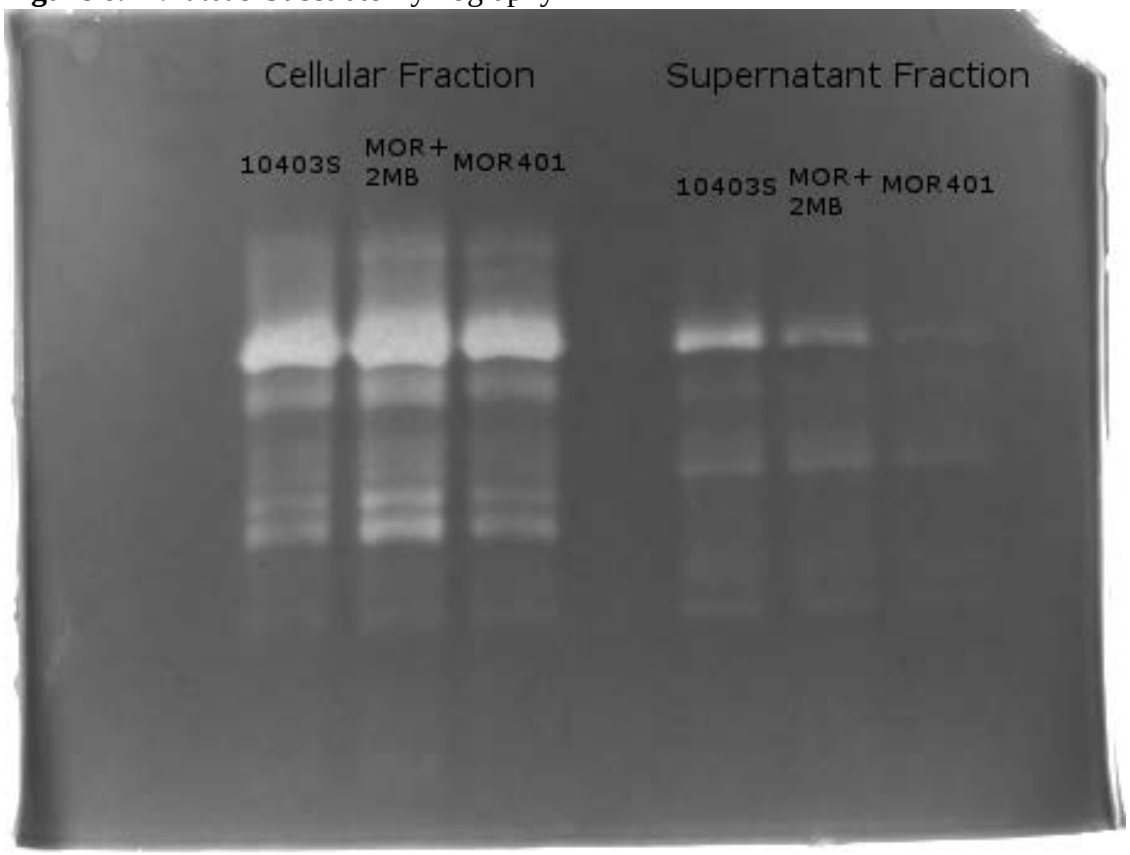
Autolysis rate of crude cell walls as measured by percent OD over time.

Peptidoglycan Hydrolase Profiles

In an effort to further characterize the autolytic properties of cell harboring the *bkd* mutation, peptidoglycan hydrolase profiles were analyzed by zymography. Hydrolases were isolated from the cellular fraction and concentrated from culture supernatant to account for spatial localization and secretion of autolysins. Purified samples were electrophoresed and renatured in paired zymograms with different substrates: one contained lyophilized *Micrococcus luteus* cells, while the other contained lyophilized *L. monocytogenes* 10403S. In the *M. luteus* zymogram, major hydrolytic bands in both cellular and supernatant fractions appear to be present in wild-type and the MOR401 strain grown in the presence or absence of 2MB (Figure 6). However, the intensity of some of these bands in the MOR401 strain, particularly the higher molecular weight enzymes in the supernatant fraction, may suggest differences in expression levels

or extracellular presence of some autolysins. In particular, an approximately 66 kDa enzyme appears significantly reduced in its intensity. The hydrolytic profile using *L. monocytogenes* cells as a substrate also looks fairly constant across both strains, with the exception of an approximately 32 kDa enzyme from the cellular fraction which is absent in the MOR401 strain (Figure 7). In addition, a higher molecular weight protein in the supernatant fraction - corresponding to approximately 104 kDa - appears to be reduced in intensity in the MOR401 strain grown in the presence or absence of 2MB.

Figure 6. *M. luteus*-Substrate Zymography



Peptidoglycan hydrolase profiles analyzed via zymography with M. luteus cells. Each lane was loaded with 50 ug of protein

Figure 7. *L. monocytogenes*-Substrate Zymography

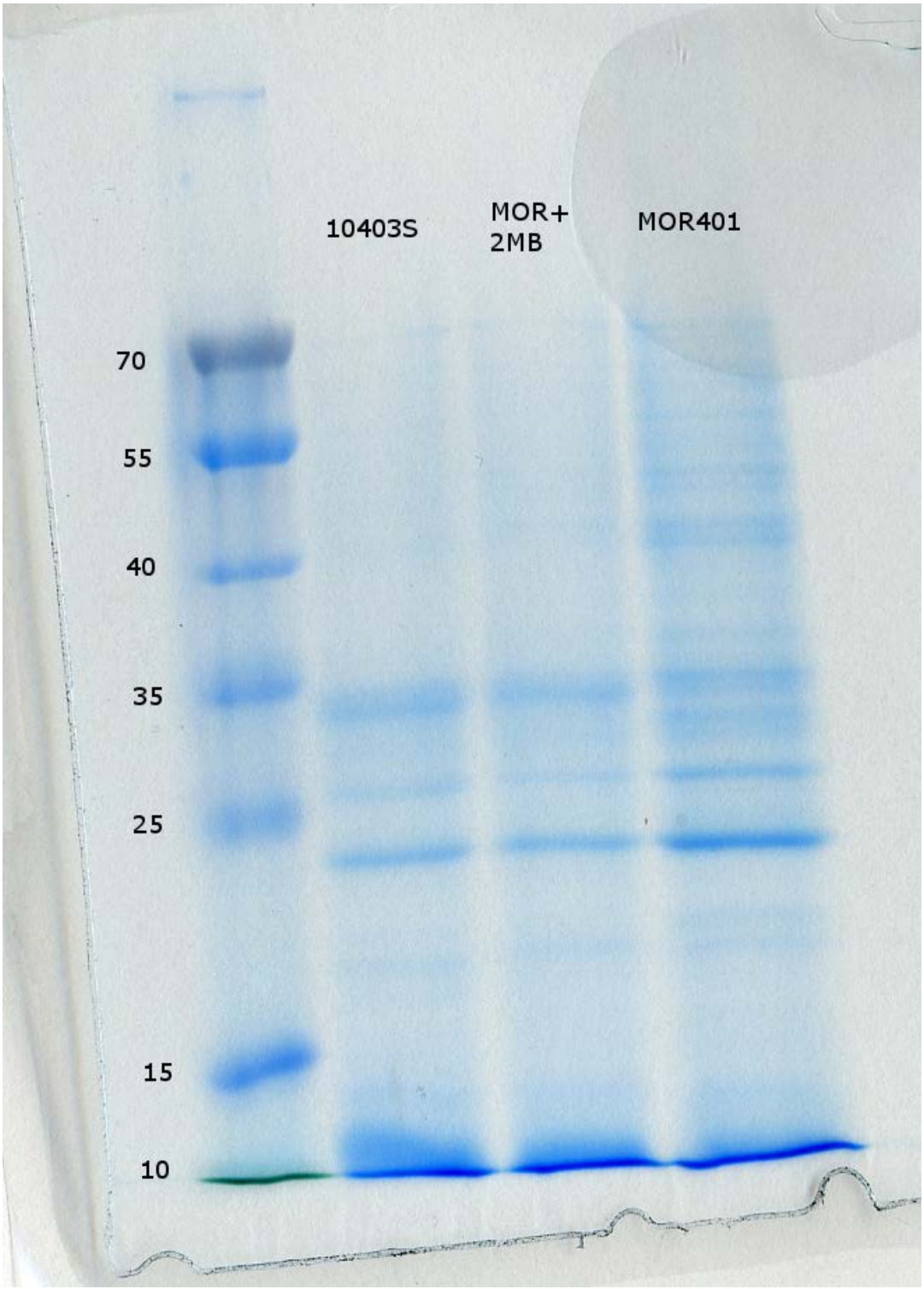
Peptidoglycan hydrolase profiles analyzed via zymography with L. monocytogenes cells. Each lane was loaded with 50 ug of protein

Cell Surface Proteins

Another potential indicator of cell wall alterations could be in the profiles of surface proteins associated with the cell wall. Traditionally, these proteins can be segregated into two groups: those which are non-covalently associated with the cell wall, and those which are covalently anchored to peptidoglycan. In the case of non-covalent cell wall proteins, mid-exponential phase cells were incubated with a PBS solution containing 2% SDS to disrupt these interactions and release these proteins. Covalently anchored proteins were released by the digestion of peptidoglycan with mutanolysin. The

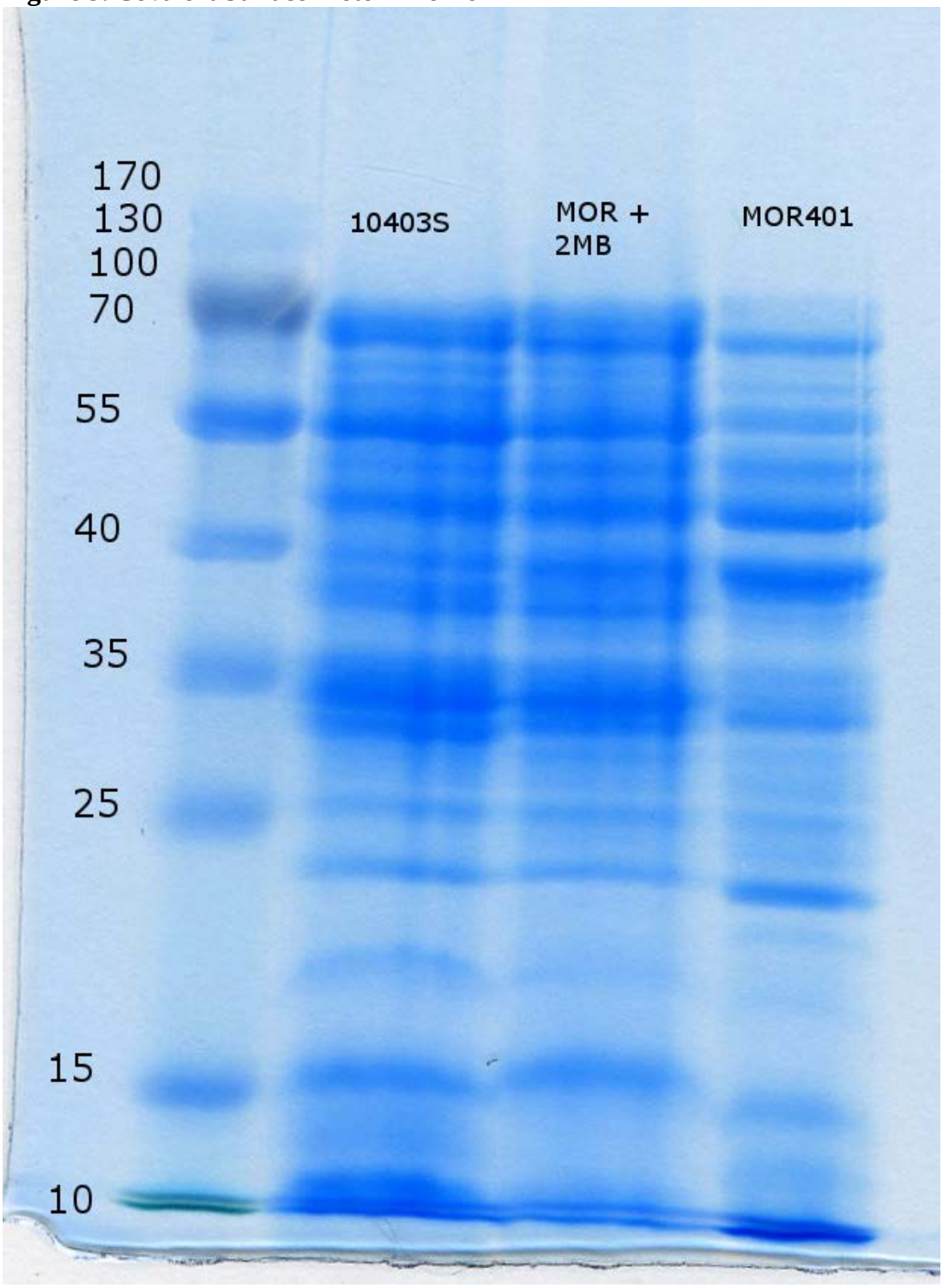
profile of MOR401 non-covalent proteins appears to possess quantitative differences in several proteins within the 40-55 kDa range, and a distinct protein at approximately 30 kDa was present that was not present in the wild-type (Figure 8). Covalently-attached proteins in the MOR401 strain also appear to display a significantly altered profile compared to the wild-type and MOR401 grown in the presence of 2MB (Figure 9). Bands of around 23 and 40 kDa appear to be enriched, while bands around 35, 55, and 70 appear reduced in intensity. A novel protein may be present at approximately 20 kDa.

Figure 8. Non-Covalent Surface Protein Profile



Non-covalently associated cell wall protein profiles visualized via SDS-PAGE. Each lane contains 150ug of protein

Figure 9. Covalent Surface Protein Profile



Covalently attached cell wall protein profiles visualized via SDS-PAGE. Each lane contains 50ug of protein.

Cell Wall Purification and Extraction of Peptidoglycan and Teichoic Acid

Initial attempts to purify cell walls often resulted in diminished yields or incompletely purified samples, prompting the desire to refine these procedures. By synthesizing several sources, certain steps were modified or added to improve the process and make it more consistent. Considering the specific goal of analyzing cell wall chemistry and structure, the inclusion of a boiling step ensured the inactivation of hydrolytic enzymes prior to protease exposure. This also improved safety through killing the bacteria. However, if interests are in the autolytic properties of crude cell walls, the boiling step should be avoided. Following breakage with the BeadBeater, care must be taken to avoid resuspension of insoluble debris and intact cells. To assist in this process, the first spin after harvesting the filtrate can be a low-speed centrifugation to pellet this debris, which can then be discarded after supernatant is transferred to a fresh tube. Incubation with nucleases and trypsin remove extraneous cellular material and proteins, while treatment with SDS further removes protein and membrane components. As SDS can interfere with downstream applications, its removal is an integral step in the procedure. Cold temperature causes SDS to precipitate and persist within the sample, necessitating room temperature reagents and conditions for wash steps. When finished, the cell wall purification process consistently yields 20-25mg of PCW per liter of mid-exponential phase culture.

To extract TA, PCW samples were originally respended in a 5% TCA solution and incubated for several hours at 60°C. After centrifugation, the supernatant containing TA was retained and the extraction repeated twice, pooling the supernatant in a 30ml Corex tube kept at 4^{o}C. Overnight incubation with ethanol was used to precipitate and purify the

TA, but only a negligible quantity of TA precipitate could be observed following two precipitations. A different extraction procedure was attempted, using a glycine/HCl (pH 2.5) buffer under boiling conditions and for a shorter duration of time. Ethanol precipitation was again used to purify the TA, and the addition of ethanol produced a more noticeable turbidity of the solution. Little precipitate could be harvested after the first incubation, but resuspension of the remaining material and addition of ethanol produced an even greater turbidity during the second incubation. A thick white precipitate could be observed to adhere all around the glass, and this material could be harvested and washed with acetone. Ultimately, this procedure yielded a significant quantity of TA precipitate compared to the original method. The presence of this precipitate also suggested the insoluble material present following extraction could be washed and used as a TA-extracted PGN sample.

CHAPTER IV

DISCUSSION

Membrane fatty acid composition and fluidity have been established as integral characteristics to several physiological processes, including nutrient uptake, protein function, and barrier properties of the membrane (13, 18). Temperature in particular is known to significantly impact membrane function through alterations in fluidity, and many microorganisms possess mechanisms to maintain appropriate fluidity under variable temperature conditions (15, 19, 21). In *L. monocytogenes*, a connection has also been forged between fatty acid composition and virulence (28, 29). However, the role these membrane characteristics may play in cell division processes remains unclear. Many critical factors involved in cell division and separation are associated with the membrane, from assembly of the cytokinetic ring to secretion of hydrolytic enzymes (30, 33, 38). Peptidoglycan and teichoic acid, two cell wall constituents intimately involved in the division process, also possess biosynthetic machinery associated with the membrane (46, 96). As such, alterations in membrane fluidity could also modulate qualities of peptidoglycan and teichoic acids which, in turn, may influence appropriate cell division. Finally, membrane-localized sortases responsible for attachment of proteins to the cell wall could also be disturbed by alterations in membrane fluidity (36). Covalently-bound proteins may be directly impacted by this disturbance, while non-covalent cell wall proteins could be disturbed by structural changes in peptidoglycan.

Our study sought to document alterations in autolytic rates, peptidoglycan

hydrolase profiles, and cell wall properties which may represent correlates of observed aberrant cell division in the BCFA-deficient *L. monocytogenes* mutant, MOR401. This mutant possesses a membrane of significantly different fatty acid composition and decreased membrane fluidity compared to the parent strain (21, 22). Consequently, it is unable to grow under low temperature (21) and extreme pH (25) conditions. During the course of morphological investigations into this mutant, it was observed to grow in moderately long chains connected by incompletely hydrolyzed cell wall material (unpublished observations). Further, mutant cells appeared to be elongated compared to wild-type cells. Addition of 2MB to the growth medium of MOR401 restored the wild-type morphology. These observations suggest appropriate membrane fluidity may be necessary for the correct assembly and function of the divisome and associated machinery.

Data accumulated during our investigation provides insight into the physiology of decreased membrane fluidity and BCFA-deficient *L. monocytogenes*. In our experiments of autolytic rates, results of decreased, increased, and no significant alteration in autolysis were all observed. Autolysis induced by membrane permeabilizing agents such as Triton X-100 and EDTA resulted in no significant difference or a decrease in autolytic rate, respectively, of the mutant strain (Figures 1 and 2). In contrast, crude cell walls of the mutant were observed to undergo increased autolysis compared to wild-type (Figure 5). Changes in the autolytic rate of crude cell walls, which retain functional hydrolytic enzymes, may suggest an alteration to cell wall chemistry or wall-associated peptidoglycan hydrolase properties. Likewise, the decreased autolysis observed in EDTA-treated whole cells could suggest modifications to native hydrolases or their

respective substrates. These results suggest alterations may exist, but offer little clarification over the mechanism. Whole cells may also possess additional confounding factors to their interpretation compared to isolated cellular components: autolysins could become sequestered or otherwise interfered with by other cellular constituents, such as positively charged phospholipids or LTAs.

Oddly, muramidase treatment of whole cells with lysozyme and mutanolysin both resulted in lysis rates comparable across all strains (Figures 3 and 4). These results on their own would suggest an absence of alteration to the glycan strand of peptidoglycan, which houses the target ß-1,4-linkage of these enzymes. However, Sun et al. (2012) (29) had previously observed both agents to reduce MOR401 viability in survival assays, implicating fatty acid composition in susceptibility to peptidoglycan hydrolases. Several explanations for our conflicting results appear possible: the conditions of our experiment using static, whole cells may not have been appropriate to see substantial differences when exposed to these surface stressors; the mechanism of observed killing in Sun et al. (2012) may be distinct from cell lysis; or susceptibility to these muramidases may be contingent upon active cellular growth and cell wall turnover. Considering the delicate balance between cell wall synthesis and hydrolysis necessary to maintain cell viability, additional stress during this process may sufficiently destabilize osmotic functions of the cell wall. In future experiments, assays utilizing purified cell walls lacking enzymatic components should be useful in isolating this phenomenon. It is also possible the mechanism of killing observed in Sun et al. (2012)

To complement our experiments of autolytic rates, peptidoglycan hydrolase profiles were assayed by zymography. Overall, the repertoire of expressed autolysins

across *L. monocytogenes* strains appeared consistent, with the exception of a low molecular weight band present in wild-type but not MOR401 grown in the presence or absence of 2MB (Figure 7). The observed pattern of lytic bands appears highly similar to those obtained by McLaughlan and Foster (97), suggesting this absent band could be a ~32 kDa enzyme. This 32 kDa enzyme appears to be species-specific and was observed to possess activity on *L. monocytogenes* and *B. subtilis*, but not *M. luteus*, cell walls (97), suggesting a distinct substrate specificity. Both *B. subtilis* and *L. monocytogenes* possess directly cross-linked m-Dpm peptidoglycan in contrast to *M. luteus*, which typically expresses an L-alanine cross-bridge linked to L-lysine and D-alanine (54, 56). This enzyme could therefore play a role in hydrolyzing the PGN cross-bridge, and potentially be involved in the cell separation processes.

The supernatant fractions of both zymograms appear to possess quantitative differences in some autolytic bands, though the most prominent ones seem to be of distinct sizes. It is perhaps telling that, during the course of these experiments, the supernatant fraction of the mutant was routinely observed to produce considerably less precipitate relative to the wild-type (personal observation). This could suggest a decrease in the quantity of proteins reaching the extracellular space of the MOR401 strain. In the zymogram containing *L. monocytogenes* cells, a protein of approximately 104 kDa appears to show moderately decreased intensity in the mutant strain (Figure 7). One of the higher molecular weight peptidoglycan hydrolases of *L. monocytogenes* is Ami, a 102.3 kDa enzyme (75). Ami is non-covalently attached to the cell wall (98) and possesses significant homology to the major *S. aureus* autolysin AtlA required for appropriate cell separation and adhesion (99, 100). However, Ami has not been

specifically implicated in the cell division process, making perturbation of this enzyme an unlikely determinant of aberrant cell division.

In the *M. luteus* zymogram, the most prominent quantitative discrepancy appears to be associated with a band approximately 65 kDa in size (Figure 6). *L. monocytogenes* expresses two peptidoglycan hydrolases of approximately this size: the amidase Auto (~64 kDa) and the muramidase MurA (~66 kDa) (41, 80). Auto itself has not been associated with cell division processes, but mutants deficient in MurA have been observed to grow in long chains of unseparated cells. While the cellular fraction does not appear to substantially reflect this alteration, MurA is a non-covalent cell wall-associated enzyme whose localization and secretion by the combined actions of DivIVA and SecA2 is known to be essential for appropriate cell division (37, 38). Therefore, the decreased extracellular presence of this enzyme (in addition to the major autolysin, p60) corresponds to a pronounced chaining phenotype. Unfortunately, p60 is reportedly not represented in zymograms utilizing these experimental conditions (97), making assessment of its role in *MOR401* physiology presently impossible. Future studies seeking to specifically characterize hydrolytic properties of p60 may be required to explore alternative techniques.

It is interesting to note the similarities and differences observed between division defects present in the MOR401 strain and those present in mutants of p60, MurA, DivIVA, and SecA2. In the latter cases, cells exhibit a pronounced chaining morphology of extreme length and incomplete cell separation (38, 41, 42). Division defects in MOR401 appear to manifest in much more intermediate-length chains, though also possess evidence of incomplete cell separation (Figure S2). This difference could suggest

the aberrant division characteristics of MOR401 be the result of physiological alterations which exist on a spectrum, rather than through the disturbance of a single discrete function. For instance, the DivIVA protein required for proper localization of the p60 and MurA autolysins is known to self-localize through membrane curvature dynamics (101). It seems possible membrane fluidity could influence the distribution of this protein by disturbing membrane curvature, thereby limiting the extent to which these enzymes reach the extracellular milieu. In the work of Halbedel et al. (2012) (38), co-culture of a *divIVA* null mutant with wild-type *L. monocytogenes* expressing both p60 and MurA markedly reduced the length of cell chains to levels more reminiscent of the MOR401 chaining phenotype. This could suggest proper localized concentrations of autolysin at the septum are necessary for appropriate separation. If supernatant concentrations of MurA or p60 are significantly reduced in the MOR401 strain, mechanisms of its localization and secretion may be worth investigating. One possibility may be confocal microscopy of fluorescently-tagged DivIVA to investigate whether its distribution within the cell is altered compared to the wild-type.

Many cell wall-associated proteins are involved in both division- and virulence-related functions, and sortases responsible for the covalent attachment of some proteins are membrane associated. Therefore, we sought to characterize the surface protein profiles of the MOR401 mutant. Non-covalent proteins are commonly associated with the cell wall through one of three mechanisms: GW (glycine and tryptophan) repeats which interact with LTA; hydrophobic tails which anchor to the membrane; or LysM repeats which interact with peptidoglycan (61). The profile of non-covalent proteins from the MOR401 mutant grown without 2MB appears to contain some quantitative

differences compared to the wild-type and MOR401 grown in the presence of 2MB (Figure 8.) In particular, several bands between 40 and 55 kDa appear more intense in the mutant. There also seems to be an area of heavier banding around 30 kDa. Alterations to the profile of these proteins may reflect chemical alterations to peptidoglycan or the influence of altered membrane dynamics disturbing proper association.

Several hydrolytic enzymes are non-covalently associated with the cell wall, including Ami (GW), p60 (LysM), MurA (LysM), Auto (GW), and p45 (LysM) (75). Both p60 and p45 are also known to localize to the extracellular space with only a minority fraction appearing to remain associated with the cell surface (102), making it difficult to definitively discern their presence. The function of p45 is currently unknown (78), while p60 has a well-characterized role in cell separation and virulence (42). In addition, the N-deacetylase PgdA, which is predicated to undergo secretion and become associated with the cell wall, may be a non-covalent surface protein (50). PgdA is reported to have a size of approximately 52 kDa, but whether this corresponds to any enriched band is difficult to discern. Since N-deacetylation is known to modulate muramidase activity, potentially including that of MurA, alteration to this modification pattern could impact the physiological activity of these enzymes (49). Larger (>60 kDa) enzymes also appear difficult to identify in our assay, and may or may not contribute to the altered expression profile. Alternatively, additional or absent bands may represent processed or degradative products of larger proteins.

In Gram-positive bacteria, LPXTG motifs characterize the most common covalent attachment mechanism of proteins to peptidoglycan (103), though other motifs are known to be involved. The protein profile obtained for this class of surface proteins appears to

exhibit significant alteration in the MOR401 strain (Figure 9). Perhaps the most notable of these proteins are many of those in the internalin family, particularly InlA (61). InlB is a non-covalent GW-motif surface protein, and the major exclusion from this categorization. InlA is known to be an essential factor mediating the invasion of epithelial cells through interaction with E-cadherin (12), and secondary internalins have been associated with host colonization functions (61). While Sun et al. (2012) (29) does not document any invasion defects related to macrophage uptake for the MOR401 mutant, fatty acids have been observed to modulate expression of InlA. It may therefore be interesting to see whether MOR401 exhibits invasion defects in non-phagocytic cells; however, differences in the surface protein profile may also reflect expression differences, rather than disturbance to the anchoring mechanism. Therefore, while changes in the covalent profile may indicate alterations to peptidoglycan or sortase activity, it would appear prudent to correlate these changes with expression levels.

Other notable surface-associated proteins could also be disturbed in these mutants. The flagellar protein FlaA, involved in the expression of flagella in *L. monocytogenes,* is typically significantly attenuated at 37°C and higher at lower temperatures (1), demonstrating its temperature-regulated expression. It has a reported size of 30.4 kDa (104). It may be possible the increased rigidity of the MOR401 membrane influences FlaA regulation in a manner similar to low temperature stress, inducing its expression. Overexpression of FlaA has been observed to reduce intracellular virulence and produce a chaining phenotype at room temperature (105). This could be related to the role of BCFAs in modulating the virulence phenotype of *L. monocytogenes,* as observed through the reduced production of LLO in this mutant (28). Similarly, the actin-polymerization

protein ActA is associated non-covalently with the cell wall through a hydrophobic tail (36), and previous work has demonstrated defects in intracellular mobility of the MOR401 strain (28). Disturbed localization of this protein due to altered membrane dynamics could potentially contribute to the explanation of this phenotype.

A final, potentially crucial factor for consideration which could not be directly assayed in this study is the role of peptidoglycan O-acetylation. This modification of NAM constituents has been associated with modulating the activity of autolytic enzymes, host immune evasion, and plays a key role in cell separation in *B. subtilis* (48, 52, 53). O-acetyltransferase enzymes responsible for this phenomenon are as yet uncharacterized, though they are predicted to be membrane associated, perhaps placing their activity within the purview of future investigations. In *B. subtilis,* O-acetylation of peptidoglycan appears to modulate the chaining phenotype in a somewhat quantity-dependent manner, as successive deletion of additional Oat genes produced longer chains than single deletions (53). While the association of O-acetylation with cell separation has not yet been made in *L. monocytogenes*, this could prove a productive avenue of inquiry. If the extent of O-acetylation correlates with the severity of cell chaining, it could represent a potential explanation for the intermediate chaining phenotype observed in MOR401.

Conclusion and Future Directions

Ultimately, our results appear to suggest alterations to either peptidoglycan hydrolase properties or cell wall structure, though it remains difficult to determine the relative contribution of each. Future investigations may seek to precisely quantitate the extracellular concentrations of proteins and hydrolases, a potential factor influencing cell separation. The peptidoglycan hydrolases p60 and MurA both have an established role in

this process and could be specifically explored for alterations in expression, extracellular presence, or activity on *L. monocytogenes* wild-type and mutant cell walls. Total cellular protein could also be a correlate of the MOR401 chaining phenotype, and this could be quantified. Assays of cytoplasmic membrane proteins could also provide information on transport efficacy and the protein repertoire of the cell. Methods detailing the isolation of this subcellular fraction have been described previously (88). Profiles of membrane proteins would complement the cell wall protein profiles performed here.

Investigations into cell wall chemistry using purified cell wall components will also prove useful in identifying potential structural alterations, either in cross-linking, N-deacetylation, O-acetylation, or other modifications. These modifications can influence the activity of autolytic enzymes and cell separation processes. PCWs can be assayed with autolytic enzymes to more precisely identify whether structural variations are influencing autolytic activity. Alterations in teichoic acid structure could also contribute to the activity and localization of autolysins. Efforts chronicled here to refine the process of cell wall purification and PGN and TA extraction should facilitate these investigations.

The specific influence of membrane fluidity on the localization of DivIVA may also be interesting to explore should future work suggest its involvement. Fluorescent tagging of DivIVA or MurA and p60 could reveal alterations in their cellular localization. Destabilization of this protein may suggest a mechanism for an apparent decrease in extracellular hydrolases necessary for cell separation.

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APPENDIX

SUPPLEMENTAL FIGURES

1. Differential interference contrast microscopy of *L.monocytogenes* 10403S

2. Differential interference contrast microscopy of *L. monocytogenes* MOR401 grown in the absence of 2MB

3. Differential interference contrast microscopy of *L. monocytogenes* MOR401 grown in the presence of 2MB

Figure S1. DIC Microscopy of *L. monocytogenes* 10403S at 100x Magnification. Image Courtesy of Suranjana Sen.

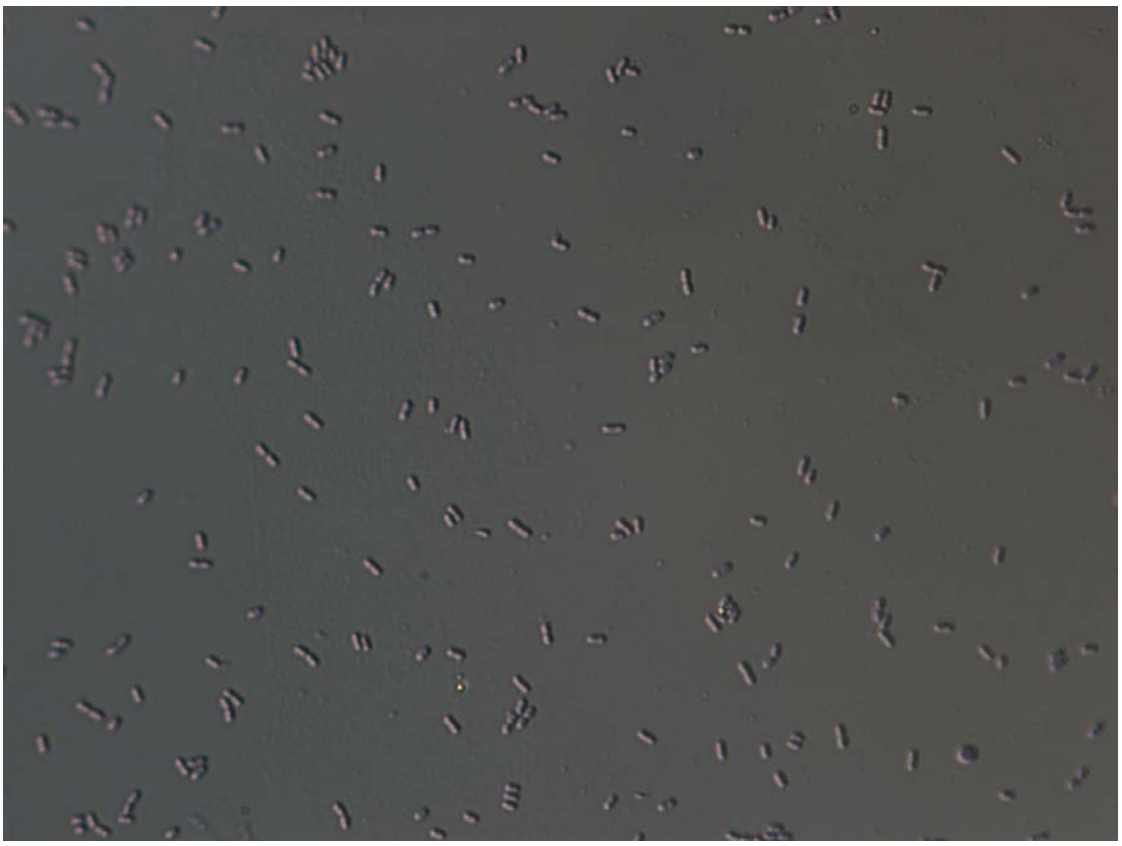

Figure S2. DIC Microscopy of *L. monocytogenes* MOR401 Grown in the Absence of 2MB at 100x Magnification. Image Courtesy of Suranjana Sen.

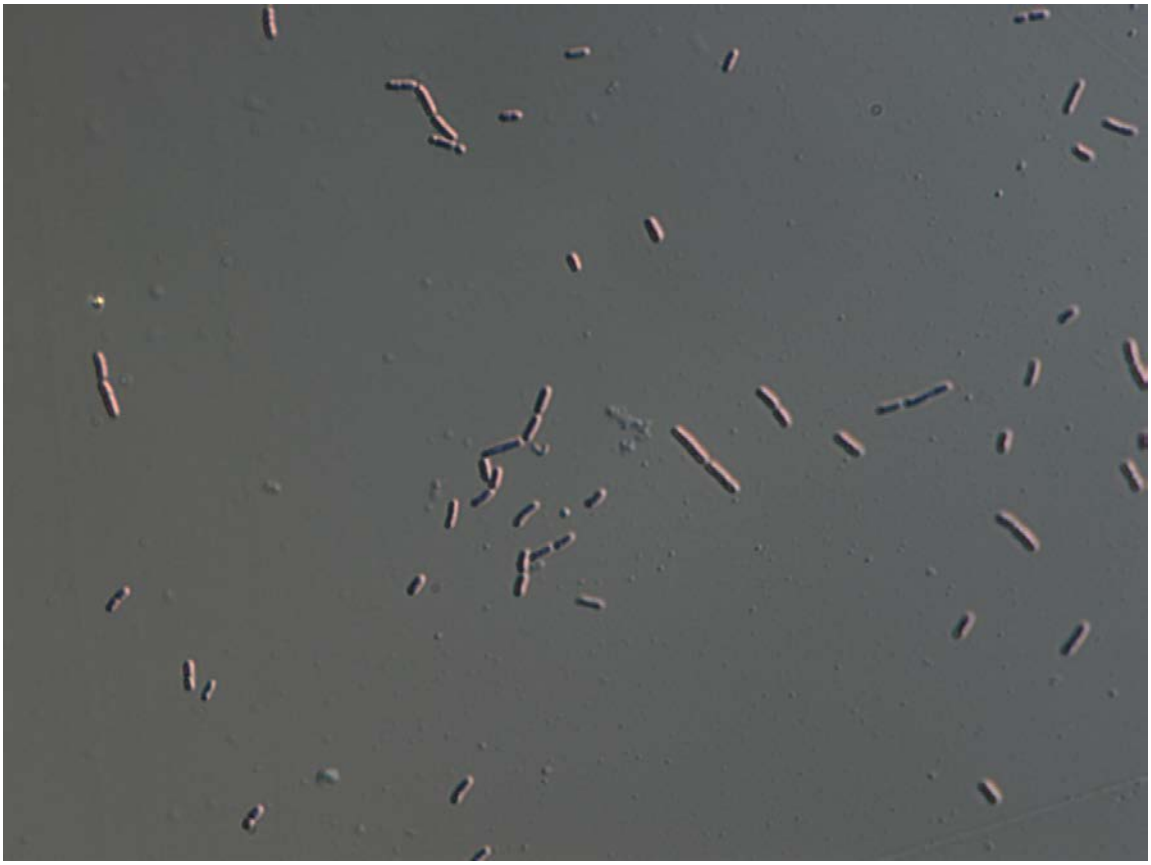

Figure S3. DIC Microscopy of *L. monocytogenes* MOR401 Grown in the Presence of 2MB at 100x Magnification. Image Courtesy of Suranjana Sen.